STEM CELLS AND PROGENITOR CELLS IN ISCHEMIC STROKE - FASHION OR FUTURE?

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STEM CELLS AND PROGENITOR CELLS IN ISCHEMIC STROKE - FASHION OR FUTURE?

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Stroke remains one of the most devastating diseases in industrialized countries. Recanalization of the occluded arterial vessel using thrombolysis is the only causal therapy available. However, thrombolysis is limited due to severe side effects and a limited time window. As such, only a minority of patients receives this kind of therapy, showing a need for new and innovative treatment strategies. Although neuroprotective drugs have been shown to be beneficial in a variety of experimental stroke models, they ultimately failed in clinical trials. Consequently, recent scientific focus has been put on modulation of post-ischemic neuroregeneration, either via stimulation of endogenous neurogenesis or via application of exogenous stem cells or progenitor cells.

Neurogenesis persists within the adult brain of both rodents and primates. As such, neural progenitor cells (NPCs) are found within distinct niches like the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus. Cerebral ischemia stimulates these astrocyte-like progenitor cells, upon which NPCs proliferate and migrate towards the site of lesion. There, NPCs partly differentiate into mature neurons, without significantly being integrated into the residing neural network. Rather, the majority of new-born cells dies within the first weeks post-stroke, leaving post-ischemic neurogenesis a phenomenon of unknown biological significance. Since NPCs do not replace lost brain tissue, beneficial effects observed in some studies after either stimulated or protected neurogenesis are generally contributed to indirect effects of these new-born cells. The precise identification of appropriated cellular mediators, however, is still elusive. How do these mediators work? Are they soluble factors or maybe even vesicular structures emanating from NPCs? What are the cues that guide NPCs towards the ischemic lesion site? How can post-ischemic neurogenesis be stimulated? How can the poor survival of NPCs be increased?

In order to support post-ischemic neurogenesis, a variety of research groups have focused on application of exogenous stem/progenitor cells from various tissue sources. Among these, cultivated NPCs from the SVZ and mesenchymal stem cells (MSCs) from the bone marrow are frequently administered after induction of stroke. Although neuroprotection after delivery of stem/progenitor cells has been shown in various experimental stroke models, transplanted cells are usually not integrated in the neural network. Again, the vast amount of grafted cells dies or does not reach its target despite profound neuroprotection, also suggesting indirect paracrine effects as the cause of neuroprotection. Yet, the factors being responsible for these observations are

under debate and still have to be addressed. Is there any "optimal" cell type for transplantation? How can the resistance of grafted cells against a non-favorable extracellular milieu be increased? What are the molecules that are vital for interaction between grafted cells and endogenous NPCs?

The present research topic seeks to answer - at least in part - some of the aforementioned questions. Although the research topic predominantly focuses on experimental studies (and reviews alike), a current outlook towards clinical relevance is given as well.

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EDITORIAL
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Editorial: Stem cells and progenitor cells in ischemic stroke—fashion or future?

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Keywords: stem cells, cell transplantation, stroke, cerebral ischemia, neurogenesis, neuroregeneration

Despite recent achievements in re-canalizing stroke therapies (Campbell et al., 2015; Goyal et al., 2015), which ensure reduction of deficits in a significant patient faction due to combined systemic thrombolysis and interventional clot removal, a major need remains for restorative therapies in patients suffering from persistent neurological deficits despite optimized treatment. Although neurogenesis persists in the adult mammalian brain within distinct niches, such as the subventricular zone (SVZ) hosting stem cells and neural progenitor cells (NPCs) alike, the restorative potential of endogenous neurogenesis is generally insufficient and thus unable to support a full recovery of lost functions following stroke. Consequently, transplantation of exogenous NPCs and various other stem cell sources has emerged as a potential stroke treatment. Although adult NPCs are not integrated into residing neural networks, solid experimental data demonstrates beneficial effects in pre-clinical stroke models (Bacigaluppi et al., 2009; Doeppner et al., 2012). Thorough insights into stem cell actions have been obtained in experimental studies in recent years, which raise the questions about the clinical potential of stem cell-based therapies.

The mechanisms underlying post-stroke neurogenesis are diverse and highly complex, involving interactions of stem cells with extracellular matrix (ECM) constituents, microvascular cells, brain parenchymal cells, and immune cells, as summarized within this research topic by Hermann et al. (2014). The pivotal role of calpains, which are activated upon post-ischemic cellular calcium influx and control ECM remodeling, in post-stroke neurogenesis was now further analyzed by Machado et al. (2015) who provide compelling evidence that deletion of the endogenous calpain inhibitor calpastatin hampers the proliferation and migration of NPCs, whereas calpain inhibition increases NPC proliferation, migration speed, and migration distance. Accordingly, the modulation of calpains might be a potent tool to boost post-stroke neurogenesis. Representing a molecular substrate of calpains, the multifunctional ECM glycoprotein tenascin-C exemplifies in a particularly multi-faceted way how the characteristics of stem cells are modified by ECM constituents upon brain injury, as outlined by Roll and Faissner (2014). Casting new light onto the role of the cerebral microvasculature for post-stroke neurogenesis, Adamczak et al. (2014) provide a detailed non-invasive analysis of the dynamics of VEGF and its receptor VEGFR2 in a mouse model of focal cerebral ischemia. The authors describe active VEGFR2 signaling for as long as 2 weeks post-stroke that is likely to promote NPC migration and proliferation. Non-invasive imaging will greatly facilitate research on neurogenesis in the near future, as stressed by Aswendt et al. (2014), who systematically reviewed the requirements, advantages, and limitations of optical imaging as compared with existing imaging techniques.

In light of the insufficient neurorestorative capacity of endogenous neurogenesis, various studies aimed to support neurogenesis by cell transplantation. Due to their low immunogenicity and easy accessibility, mesenchymal stem cells (MSCs) were by far most often used in experimental stroke studies followed by NPCs. Yet, recovery-promoting actions can be achieved by various cell sources, as shown in the present research topic for amniotic fluid-derived stem cells, which protect the brain against ischemic injury (Tajiri et al., 2014). Yet, several open questions and

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pitfalls still have to be overcome to enable the translation of stem cell therapy from bench to bedside. These include the differentiation, fate and safety of transplanted cells as well as the contamination of grafted cells with feeder cells that could also pose a significant hazard to the recipient, as described by Ikegame et al. (2014) and Molcanyi et al. (2014). As neither endogenous nor exogenous adult stem cells are integrated into residing neural networks, it stands to reason whether or not transplantation of stem cells is mandatory for induction of neuroprotection. Indeed, recent evidence suggests that extracellular vesicles (e.g., exosomes) containing non-coding RNAs might be the biologically active mediator of stem cell-induced neuroprotection and brain plasticity (Xin et al., 2014). Extracellular vesicles might allow for evading cell-based safety issues. This concept deserves further in-depth evaluation in experimental systems and might offer itself for subsequent proof-of-concept studies in human stroke patients.

