

# Stem cells for brain repair in neonatal hypoxia–ischemia

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**Abstract** Neonatal hypoxic–ischemic insults are a significant cause of pediatric encephalopathy, developmental delays, and spastic cerebral palsy. Although the developing brain’s plasticity allows for remarkable self-repair, severe disruption of normal myelination and cortical development upon neonatal brain injury are likely to generate life-persisting sensory-motor and cognitive deficits in the growing child. Currently, no treatments are available that can address the long-term consequences. Thus, regenerative medicine appears as a promising avenue to help restore normal developmental processes in affected infants. Stem cell therapy has proven effective in promoting functional recovery in animal models of neonatal hypoxic–ischemic injury and therefore represents a hopeful therapy for this unmet medical condition. Neural stem cells derived from pluripotent stem cells or fetal tissues as well as umbilical cord blood and mesenchymal stem cells have all shown initial success in improving functional outcomes. However, much still remains to be understood about how those stem cells can safely be administered to infants and what their repair mechanisms in the brain are. In this review, we discuss updated research into pathophysiological mechanisms of neonatal brain injury, the types of stem cell therapies currently being tested in this context, and the potential mechanisms through which exogenous stem cells might interact with and influence the developing brain.

**Keywords** Neonatal hypoxia–ischemia · Stem cell transplantation · White matter injury · Brain repair · Myelination

## Introduction

Despite major advances in monitoring technology and knowledge of fetal and neonatal pathologies, hypoxic–ischemic (HI) strokes remain the most common form of damage to the neonate brain [51], causing significant mortality and persistent neurobiological morbidity. In most cases, exact timing and underlying causes of the injury are unknown. Etiologies are complex and most often multifactorial. Reported precipitating insults include placental abnormalities [21], intrauterine growth restriction [115], preeclampsia [115], maternal infections [15, 33, 72], circulation disorders [34, 61, 114], and perinatal asphyxia [87]. Genetic makeup, sex, and degree of brain development also affect vulnerability and the mechanisms of brain injury [44, 105]. Neonatal HI occurs in 1–3 per 1,000 live full-term births and dramatically increases to 40 per 1,000 in preterm children with very low birth weight [33, 47]. Of affected newborns, 25 % develop severe and persistent neuropsychological impairments, including mental retardation, motor deficits, cerebral palsy, and epilepsy [104].

Upon neonatal HI insult, oxygen and glucose supplies are transiently depleted from the brain, causing an energy failure and initiating a cascade of biochemical events leading to cell dysfunction and oxidative stress [97]. Depending on the strength and duration of this initial insult, secondary injuries are likely to follow which include mitochondrial dysfunction, apoptosis, and excitotoxicity [97]. Tertiary effects may persist in the brain such as sensitization to inflammation, impaired oligodendrocyte maturation/myelination, persistent gliosis, and epigenetic changes [14, 26, 97]. Although those enduring damages might predispose patients to developmental disruption and sensitization to further injury, this also creates an extended window of opportunity for further treatment.

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## White matter damages in the developing brain

Brain white matter consists of glia and myelinated axons and plays a crucial role in fast signal transmission throughout the CNS. The process of active myelination reflects the progression of functional brain maturation and connectivity in the first years of life and renders the infant brain particularly vulnerable to injuries such as pediatric stroke and epilepsy [73]. Consequently, white matter injury and disruption of normal myelination is a hallmark of neonatal HI. Clinical diffusion tensor imaging (DTI) studies have shown that the majority of children with spastic types of cerebral palsy present white matter injuries and the importance of myelin loss correlates with the severity of motor impairments and learning disabilities [65, 80]. Together with perinatal inflammation, hypoxia–ischemia is considered the principal initiating mechanism for pathogenesis of periventricular leukomalacia (PVL), a condition characterized by coagulation and necrosis of white matter near the lateral ventricles, accompanied by gliosis/necrosis evolving from diffuse to focal cyst formation [45]. Non-cystic diffuse PVL accounts for most of the cerebral white matter injury in the newborn and is associated with loss of pre-myelinating oligodendrocytes, astrogliosis, and microglial infiltration [72]. Studies indicate that pre-myelinating late oligodendrocyte progenitors (pOPC) are crucial in renewing the pool of oligodendrocyte progenitor (OPC) and driving their differentiation towards myelinating oligodendrocytes. These pOPC are particularly vulnerable to the hypoxic stress generated during ischemic insults, and hence, rapidly undergo apoptosis in the injured environment [4, 85]. Systemic upregulation of pro-inflammatory cytokines associated with astrogliosis and microglial infiltration as well as resulting oxidative stress are also likely to restrict white matter development and affect existing and forming myelin fibers [47, 109].