The vast majority of pre-clinical stroke studies were hitherto limited to adolescent, otherwise healthy rodents, which poorly reflects the clinical situation of elderly, multimorbid stroke patients. In order to analyze consequences of arterial hypertension, a particularly prevalent stroke risk factor, for responses to stem cell therapy, Diederich et al. (2014) evaluated effects of granulocyte-colony stimulating factor (G-CSF) and bone marrow derived mononuclear cells (BM-MNCs) in spontaneously hypertensive rats exposed to stroke (Diederich et al., 2014). In their study, the combined delivery of G-CSF and BM-MNCs was not superior to G-CSF alone. Most importantly, single treatment with BM-MNCs did not yield any therapeutic effect, in line with earlier data from this group (Minnerup et al., 2014). The evaluation of risk factors, such as arterial hypertension, will require intensified research in the future. Beside co-morbidities, age-related changes of the cerebral microenvironment have a strong impact on post-stroke brain remodeling according to Popa-Wagner et al. (2014) who claim that the aged brain is not refractory to post-stroke plasticity after cell grafting. Yet, significant age-related changes have been identified, i.e., a higher vulnerability to ischemic insults, a reduced rate of neurogenesis, and a delayed initiation of neurological recovery, which should carefully be considered in the implementation of clinical proof-of-concept studies. In view of age-related specificities, the neonatal mammalian brain might provide a particularly suitable environment for cell transplantation studies according to van Velthoven et al. (2014).

In the context of cell therapy, the selection of behavioral tests has repeatedly been criticized. Thus, it was objected that behavioral test batteries are optimized to detect functional neurological improvements, the significance of which under clinical conditions remains obscure. In a systematic study including as many as 12 motor-coordination and cognitive tests, Doeppner et al. (2014) now refute these criticisms, demonstrating consistent improvement of neurological function in response to NPC delivery across a large variety of tests. These data provide strong evidence regarding the potency of stem cells in experimental stroke settings, supporting the contributors' overall view that cell-based therapies have true potential for clinical translation. In light of a plethora of pre-clinical studies demonstrating successful post-stroke neurological recovery and brain remodeling after stem cell transplantation, first clinical trials have already been performed on small patient cohorts, as summarized by Doeppner and Hermann (2014). Promising data were obtained particularly following intravenous MSC delivery that until now, however, lack appropriate control groups. Stringent proof-of-concept strategies including clear-defined goals, measures, and actions will now have to be implemented, which further bridge the gap between the laboratory bench and the clinical setting. With such information, controlled clinical proof-of-concept studies may then be scheduled providing ultimate proofs whether cell-based therapies are able to enhance neurological recovery post-stroke.

References

Adamczak, J. M., Schneider, G., Nelles, M., Que, I., Suidgeest, E., van der Weerd, L., et al. (2014). *In vivo* bioluminescence imaging of vascular remodeling after stroke. *Front. Cell. Neurosci.* 8:274. doi: 10.3389/fncel.2014.00274

Aswendt, M., Adamczak, J., and Tennstaedt, A. (2014). A review of novel optical imaging strategies of the stroke pathology and stem cell therapy in stroke. *Front. Cell. Neurosci.* 8:226. doi: 10.3389/fncel.2014.00226

Bacigaluppi, M., Pluchino, S., Peruzzotti-Jametti, L., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132(Pt 8), 2239–2251. doi: 10.1093/brain/awp174

Campbell, B. C., Mitchell, P. J., Kleinig, T. J., Dewey, H. M., Churilov, L., Yassi, N., et al. (2015). Endovascular Therapy for ischemic stroke with perfusion-imaging selection. *N. Engl. J. Med.* 372, 1009–1018. doi: 10.1056/NEJMoa1414792

Diederich, K., Schmidt, A., Beuker, C., Strecker, J. K., Wagner, D. C., Boltze, J., et al. (2014). Granulocyte colony-stimulating factor (G-CSF) treatment in combination with transplantation of bone marrow cells is not superior to G-CSF treatment alone after cortical stroke in spontaneously hypertensive rats. *Front. Cell. Neurosci.* 8:411. doi: 10.3389/fncel.2014.00411

Doeppner, T. R., Ewert, T. A., Tönges, L., Herz, J., Zechariah, A., ElAli, A., et al. (2012). Transduction of neural precursor cells with TAT-heat shock protein 70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. *Stem Cells* 30, 1297–1310. doi: 10.1002/stem.1098

Doeppner, T. R., and Hermann, D. M. (2014). Stem cell-based treatments against stroke: observations from human proof-of-concept studies and considerations regarding clinical applicability. *Front. Cell. Neurosci.* 8:357. doi: 10.3389/fncel.2014.00357

Doeppner, T. R., Kaltwasser, B., Bähr, M., and Hermann, D. M. (2014). Effects of neural progenitor cells on post-stroke neurological impairment-a detailed and comprehensive analysis of behavioral tests. *Front. Cell. Neurosci.* 8:338. doi: 10.3389/fncel.2014.00338

Goyal, M., Demchuk, A. M., Menon, B. K., Eesa, M., Rempel, J. L., Thornton, J., et al. (2015). Randomized assessment of rapid endovascular treatment of ischemic stroke. *N. Engl. J. Med.* 372, 1019–1030. doi: 10.1056/NEJMoa1414905

Hermann, D. M., Peruzzotti-Jametti, L., Schlechter, J., Bernstock, J. D., Doeppner, T. R., and Pluchino, S. (2014). Neural precursor cells in the ischemic brain - integration, cellular crosstalk, and consequences for stroke recovery. *Front. Cell. Neurosci.* 8:291. doi: 10.3389/fncel.2014.00291

Ikegame, Y., Yamashita, K., Nakashima, S., Nomura, Y., Yonezawa, S., Asano, Y., et al. (2014). Fate of graft cells: what should be clarified for development of mesenchymal stem cell therapy for ischemic stroke? *Front. Cell. Neurosci.* 8:322. doi: 10.3389/fncel.2014.00322

Machado, V. M., Morte, M. I., Carreira, B. P., Azevedo, M. M., Takano, J., Iwata, N., et al. (2015). Involvement of calpains in adult neurogenesis: implications for stroke. *Front. Cell. Neurosci.* 9:22. doi: 10.3389/fncel.2015.00022

Minnerup, J., Wagner, D. C., Strecker, J. K., Pösel, C., Sevimli-Abdis, S., Schmidt, A., et al. (2014). Bone marrow-derived mononuclear cells do not exert acute neuroprotection after stroke in spontaneously hypertensive rats. *Front. Cell. Neurosci.* 7:288. doi: 10.3389/fncel.2013.00288

Molcanyi, M., Mehrjardi, N. Z., Schäfer, U., Haj-Yasein, N. N., Brockmann, M., Penner, M., et al. (2014). Impurity of stem cell graft by murine embryonic fibroblasts - implications for cell-based therapy of the central nervous system. *Front. Cell. Neurosci.* 8:257. doi: 10.3389/fncel.2014.00257

Popa-Wagner, A., Buga, A. M., Doeppner, T. R., and Hermann, D. M. (2014). Stem cell therapies in preclinical models of stroke associated with aging. *Front. Cell. Neurosci.* 8:347. doi: 10.3389/fncel.2014.00347

Roll, L., and Faissner, A. (2014). Influence of the extracellular matrix on endogenous and transplanted stem cells after brain damage. *Front. Cell. Neurosci.* 8:219. doi: 10.3389/fncel.2014.00219

Tajiri, N., Acosta, S., Portillo-Gonzales, G. S., Aguirre, D., Reyes, S., Lozano, D., et al. (2014). Therapeutic outcomes of transplantation of amniotic fluid-derived stem cells in experimental ischemic stroke. *Front. Cell. Neurosci.* 8:227. doi: 10.3389/fncel.2014.00227

van Velthoven, C. T., Gonzalez, F., Vexler, Z. S., and Ferriero, D. M. (2014). Stem cells for neonatal stroke- the future is here. *Front. Cell. Neurosci.* 8:207. doi: 10.3389/fncel.2014.00207

Xin, H., Li, Y., and Chopp, M. (2014). Exosomes/miRNAs as mediating cell-based therapy of stroke. *Front. Cell. Neurosci.* 8:377. doi: 10.3389/fncel.2014.00377

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Neural precursor cells in the ischemic brain – integration, cellular crosstalk, and consequences for stroke recovery

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After an ischemic stroke, neural precursor cells (NPCs) proliferate within major germinal niches of the brain. Endogenous NPCs subsequently migrate toward the ischemic lesion where they promote tissue remodeling and neural repair. Unfortunately, this restorative process is generally insufficient and thus unable to support a full recovery of lost neurological functions. Supported by solid experimental and preclinical data, the transplantation of exogenous NPCs has emerged as a potential tool for stroke treatment. Transplanted NPCs are thought to act mainly via trophic and immune modulatory effects, thereby complementing the restorative responses initially executed by the endogenous NPC population. Recent studies have attempted to elucidate how the therapeutic properties of transplanted NPCs vary depending on the route of transplantation. Systemic NPC delivery leads to potent immune modulatory actions, which prevent secondary neuronal degeneration, reduces glial scar formation, diminishes oxidative stress and stabilizes blood–brain barrier integrity. On the contrary, local stem cell delivery allows for the accumulation of large numbers of transplanted NPCs in the brain, thus achieving high levels of locally available tissue trophic factors, which may better induce a strong endogenous NPC proliferative response. Herein we describe the diverse capabilities of exogenous (systemically vs. locally transplanted) NPCs in enhancing the endogenous neurogenic response after stroke, and how the route of transplantation may affect migration, survival, bystander effects and integration of the cellular graft. It is the authors' claim that understanding these aspects will be of pivotal importance in discerning how transplanted NPCs exert their therapeutic effects in stroke.

Keywords: cell therapy, neurogenesis, stroke, blood–brain barrier, brain plasticity, neuroprotection

INTRODUCTION

Ischemic stroke represents the most common cause of serious morbidity and the second most common cause of mortality in industrialized countries (Donnan et al., 2008). While a certain degree of spontaneous recovery of lost functions takes place in some stroke patients, the majority never regain full functional independence and ultimately suffer from a reduced quality of life (Lloyd-Jones et al., 2009). Clearly, this health burden represents a major unmet clinical need that may in part be fulfilled via a detailed understanding of the mechanisms driving neurological recovery after stroke.

In animal models, the mobilization and recruitment of NPCs from the major stem cell niches within the central nervous system [CNS; i.e., the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone of the dentate gyrus (DG)] are essential compensatory responses after an ischemic insult (Arvidsson et al., 2002). However, while it is known that endogenous NPCs do positively enhance the brain's own restorative potential via trophic influences on the ischemic microenvironment, the overall neurogenic response after stroke is insufficient for a number of reasons, which include the limited survival of NPCs, their transient mobilization from the neurogenic niches and their incomplete integration within damaged brain circuitries (Thored et al., 2007).

The observation that NPCs can be harvested from the adult brain and used therapeutically in animal models of stroke argues in favor of the potential utility of cell-based therapies in ischemic stroke. We have shown that the injection of somatic mouse NPCs ameliorates the clinicopathological features of stroke in relevant murine models by reducing secondary neurodegeneration, decreasing glial scar formation, promoting endogenous neurogenesis and stabilizing blood–brain barrier (BBB) integrity (Bacigaluppi et al., 2009; Doeppner et al., 2012). Grafted NPCs adapt to the ischemic microenvironment and facilitate homeostasis via the secretion of numerous tissue trophic factors that have

Abbreviations: BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; CCL, CC chemokine ligands; CSPGs, chondroitin sulfate proteoglycans; CNTF, ciliary neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; HO-1, heme oxygenase 1; IFN-γ, interferon-γ; IGF1, insulin-like growth factor-1; IL, interleukin; LIF, leukemia inhibitory factor; MMPs, matrix metalloproteinases; NGF, nerve growth factor; NO, nitric oxide; NPCs, neural precursor cells; NT-3, neurotrophin-3; PGE, prostaglandin E; SDF-1α, stromal cell-derived factor-1α; SHH, sonic hedgehog homolog; SVZ, subventricular zone; TIMPs, tissue inhibitors of metalloproteinases; TN-C, tenascin-C; TNF-α, tumor necrosis factor-α; TSP, thrombospondin; VEGF, vascular endothelial growth factor.

beneficial effects on endogenous brain cells, as well as modulatory actions on both innate and adaptive immune responses (Pluchino and Cossetti, 2013). This concept has now come to be known as functional stem cell multipotency, and it is one of the core tenants behind the use of NPC grafts in attempt to boost the recovery potential of the ischemic brain (Martino and Pluchino, 2006).

However, there remains an enormous need to understand how the complex interactions of stem cell grafts with the ischemic brain may be affected by the route and timing of cell delivery. In particular, we still have to define how NPCs should best be administered in order to enhance endogenous restorative responses that depend on (i) the homing, survival and integration of transplanted NPCs, (ii) the proliferation of the host's endogenous NPCs, (iii) the modification of the cerebral microenvironment, and (iv) the remodeling of ischemic tissue via actions that include the modification of glial responses and the promotion of neuronal plasticity.

Herein, we summarize current knowledge with regard to how transplanted NPCs interact with host tissues, aiming to identify how exogenously delivered NPCs may eventually be used to promote neurological recovery in mouse models of ischemic stroke.