While experimental models have been proposed to prevent or ameliorate white matter injury-associated mechanisms separately [45], therapies targeting the overall neurogenic niche are likely to be the most effective in restoring progression of white matter formation and alleviate long-term disabilities following neonatal HI injuries.

## Preclinical approach

### Animal models

Because of the large heterogeneity in factors potentially contributing to cerebral palsy, it has been difficult to achieve a reliable animal model. The Levine and Rice-Vannucci model of neonatal HI [54, 78, 103], involving unilateral temporary or permanent ligation of the common carotid artery and subsequent exposure to hypoxic condition (8–10% O<sub>2</sub>) on postnatal day 7 (P7) animals, is the most commonly used paradigm to model

neonatal HI and neurobehavioral outcomes reminiscent of cerebral palsy. Multiple variations of this model have been explored including global hypoxia without carotid artery ligation, bilateral carotid occlusion, and temporary carotid occlusions using aneurysm clips. The neonatal hypoxia model has been applied in rats, mainly Sprague Dawley and Wistar rats. It has been shown that a more consistent injury was achieved in Wistar rats as compared to Sprague Dawley rats probably because of differences in collateral circulation [69]. Other adjunct interventions have been explored such as the combination with low doses of lipopolysaccharide—a potent trigger of the innate immune system—which dramatically increased injury response to HI challenge [53, 110] and suggested that inflammation may further sensitize the immature central nervous system.

## Stem cell therapy

First studies on cell therapy for neonatal HI were designed to evaluate the ability of transplanted cells in replacing damaged tissue and utilized fetal cortical grafts or polymer scaffolds seeded with neural stem cells [22, 43, 71]. However, several reports subsequently demonstrated that transplanted stem cells promote CNS tissue repair not merely through cell replacement but by providing trophic and immunomodulatory support for endogenous repair mechanisms [60]. Different types of stem cells have been considered to mediate brain repair including stem cells derived from umbilical cord blood, bone marrow, fetal central nervous system, embryonic tissues as well as reprogrammed somatic [99] cells. Because each stem cell type have unique characteristics, it is likely that they use distinct machineries to interact with the ischemic environment and trigger regenerative mechanisms. On the other hand, some mechanisms are common to all stem cell types, and those might be particularly relevant for the induction of repair mechanisms in the brain. In the following paragraphs, we will review cell type-specific characteristics that might be relevant to regeneration in the injured neonatal brain. We will also discuss current concepts of the potential mechanisms of action that induce repair/regeneration of the injured CNS.

### Umbilical cord blood-derived mesenchymal stem cells

The human umbilical cord is a rich source of stem and progenitor cells including mesenchymal stem cells (MSC) and hematopoietic stem/progenitor cells (HPC). Umbilical cord cells are easily available via noninvasive procedures and considered less immunogenic than alternative adult stem cell sources such as bone marrow. MSC derived from cord blood have shown to generate neural stem cells *in vitro* [19, 28] and to lead to functional improvement in rodent models of perinatal brain injury [55, 116]. Thus, there is a growing

interest in studying the potential of umbilical cord-derived stem cells for the treatment of brain diseases [60, 99, 101]. MSC have demonstrated potent trophic support [8] and immunomodulatory properties in vitro [98, 112], and therefore, appear as particularly interesting candidates to modulate glial activation and pro-inflammatory mechanisms that accompany neonatal ischemic injuries. Furthermore, as those cells are isolated at birth following clamping and elimination of the umbilical cord, they present the considerable advantages of being autologous and freshly available after sorting and expansion. Short-term exposure to mild hypoxia has shown to optimize MSC functions [40], which might further emphasize their therapeutic relevance in the context of HI injury. Besides MSC, other cells can be isolated from the mononuclear fraction of umbilical cord blood, with a relative immature and naive phenotype. Those include HPC and endothelial progenitors, which derive from a common precursor cell called the hemangioblast [52]. Interestingly, while intravenous injection of HPC in the ischemic brain has demonstrated neuroprotective and immunomodulatory effects [84], transplantation of a cord blood fraction enriched in hemangioblastic cells induced neovascularization in a mouse model of stroke [64, 93]. This might further argue that different stem cell types support distinct repair mechanisms depending on their respective lineage, and combinational therapies might be appropriate in major injuries.