REGULATION OF ENDOGENOUS NEUROGENESIS AFTER STROKE

The SVZ is situated within the lateral walls of the lateral ventricles and is composed of four main cell types: ciliated ependymal cells (type E), slowly proliferating stem cells (type B), transient amplifying progenitors (type C) and proliferating neuroblasts (type A; Mirzadeh et al., 2008). After an ischemic stroke that involves the striatum, the number of type A and C cells in the SVZ is persistently increased, while type B and E cells undergo a period of transient proliferation (Zhang et al., 2004, 2007). Increases in mitotic activity within the SVZ appear to peak between 7 and 10 days, subsequently decrease during weeks 3–5 post-stroke, and thereafter continues at lower levels over the course of the following year (Arvidsson et al., 2002; Parent et al., 2002; Thored et al., 2006). This suggests that the SVZ may serve as a constant reservoir of new neurons after stroke even in the chronic phases of recovery, and thereby offers an extended window of opportunity for therapeutic intervention.

Signals that stimulate the stroke-induced neurogenic response have yet to be fully elucidated, but likely involve the interplay of morphogens, growth factors, and inflammatory mediators. Several groups have found that the notch pathway stimulates SVZ cell proliferation and neurogenesis after stroke (Androutsellis-Theotokis et al., 2006; Wang et al., 2009). Other signaling pathways that appear to be important for stroke-induced neurogenesis include retinoic acid (RA), sonic hedgehog (SHH), and bone morphogenic protein (BMP; Chou et al., 2006; Plane et al., 2008; Sims et al., 2009; Kernie and Parent, 2010). Soluble growth factors, such as basic fibroblast growth factor (bFGF), BDNF, epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF), erythropoietin (EPO), CNTF, transforming growth factor (TGF)-α, VEGF, and granulocyte-colony stimulating factor (G-CSF), have also been inextricably linked to stroke-induced neurogenesis (Kokaia et al., 1995; Planas et al., 1998; Kitagawa et al., 1999; Sun et al., 2003; Schneider et al., 2005; Tureyen et al., 2005; Leker et al., 2009; Kang et al., 2012). Inflammatory mediators have been shown to have variable effects on NPC proliferation, migration, survival, and incorporation within injured CNS circuitries (Peruzzotti-Jametti et al., 2014). Some studies have indeed reported that activated microglial cells can reduce NPC viability through the secretion of soluble molecules such as IFN-γ, IL-1β, IL-6, and tumour necrosis factor (TNF)-α or by direct cell-to-cell contact (Ben-Hur et al., 2003; Cacci et al., 2008). Other studies suggest instead that microglial cells can increase neurogenesis via TNF-α/ TNF-R2 interaction or insulin-like growth factor (IGF)-1 secretion (Heldmann et al., 2005; Thored et al., 2009). These differential effects of inflammation, which appear to either support or impair the adult neurogenic response, most likely depend on the phenotype of the inflammatory cells (and their cytokine production profile; Ekdahl et al., 2009).

Since NPCs can secrete many of the factors that regulate neurogenesis (Drago et al., 2013), and are also able to beneficially modulate inflammatory responses after CNS damage (Bacigaluppi et al., 2009; Cusimano et al., 2012), the possibility of exploiting NPC transplantation in an effort to augment endogenous neurogenesis and the brain's spontaneous reparative processes (e.g., plasticity) after stroke is readily apparent. In this review, cellular and molecular interactions of NPCs with the brain environment have been illustrated in **Figure 1**. Effects of exogenously delivered NPCs in experimental models of stroke have been summarized in **Table 1**.

EFFECTS OF TRANSPLANTED NPCs ON ENDOGENOUS NEUROGENESIS

In view of their intrinsic actions, the possibility of exploiting NPCs in an effort to augment *endogenous* neurogenesis has been the focus of intensive research efforts. Among the different routes of NPC delivery, there are several studies suggesting that the local (i.e., intracerebral or intracerebroventricular) administration of NPCs has the most relevant effects on endogenous neurogenesis. The local route of cell delivery indeed allows for a large number of cells to be administered, which facilitates the secretion of high concentrations of growth factors that ultimately promote the endogenous neurogenic response (Hao et al., 2014). In line with the need for an efficient accumulation of NPCs within the ischemic parenchyma, the intravenous administration of NPCs may be inferior with regard to the stimulation of neurogenesis. As such, when mouse embryonic stem (ES)-derived NPCs were transplanted 24 h after photothrombotic stroke in adult immunosuppressed rats, no effect on post-stroke neurogenesis of the SVZ was shown, and a decrease in newly generated neurons in the DG was observed (Minnerup et al., 2011).

The early transplantation of human NPCs has instead been proven highly effective in stimulating endogenous neurogenesis in rats when cells were delivered directly into the ischemic brain parenchyma. Human fetal NPCs, injected in the cortical peri-infarct tissue 24 h after permanent middle cerebral artery occlusion (MCAO) promoted cell proliferation in the SVZ (up

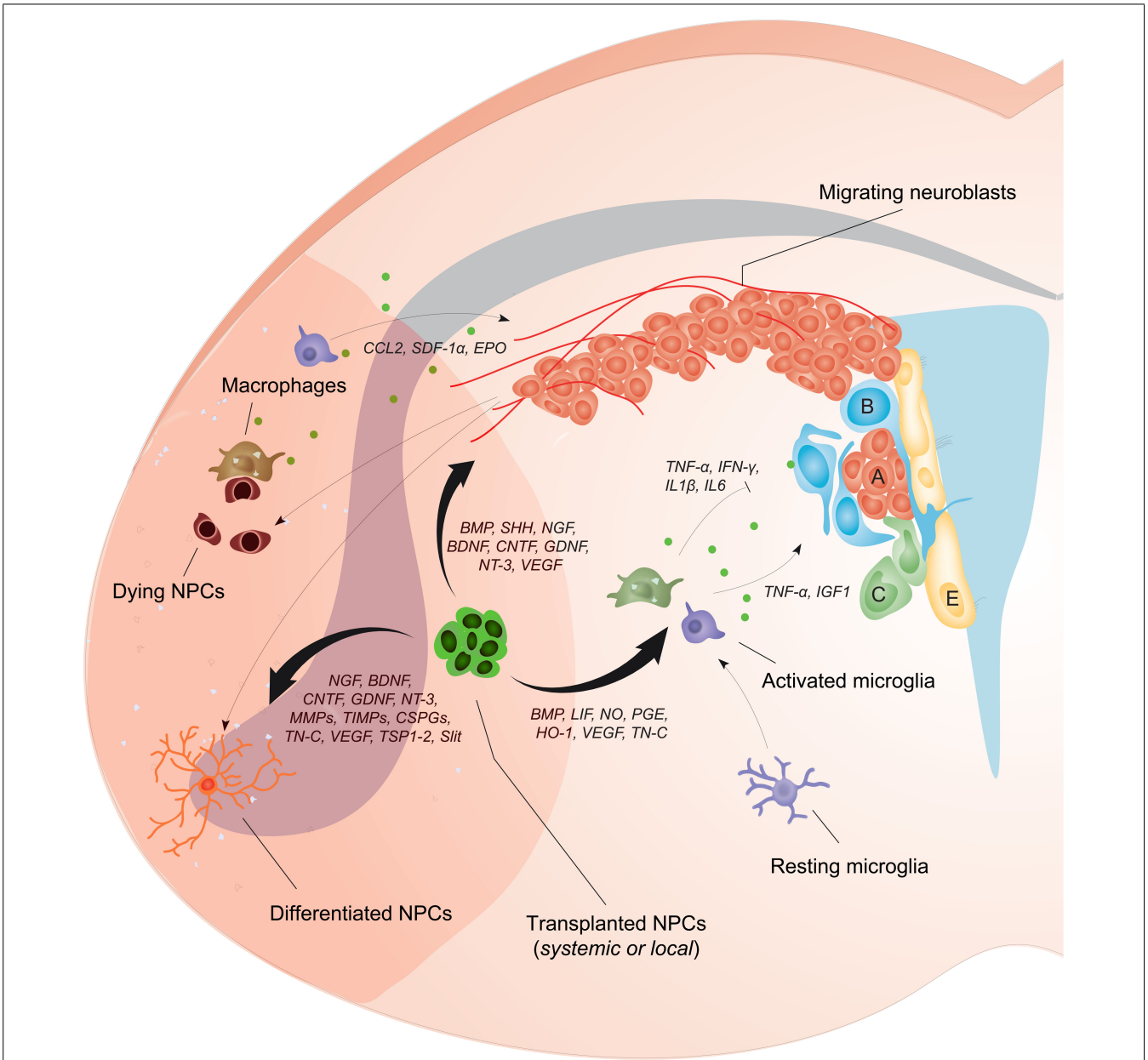

FIGURE 1 | Neural precursor cell (NPC) transplantation boosts the endogenous neurogenic response after stroke. NPC proliferation within the SVZ is augmented after stroke leading to the generation of newly formed neuroblasts that migrate along vessels toward gradients of chemokines that are produced locally by glial and inflammatory cells (e.g., CCL2, SDF1-α, and EPO). The bystander effects of transplanted NPCs depend on the release of several factors (e.g., BMP, SHH, NGF, BDNF, CNTF, GDNF, NT-3, and VEGF) that can directly increase cell proliferation within the SVZ, potentiate neuroblasts migration, and augment peri-ischemic angiogenesis. Transplanted NPCs can also positively affect the differentiation of endogenous neuroblasts and plasticity within the ischemic tissue (via the secretion of NGF, BDNF, CNTF, GDNF, NT-3, MMPs, TIMPs, CSPGs, TN-C, VEGF, TSP1-2, Slit), or directly differentiate into post-mitotic neurons, astrocytes, or oligodendrocytes. Most importantly, transplanted NPCs secrete a plethora of soluble molecules that modulate the activation of host microglia/macrophages (e.g., BMP, LIF, NO, PGE, HO-1, VEGF, TN-C), thus modifying the release of inflammatory mediators that inhibit (e.g., TNF-α, IFN-γ, IL1β, IL6) or increase (e.g., TNF-α, IGF1) endogenous neurogenesis. Green dots represent mediators secreted by inflammatory cells.

to 15 days post-stroke) and increased angiogenesis in peri-infarct regions (Zhang et al., 2011). Intraparenchymal cell delivery has also yielded possible evidence of therapeutic potential when administered in the subacute and chronic phases of the stroke. A study with human fetal NPCs transplanted 2 days after MCAO showed that the intraparenchymal injection of NPCs was able to promote endogenous neurogenesis in multiple ways (Mine et al., 2013). Transplanted cells were able to induce a prolonged increase in $Ki67^{+}$ proliferating cells within the SVZ, which was associated with an increase in the number of endogenous neuroblasts

Table 1 | Effects of exogenously delivered NPCs in experimental stroke models.

Study	Experimental paradigm	Observations
Andres et al. (2011a)	Intraarterial delivery of adult NPCs obtained from CCR2+/+ and CCR2-/- mice 24 h after transient (30 min) common carotid artery occlusion combined with 8% hypoxia in CCL2+/+ and CCL2-/- mice	Decreased homing of CCR2-/- NPCs compared with CCR2+/+ NPCs in CCL2+/+ mice. Decreased homing of CCR2+/+ NPCs in CCL2-/- compared with CCL2+/+ mice. Mice receiving CCR2+/+ NPCs showed significantly better neurological recovery than animals receiving CCR2-/- NPCs.
Andres et al. (2011b)	Intracerebral delivery of fetal human NPCs 7 days after permanent distal MCAO combined with transient (30 min) bilateral common carotid artery occlusion in rats	Increased dendritic plasticity in ipsi- and contralesional cortex after NPC delivery that coincided with functional neurological recovery. Increased corticocortical, corticostriatal, corticothalamic and corticospinal axonal sprouting from the contralesional hemisphere associated with transcallosal and corticospinal axonal sprouting. Reduced brain amyloid precursor protein accumulation.
Bacigaluppi et al. (2009)	Intravenous delivery of adult mouse NPCs 3 days after transient (45 min) proximal (intraluminal) MCAO in mice	Improved neurological recovery after NPC delivery. Small percentage of transplanted NPCs (< 1%) accumulated in the brain, integrating mainly in the infarct boundary zone, where most of the NPCs remained undifferentiated. Reduced secondary striatal and corpus callosum atrophy associated with downregulation of markers of inflammation, glial scar formation and neuronal apoptotic death.
Darsalia et al. (2011)	Intracerebral delivery of fetal human NPCs 48 h or 6 weeks after transient (30 min) proximal MCAO in rats	Better NPC survival after early than late NPC transplantation. Magnitude of NPC proliferation, migration, and neuronal differentiation was not influenced by transplantation time. Greater numbers of grafted NPCs did not result in greater numbers of surviving NPCs or increased neuronal differentiation.
Doeppner et al. (2012)	Intravenous or intracerebral delivery of adult mouse NPCs 6 h after transient (30 min) proximal (intraluminal) MCAO in mice	Intravenous and intracerebral NPC delivery similarly induced neurological recovery, but only intravenous NPC delivery yielded sustained neuroprotection that persisted in the post-acute stroke phase. Intracerebral NPF delivery associated with higher brain concentrations of BDNF, FGF, and VEGF. Intravenous, but not intracerebral NPC delivery stabilized blood–brain barrier, reduced activation of MMP9 and decreased formation of reactive oxygen species.
Hassani et al. (2012)	Intracerebral delivery of human conditionally immortalized neural stem cells CTX0E03 4 weeks after transient (60 min) proximal (intraluminal) MCAO in rats	Increased endogenous NPC proliferation in striatum of NPC treated rats. Significant proportion of proliferative cells expressed immature neuronal marker doublecortin. Increased proliferation of CD11b + microglial cells in NPC treated rats.
Jin et al. (2005)	Intravenous, intracerebral or intracerebroventricular delivery of embryonic mouse NPCs 24 h after permanent distal MCAO in rats	Brain entry of NPCs with accumulation in ischemic striatum and cortex observed using all three delivery strategies, intrastriatal transplants resulting in highest and intravenous transplants in lowest cell densities. Majority of cells expressing undifferentiated neuroepithelial (nestin) or neuronal (doublecortin) markers.
Mine et al. (2013)	Intracerebral delivery of fetal human NPCs 48 h after transient (60 min) proximal MCAO in T cell deficient rats	Subpopulation of NPCs exhibited differentiated neuronal phenotype at 6 and 14 weeks. Numbers of proliferating endogenous NPCs were elevated, and numbers of activated microglia/macrophages were reduced in ischemic striatum of NPC treated rats. Some grafted NPCs projected axons from striatum to globus pallidus. NPC treated rats showed improved neurological recovery.
Minnerup et al. (2011)	Intravenous delivery of embryonic mouse NPCs 24 h after photothrombotic stroke in immunosuppressed (cyclosporine A) rats	Improved neurological recovery associated with increased dendritic growth and branching, reduced endogenous neurogenesis and increased microglial activation in NPC treated rats.
Tang et al. (2014)	Intracerebral delivery of embryonic mouse NPCs 24 h after transient (120 min) proximal MCAO in young-adult (3 month-old) and aged (24 month-old) rats	Aged rats developed larger infarcts with worse neurological deficits than young-adult rats. Brain infarction and neurologic deficits were attenuated by NPC delivery in aged and young-adult rats. Number of surviving NPCs was similar in both age groups. Angiogenesis and neurogenesis were enhanced by NPCs in aged and young-adult rats.
Zhang et al. (2011)	Intracerebral delivery of fetal human NPCs 24 h after permanent distal MCAO in rats	Increased proliferation of endogenous NPCs in ipsilesional (ischemic) subventricular zone of rats receiving NPC grafts. Enhanced angiogenesis in peri-infarct cortex.