#### Fetal/adult neural stem/progenitor cells

Neural stem/progenitor cells (NPC) can be found in endogenous neurogenic areas such as the subventricular zone or the dentate gyrus of the hippocampus. In the case of human NPC, the primary source can either be donated fetal tissue [25, 92, 95, 107, 108] or adult post-mortem brains [70]. Fetal-derived NPC are typically isolated from the brain tissue by fluorescent-activated cell sorting and then grown as neurospheres under proliferative conditions [95]. Adult human neural stem cells have been isolated from brain tissue obtained from patients undergoing surgical procedures involving removal of brain tissue for the treatment of epilepsy, tumors, or trauma [3, 9, 32]. These studies demonstrate that the adult human brain contains a renewable source of NPC, which can be successfully isolated through various surgical techniques. Regardless of source, these cells can differentiate into oligodendrocytes, astrocytes, and neurons. NPC demonstrated potent ability to migrate in response to endogenous chemokines [41] and can move at a rate of 100–125  $\mu\text{m}/\text{day}$  towards the area of injury in the neonatal brain where they can survive for up to 52 weeks following transplantation [67]. As for MSC, mild hypoxia has shown to enhance proliferation and differentiation of a human NPC line towards neuronal and oligodendroglia lineages, emphasizing the potential of fetal NPC to mediate brain repair

in HI conditions [83]. Most stem cell therapy studies in neonatal HI utilized rodent NPCs. The only study to date using human NPCs found a change in activation of resident microglia, and most interestingly, a change in gene expression in the brain after cell engraftment, suggesting a potent cross-talk between transplanted and intrinsic cells [13]. In this study, the increase in transcripts for growth factors GDNF, IGF-1, and FGF2; neuronal marker doublecortin; and oligodendrocyte markers Olig2 and myelin basic protein suggest that widespread changes in the brain could be driving the functional improvements seen following NPC engraftment [13].

#### Embryonic stem cell-derived neural stem cells

Embryonic stem cells (ESC) are derived from the microscopic cluster of cells populating the blastocyst cavity a couple of days following fertilization [96]. ESC have been reported to differentiate into various cell types including NPC and oligodendroglial progenitors [10, 46] and are therefore considered as a potential source for cell replacement therapy in CNS diseases. Among all stem cell types, ESC are the most truly self-renewable and pluripotent populations. These properties confer them the considerable advantages of providing an almost unlimited supply of cells and differentiating toward a whole spectrum of distinct cell types. For instance, human embryonic stem cells have proven to differentiate in vitro towards oligodendrocyte progenitor cells, motoneurons, dopaminergic neurons, astrocytes, and peripheral sensory neurons (reviewed in [24]). However, counterparts to those properties are increased risks of neoplastic transformation (“graft overgrowth”) and multigerm layer teratoma formation following transplantation, events which are likely to be supported by factors released by the injured brain, thus raising major safety issues [42, 79, 86]. Furthermore, relative immaturity of ESC-derived lineages might confer protracted development requiring several months of further in vitro or in vivo maturation before demonstrating therapeutic potential [7, 63]. Last but not least, human ESC research is a rather controversial issue, as creation of an ESC line requires the destruction of a human embryo [96].

#### Induced pluripotent stem cells

Induced pluripotent stem cells (iPSC) are adult somatic cells that have been reprogrammed to an embryonic stem cell-like state by enforced expression of pluripotency transcription factors, mainly Oct3/4, Sox2, Klf4, and c-myc [94]. As iPSC capture the genetic diversity of the donor, provide access to the earliest stages of development, and virtually generate unlimited numbers of patient-specific cells, they are particularly considered as a valuable tool to model human genetic diseases in vitro [59, 74, 91]. In the field of stem cell transplantation, they present the advantages to overcome ethical limitations and to allow for generation of autologous cellular products, while

behaving similar to ESC in morphology, gene expression, and differentiation potential. Although this new field has generated quite some excitement within the scientific community, major safety issues remain associated with the use of iPSC. First, the ectopic expression of the chosen set of pluripotency factors can lead to neoplastic transformation of cells derived from these iPSC [6, 31, 38, 68]. Furthermore, in most existing procedures, these genes are introduced via DNA integrating viruses, which are likely to maintain or reactivate their transcriptional activity in the pluripotent cells or their progeny [68]. Also of major safety concern, there is a growing body of evidence demonstrating genetic and epigenetic instability in human iPSC, likely to originate during *in vitro* selection of the reprogrammed clones [66] and the reprogramming procedure *per se* [31]. If iPSC are to be used for clinical regenerative purposes, careful genetic integrity controlling is an absolute requirement. Considerable efforts are currently being made to develop safer and more efficient methods to generate iPSC. For instance, recent promising procedures focus on the use of non-integrating Sendai viruses that can further be removed through temperature shift [5].

Future should confirm the potential use of iPSC in stem cell therapies in the context of stroke. On an optimistic note, a recent study in a rat experimental stroke model has reported that iPSC-derived NPC are effective in reducing stroke-induced inflammatory response, gliosis, and apoptosis, contributing to endogenous neurogenesis and inducing behavioral recovery [11].

### Potential therapeutic mechanisms of action

#### Neuroprotection and neuroplasticity

Although stem cell-induced endogenous repair mechanisms are largely unknown, it is likely that transplanted cells and resident neuronal and glial lineages will mutually interact to support therapeutic effect. In support for the present hypothesis, we have recently demonstrated that NPC-secreted vascular-endothelial growth factor (VEGF) is necessary and sufficient to regulate endogenous microglial proliferation, activation, and phagocytic properties [62]. Although this study was performed with mouse NPC, it strongly suggests that exogenous stem cells assume a role in maintaining tissue integrity and immune function in the CNS apart from their purpose to merely produce and replace neural cells. Neonatal brain injury is accompanied by activation of pathways of oxidative stress, inflammation, and excitotoxicity that can lead to damages progressing over a long period of time and causing persisting disabilities in the growing child. In addition to neuronal damages, injury to non-neuronal types such as oligodendrocytes [4, 85, 89] and astrocytes [89] might also impair neurodevelopment. Neuroprotective and antiapoptotic effects mediated by NPC and stem cells in general have been recognized and largely reviewed in the context of different CNS

disorders including neonatal stroke [16, 48, 76, 99]. Based on previous data, it sounds reasonable to postulate that the transplants induce a greater survival of intrinsic brain populations. For instance, it has been shown that intravenously administrated marrow stem cells increase the expression of critical neurotrophic growth factors in the rat brain after traumatic injury [58]. Transplantation of human neural stem cells in a rat model of adult stroke has also shown to modulate dendritic plasticity and axonal transport, mostly through non-cell autonomous secretion of VEGF and thrombospondins [2].

#### Oligodendrogenesis and white matter regeneration

Brain anatomy is characterized by a dramatic growth from the end of the second trimester through the neonatal stage, with whole brain volume increasing almost 17 times [39]. Brain structures have shown to develop disproportionately during this period, and this might partly explain the extensive inhomogeneity of white matter injuries and their outcomes observed in cerebral palsy patients. Together with the reported increased sensitivity of pOPC to HI insults [4, 85], these observations place oligodendrogenesis and myelination as core processes underlying early brain injuries and life-persisting symptoms. A number of molecules have been critically involved in oligodendrogenesis, including neurotrophic factors such as platelet-derived growth factor- $\alpha$  (PDGF $\alpha$ ) [27], insulin-like growth factor-1 (IGF-1) [50], brain-derived neurotrophic factor [102], and erythropoietin [117]. Even though different stem cell types have not been compared side by side for their expression of neurotrophic factors, it is likely that secretion of some of those critical factors by NPC will partly explain the bystander effects of stem cell therapies [2, 48, 56]. In a model of multiple sclerosis recapitulating demyelination, it has been demonstrated that NPC induced OPC proliferation and maturation via secretion of PDGF $\alpha$  and FGF2 specifically [20]. In a rat model of neonatal hypoxia-ischemia, a study reported that human NPC graft specifically enhanced axonal transport and sprouting [13] through upregulation of oligodendrogenesis, myelination as well as an increase in neurotrophic factors. Once again, this strongly argues for cross-talk mechanisms between exogenous NPC and intrinsic cells, here the oligodendrocytes, in mediating brain repair and functional recovery through multiple modalities.