List of major studies using ischemic stroke models, in alphabetic order.

survival, migration and maturation in the striatum. This prolonged endogenous neurogenic response, which persisted up to 14 weeks, was accompanied by the long-term suppression of microglia/macrophage driven inflammatory responses (Mine et al., 2013). Similarly, when human conditionally immortalized NPCs (cell line CTX0E03) were transplanted in immunosuppressed rats 4 weeks after MCAO, the number of proliferating doublecortin $(DCX)^+$ neuroblasts considerably increased in the ischemic striatum (Hassani et al., 2012). Interestingly, the authors attributed this finding to an increase (rather than a decrease) of proliferating microglia in the striatum (Hassani et al., 2012).

Despite these data, which highlight the promising effects of intraparenchymal NPC delivery, comparative studies of different routes and times of transplantation (without the use of confounding immunosuppressive regimens) must be performed in order to determine the optimal spatiotemporal settings that would allow for the ideal stimulation of endogenous neurogenesis in stroke. Additionally, given that the neurogenic potential of adult NPCs declines with age (Conover and Shook, 2011), the effect of NPC transplantation in aged brains also warrants investigation. Interestingly, a recent study has shown that the local NPC transplantation (24 h post-ischemia) is capable of similar increases in neurogenesis and angiogenesis in the ischemic striatum of both young and aged mice (Tang et al., 2014). The aforesaid, coupled with recent data showing increased neurogenesis in the SVZ of both young and aged animals following the local administration of human ES-derived NPCs, suggests that the ischemic environment may effectively be modified in order to restore deficient neurogenic responses in the aged brain as well (Jin et al., 2011).

HOMING AND SURVIVAL OF TRANSPLANTED NPCs

In a comparative study evaluating intrastriatal, intracerebroventricular and intravenous NPC delivery (24 h after MCAO), the intrastriatal transplant of NPCs yielded the highest numbers of grafted cells within the ischemic brain (Jin et al., 2005). Intracerebroventricular transplantation into the lateral ventricle led to the survival of less cells, yet more cells were found when compared to intravenous NPC delivery (Jin et al., 2005). The reasons behind these observations are manifold in nature and may be linked to both the differential homing of grafted cells and their distinct survival profiles within the ischemic brain.

The mechanisms that regulate the homing of transplanted cells to the ischemic lesion are extremely similar to those that regulate the migration from within the endogenous NPC compartment (Jin et al., 2003; Zhang et al., 2009). Upon ischemia, EPO activates endothelial cells, which promote endogenous neuroblasts migration by secreting MMPs that degrade extracellular matrix (ECM) components (Wang et al., 2006). Migration of neuroblasts along the vessels is then modulated by the interaction of chemokine receptors on NPCs [e.g., C-X-C motif chemokine receptor (CXCR)-4 and C-C motif chemokine receptor (CCR2)] with molecules secreted by activated neuronal and glial cells within the ischemic lesion [e.g., stromal-derived factor (SDF)-1α and C-C chemokine ligand (CCL)-2, respectively; Robin et al., 2006; Yan et al., 2007].

When exogenous NPCs are administered intravenously, the crossing of the BBB involves a further degree of complexity. The CCL2/CCR2 interaction has been demonstrated to be critical for transendothelial recruitment of intraarterially delivered NPCs in response to ischemic injury (Andres et al., 2011a). Stem cells in circulation directly enter the injured brain through endothelial rolling and adhering on cadherins (VCAM-1) and integrins that become selectively up-regulated within the zone of ischemic damage (Mueller et al., 2006). This phenomenon, coupled with the disturbance of BBB integrity that occurs as a consequence of ischemia, is responsible for the *pathotropism* of transplanted NPCs for ischemic tissue that occurs after systemic delivery. Interestingly, intravenous transplantation of NPCs has the unique advantage of stabilizing the BBB, via mechanisms that involve a reduction of MMP9 expression and reactive oxygen species (ROS), as we recently showed after the transplantation of adult NPCs at 6 h after transient MCAO (Doeppner et al., 2012). Despite the fact that systemic NPC transplantation yields fewer cells in the ischemic brain parenchyma, current evidence suggests that intravenous injection is clearly able to promote neuronal/glial survival at delayed time-points (Bacigaluppi et al., 2009; Doeppner et al., 2012). As such, while intracerebrally transplanted adult mouse NPCs transiently improved motor coordination during the first 1–2 weeks after MCAO in mice, intravenous NPC transplantation resulted in persistent motor coordination improvements that persisted over at least 8 weeks post-stroke (Doeppner et al., 2012).