#### Immunomodulation

Several independent observations suggest a pivotal role for the immune system in shaping the central nervous system and contributing to its recovery upon injuries. Firstly, it has been recently demonstrated that immune myeloid cells home to the central nervous system before neurogenesis occurs [30]. Second, brain architecture and myelination are severely compromised in microglia-depleted animals [23]. Third, most of the reported stem cell-secreted factors are potent triggers of

immune-related pathways, including molecules directly involved in chemo-attraction and modulation of inflammation and phagocytosis [1, 13, 62, 77]. It appears that there are direct NPC-mediated immunomodulatory effects on the brain resident immune cells as well as modulation of the systemic immune system. Using NPC transplantation, several groups have demonstrated that induced recovery is partly mediated through modulation of microglial phagocytic activities [13, 62]. Studies in multiple sclerosis and spinal cord injury also emphasize the role of the innate immune system, principally macrophages/microglia, in supporting remyelination processes via phagocytosis of myelin debris [49, 82, 88]. Interestingly, transplantation of HPC, the progenitors of the immune and endothelial lineages, has also proven beneficial in animal models of stroke without reported cell fusion events [84]. In the field of neurodevelopmental disorders, recent studies have also started to unravel the beneficial role of bone marrow transplantation. For instance, in a genetic rodent model of Rett’s syndrome, a group strikingly identified microglial phagocytic activity as a major mediator of functional recovery [18]. Interestingly, different types of stem cells have intrinsic phagocytic properties [57, 100], and this might contribute to their beneficial effects in the injured brain.

A second immunomodulatory effect appears to be systemic. Cord blood stem cell infusion in a model of acute stroke has shown to mediate brain protection through specific modulation of the splenic release of inflammatory cells [106]. The same has been shown for stem cell-induced recovery in traumatic brain injury [111]. In this study, stem cell injection led to a preservation of blood brain barrier integrity through modulation of the immune cell response in the spleen. A pharmacologic correlate to this phenomenon relies on the use of sphingosine-1 phosphate receptor agonist fingolimod (FTY720)—a multiple sclerosis market drug. Its main action is to segregate inflammatory cells within the spleen, and it has also demonstrated long-term protection in rodent models of cerebral ischemia [113]. Taken together, these findings support the concept of NPC-mediated local and systemic modulation of the immune response as a potentially important mechanism of action.

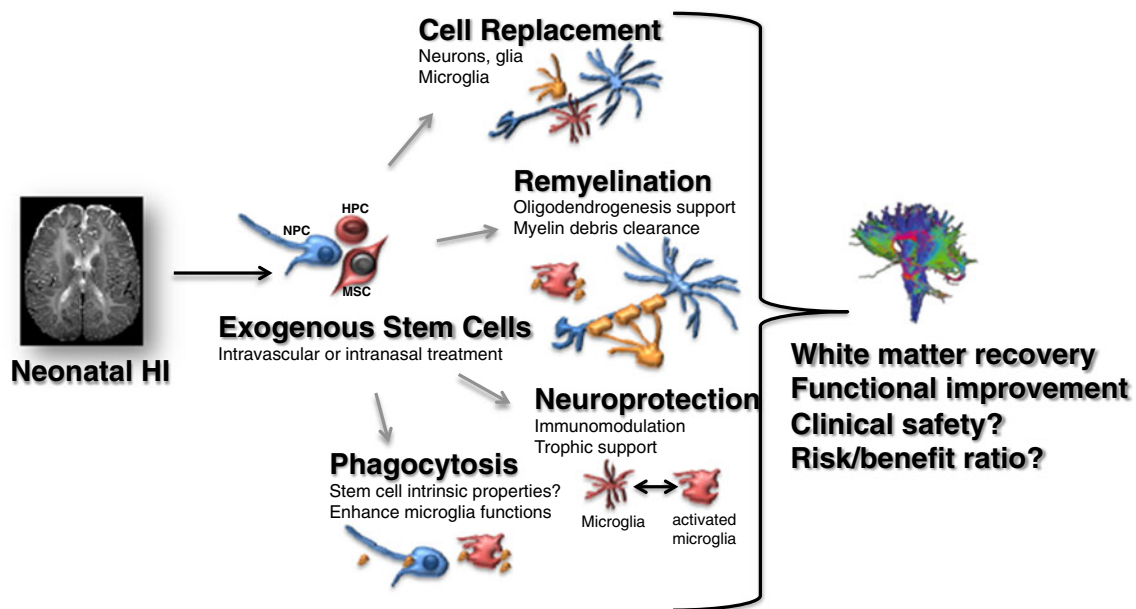
**Methodologies**

Cell delivery and timing

Aside from the type of stem cells used for transplantation in neonatal HI, the route of administration and timing are likely to play crucial roles in the efficacy of the treatment. Stem cells can be delivered systemically or directly into the brain. Our laboratory previously demonstrated that delivery of stem cells to the injured brain via intravascular treatment allows for a widespread distribution of cells in the brain by means of a