We have previously shown that upon the intravenous transplantation of adult mouse NPCs in the subacute stroke phase (72 h post-ischemia) only a small minority of transplanted cells (approximately 0.3% based on systematic counts) accumulate in the brain (Bacigaluppi et al., 2009). Notably, within the first 72 h post transplantation, intravenously injected NPCs were found both in the ischemic and contralesional non-ischemic hemisphere (Bacigaluppi et al., 2009). In the subsequent 7 days the NPCs in the contralesional hemisphere disappeared, whereas those in the ischemic hemisphere steadily increased in number in a narrow rim around the lesion border. Given that a significant proportion (25%) of transplanted NPCs in the mouse brain expressed the proliferation marker Ki67 at 3 days post-transplantation, this selective accumulation may be explained in part by the local proliferation of transplanted cells.

Beyond the proliferation of grafted NPCs, their emergence from peripheral organs (where they may have previously homed), such as the lungs, liver and spleen (Lappalainen et al., 2008), may also contribute to the delayed accumulation of systemically injected cells around the stroke lesion. While the localization of cells of a neural lineage within peripheral organs may carry health risks related to malignant transformation, the idea that NPCs have the capacity to exert their therapeutic efficacy via peripheral actions is intriguing. When fetal human NPCs were delivered intravenously in rats submitted to collagenase-induced intracerebral haemorrhage (ICH), these cells were identified primarily in secondary lymphoid organs where they induced a reduction in the levels of inflammatory mediators and activated macrophage numbers (Lee et al., 2008). This effect was found to be therapeutically relevant as splenectomy, performed before ICH, abolished

the effects of NPC transplantation on both brain oedema and inflammatory infiltrates (Lee et al., 2008). This finding, combined with observations that transplanted NPCs can hamper the activation of myeloid dendritic cells (DCs) and restrain the expansion of antigen-specific T cells in inflammatory CNS conditions (Pluchino et al., 2005, 2009), supports the putative ability of systemically delivered NPCs in modulating important aspects of the stroke-induced activation of innate and adaptive immune responses.

It is becoming clear that the therapeutic effect of intravenous cell delivery is independent of the amount of NPCs that is achieved within the brain, while local intracerebral NPC transplantation outcomes strictly depend on the amount of NPCs that reside within the lesion site. Consequently, intracerebral transplantation of NPCs is successful only if grafted NPCs within the ischemic brain survive in adequate quantity. The major determinant of the survival of locally transplanted cells within the ischemic brain is the timing of delivery. In a recent study it has been shown that while NPC proliferation, migration, and neuronal differentiation did not differ when cells were intrastriatally transplanted in the subacute (48 h) or chronic phase (6 weeks) after stroke, NPC survival was strikingly reduced following delayed cell delivery (Darsalia et al., 2011). The reasons underlying these findings are largely unknown. It is known that the majority (approximately 80%) of adult-born neurons arising from the endogenous neurogenic niche after stroke die before integrating in the ischemic tissue (Arvidsson et al., 2002), and that the inflammatory milieu plays a pivotal role in this phenomenon. As such, the treatment with anti-inflammatory agents (e.g., indomethacin or minocycline) that suppress microglial activation result in the preservation of these newly formed neurons (Hoehn et al., 2005; Liu et al., 2007). Similarly, the survival of intrastriatal grafts may be strictly dependent on the local inflammatory milieu and, as such, intraparenchymal transplantation should take place before microglial cells are fully activated.

FUNCTIONAL INTEGRATION OF TRANSPLANTED NPCs

The route of NPC administration has little effect on the phenotype of cells that accumulate inside the ischemic brain, as transplanted NPCs via local or systemic routes retain a profile of characteristics that is similar to those observed *in vitro* prior to transplantation (Jin et al., 2005). This may either be interpreted as evidence that NPCs are able to retain their character, despite the manipulations brought about by transplantation, or that the ischemic microenvironment arrests the capacity of the transplanted NPCs to differentiate into mature neurons or glia. It has indeed been shown that when neuroinflammation predominates, transplanted cells retain an undifferentiated phenotype as a result of the release of soluble mediators (e.g., noggin) by blood-borne inflammatory cells, activated endothelial cells, and astrocytes (Pluchino et al., 2005; Martino and Pluchino, 2006). It is therefore reasonable to speculate that when inflammatory signals begin to fade, transplanted cells are subsequently enabled to differentiate into post-mitotic CNS cells.

We have shown that at 3 and 10 days post transplantation the majority of intravenously transplanted NPCs exhibit an undifferentiated phenotype, lacking lineage-specific markers such as microtubule-associated protein (MAP)-2, DCX, glial fibrillary protein (GFAP), and the oligodendroglial transcription factor (Olig)-2 (Bacigaluppi et al., 2009). Interestingly at 30 days post transplantation, when inflammation was down regulated, the number of transplanted NPCs expressing Olig2 and DCX increased (albeit to only 4.4 and 0.8%, respectively, of the total), whereas the majority of the transplanted cells still exhibited an undifferentiated morphology in the brain tissue. Understanding the mechanisms which foster graft differentiation and reduce the quantity of NPCs restricted to an undifferentiated state (once their therapeutic bystander effects have been fully exploited) is of pivotal importance for future cell-replacement therapies in stroke.

Valuable insights into the abovementioned may eventually come from the observation of the spontaneous differentiation of endogenous neuroblasts after ischemic stroke (Kernie and Parent, 2010). It has been reported that the majority of neuroblasts after ischemia give rise to striatal medium spiny neurons (Parent et al., 2002). This response can be modulated by the addition of growth factors (such as angiopoietin or EGF), which increase the number of differentiated neurons and/or drive the fating of specific neuronal subtypes (i.e., parvalbumin-expressing interneurons; Teramoto et al., 2003; Liu et al., 2009). The potential of pushing NPCs via growth factors toward specific neuronal subtypes has been exploited by a recent study on induced human pluripotent stem (iPS) cells, which were fated before local transplantation to obtain functional cortical neurons *in vivo* (Tornero et al., 2013). Despite major methodological advances associated with this approach, definitive proof regarding the additional value of cell fating on behavioral recovery, when compared to non-fated cellular grafts, is still lacking (Pluchino and Peruzzotti-Jametti, 2013).