**Table 1** Clinical trials using stem cells for treatment of neonatal stroke-related disorders. AUCB: autologous umbilical cord blood stem cells, IV: intravenous

Clinical trial ID	Cell type	Specific pathology	Endpoint classification	Patients	Age	Delivery	Completion	Location	Clinical trial title
NCT01147653	AUCB	Cerebral palsy	Efficacy	120	12 months–6 years	IV	January 16	USA	A Randomized Study of Autologous Umbilical Cord Blood Reinfusion in Children With Cerebral Palsy
NCT01072370	AUCB	Cerebral palsy	Safety/efficacy	40	1–12 years	IV	February 14	USA	Safety and Effectiveness of Cord Blood Stem Cell Infusion for the Treatment of Cerebral Palsy in Children
NCT00593242	AUCB	Neonatal encephalopathy	Safety	25	<14 days	IV	December 13	USA	Cord Blood for Neonatal Hypoxic-ischemic Encephalopathy
NCT01700166	AUCB	Arterial ischemic stroke (AIS)	Safety/Efficacy	10	6 weeks–6 years	IV	December 15	USA	Umbilical Cord Blood in the Treatment of Stroke in Children (Pedi Stroke)
NCT01506258	AUCB	Neonatal asphyxia	Safety/Efficacy	20	37 weeks–42 weeks	IV	April 13	Mexico	Autologous Stem Cells in Newborns With Oxygen Deprivation



**Fig. 1** Potential stem cell-induced brain repair mechanisms. Schematic illustrating main candidate mechanisms of action in stem cell-mediated white matter repair following neonatal hypoxic–ischemic injuries. For detail, cf. text

highly feasible, safe, repeatable, and minimally invasive approach [12, 35, 36, 75]. Our studies suggest an active mechanism of cell recruitment to the brain through cellular expression of adhesion molecules [36] and chemokine receptors [1, 81]. While previous studies involved transplantation of human stem cell grafts 24 h after neonatal hypoxia–ischemia injury [13], we established that intra-arterial transplantation of NPC 3 days after the insult resulted in higher cell engraftment and survival when compared with earlier (6–24 h) or later (7–14 days) time-points [81]. Interestingly, day 3 post-injury correlated with the highest expression of signaling molecules such as VCAM-1, CCL2, and CXCL12, here again supporting a cross-talk hypothesis, where the injured environment is likely to dictate efficiency of exogenous stem cell treatment. Current clinical trials will be using early (48 h and 2 weeks after birth) and chronic time points (up to 12 years of age) for intravenous cell delivery (Table 1).

### Imaging

Noninvasive approaches to monitor stem cells upon transplantation are pivotal to therapeutic success and to prediction of graft viability and potential complications. High field magnetic resonance imaging (MRI) remains the modality of choice to evaluate pathogenesis, severity, and evolution of neonatal HI [17]. To evaluate white matter tract integrity and repair, MRI-based DTI has become the main imaging modality [90]. MRI can not only monitor evolving neonatal cerebral injury but also track migration and location of iron oxide nanoparticle (SPIO) labeled exogenous stem cells for prolonged time periods [13, 37, 67, 75]. Other

preclinical stem cell imaging approaches have included reporter genes for bioluminescence, proton emission tomography, single photon emission tomography, radioactive tracers, and fluoride labeling for fluoride MR-based imaging (for review, see [29]). To date, however, there is no modality for clinical stem cell imaging.

### Conclusions and perspectives

Increased understanding in the pathophysiology of perinatal injuries leading to cerebral palsy has placed white matter injury and myelin loss at the core of the disorder. With their unique regenerative properties, stem cells appear as a promising and versatile tool for therapeutic approaches in pediatric neurology. Moreover, repair mechanisms might be facilitated by the immaturity and plasticity of the neonatal developing brain.

Proof of principle for stem cell-mediated brain repair and functional recovery has been shown with different types of cells in the Rice-Vannucci animal model of neonatal hypoxia–ischemia. Most of those studies argue in favor of a strong communication between transplanted cells and intrinsic brain lineages. More than cell replacement, it becomes more and more obvious that exogenous stem cells are acting as bystander cells, providing a favorable niche for neuroprotection and immunomodulation (Fig. 1). However, knowledge obtained from preclinical models is unable to totally predict clinical outcomes in pediatric patients. Several clinical trials are underway to evaluate the safety and efficacy using autologous cord blood intravenous transplantation (Table 1), and results of

those should partly address safety of cell transplantation in the acute and chronic phase, as well as efficacy of the procedure for the growing child.

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