BYSTANDER EFFECTS OF TRANSPLANTED NPCs

Functional recovery of stroke-induced deficits has similarly been reported after intracerebral, intracerebroventricular, or intravenous NPC delivery (Bliss et al., 2007; Doeppner et al., 2012). As already noted, the common finding to all routes of cell delivery is the undifferentiated state in which the majority of transplanted cells are found within the brain parenchyma (Jin et al., 2005). As a matter of fact, the therapeutic potential of NPCs seems to be initially independent of cell differentiation and rather relies on the multiple bystander mechanisms exerted by adult NPCs, which serve to boost restorative responses in the brain and modulate the injured microenvironment (Martino et al., 2011).

We have shown that adult mouse NPCs reduced inflammatory responses in the ischemic brain, thereby preventing delayed neuronal degeneration and brain atrophy, even when transplanted intravenously as late as 3 days post MCAO (Bacigaluppi et al., 2009). Iba-1$^+$/MHC class II$^+$ microglial activation was reduced upon transplantation of adult NPCs, as was GFAP$^+$ astroglial scar formation in the infarct rim (Bacigaluppi et al., 2009). On the histochemical level, dopamine-2 receptor$^+$, and cAMP regulated phosphoprotein (DARPP)-32$^+$ medium spiny neurons were protected against delayed degeneration in the striatum, which is particularly sensitive to intraluminal MCAO

(Bacigaluppi et al., 2009). Diminished delayed neuronal degeneration was noticed not only inside the ischemic lesion but also at distance to it, as the corpus callosum (CC) of mice receiving transplants of adult mouse NPCs was significantly thicker than those of control mice at 30 days post transplantation (Bacigaluppi et al., 2009).

Further, transplanted NPCs were identified in close contact with von Willebrand factor$^+$ endothelial cells, CD45$^+$ leucocytes and F4/80$^+$ macrophages (Bacigaluppi et al., 2009). On the molecular level, pronounced down-regulation of messenger transcripts of inflammatory signals (*IFN-γ*, *TNF-α*, *IL-1β*), regulators of glial proliferation and reactivity (*bFGF*, *vimentin*) and neuronal death and plasticity (*caspase-3*, *growth associated protein-43*, *versican*) was observed in the brains of ischemic mice receiving intravenous transplantation of adult mouse NPCs while a single transcript was up-regulated by adult mouse NPCs, which was the spiny neuron marker *DARPP-32* (Bacigaluppi et al., 2009). These data clearly suggest that NPCs modulate their microenvironment via inhibition rather than activation of transcriptional processes.

Reduced delayed neuronal degeneration and CC atrophy were also noticed with the grafting of human fetal NPCs into the ipsilesional cortex of rats at 7 days following distal MCAO (Andres et al., 2011b). Dendritic branching, as evaluated by Golgi staining, was enhanced by human fetal NPC transplantation, as was contralesional corticospinal axonal sprouting (Andres et al., 2011b). Accumulation of amyloid precursor protein was reduced by human fetal NPC transplantation, pointing toward the restoration of axonal transport processes (Andres et al., 2011b).

Although both systemic and intracerebral transplantation improve neurological recovery, the restorative effects of each route of transplantation exhibit important differences with regard to the potential to influence injured tissue via bystander effects. The local intracerebral grafting of adult mouse NPCs in the brain parenchyma is associated with elevated brain concentrations of BDNF, FGF, and VEGF in the subacute stroke phase, i.e., at 4 days after MCAO in mice (Doeppner et al., 2012). Notably, such elevated growth factor levels could not be observed in the ischemic brain after systemic intravenous NPC delivery, but were still present for as late as 2 months after intracerebral transplantation of NPCs transduced with heat shock protein (Doeppner et al., 2012).

CONCLUSION

Considering the lack of therapeutic options that promote brain remodeling and neurological recovery after stroke, there is a clear need to reevaluate therapeutic strategies and treatment modalities within the stroke field. Accumulating evidence suggests that beyond the recanalization of blood vessels (by means of thrombolytic therapies), it will not be possible to promote neurological recovery post-ischemia via the modulation of single targets (Hermann and Chopp, 2012).

NPCs possess unique characteristics that differ drastically from conventional therapies (namely pharmacological small molecule compounds, recombinant growth factors and/or antibody-based therapeutics). Transplanted stem cells can promote tissue regeneration by sensing diverse signals in the brain microenvironment, migrating to specific sites of damage, integrating inputs and executing complex response behaviors all aimed at the remodeling/protection of injured ischemic tissue (Fischbach et al., 2013).

While the local delivery of NPCs is able to achieve high levels of protective mediators (e.g., growth factors) in the ischemic brain tissue, questions remain about the feasibility of such surgically invasive procedures in the clinical setting. One might therefore consider systemic NPC transplantation, which is minimally invasive. In view that systemically administered NPCs do possess potent anti-inflammatory effects, promote brain remodeling and induce functional neurological recovery in rodents, this option could be indeed of clinical value.

Future studies will have to clearly define the safety and efficacy of NPC transplantation after both systemic and local delivery. Such knowledge will increase our understanding of cellular therapies and in turn guide future translational strategies that are urgently needed to promote brain remodeling and repair in stroke patients.

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REFERENCES

Andres, R. H., Choi, R., Pendharkar, A. V., Gaeta, X., Wang, N., Nathan, J. K., et al. (2011a). The CCR2/CCL2 interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain. *Stroke* 42, 2923–2931. doi: 10.1161/STROKEAHA.110.606368

Andres, R. H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., et al. (2011b). Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain* 134, 1777–1789. doi: 10.1093/brain/awr094

Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoeppner, D. J., Ravin, R., Poser, S. W., et al. (2006). Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. *Nature* 442, 823–826. doi: 10.1038/nature04940

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Bacigaluppi, M., Pluchino, S., Peruzzotti-Jametti, L., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251. doi: 10.1093/brain/awp174

Ben-Hur, T., Ben-Menachem, O., Furer, V., Einstein, O., Mizrachi-Kol, R., and Grigoriadis, N. (2003). Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells. *Mol. Cell. Neurosci.* 24, 623–631. doi: 10.1016/S1044-7431(03)00218-5

Bliss, T., Guzman, R., Daadi, M., and Steinberg, G. K. (2007). Cell transplantation therapy for stroke. *Stroke* 38, 817–826. doi: 10.1161/01.STR.0000247888.25985.62

Cacci, E., Ajmone-Cat, M. A., Anelli, T., Biagioni, S., and Minghetti, L. (2008). *In vitro* neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. *Glia* 56, 412–425. doi: 10.1002/glia.20616

Chou, J., Harvey, B. K., Chang, C. F., Shen, H., Morales, M., and Wang, Y. (2006). Neuroregenerative effects of BMP7 after stroke in rats. *J. Neurol. Sci.* 240, 21–29. doi: 10.1016/j.jns.2005.08.015

Conover, J. C., and Shook, B. A. (2011). Aging of the subventricular zone neural stem cell niche. *Aging Dis.* 2, 149–163.

Cusimano, M., Biziato, D., Brambilla, E., Donega, M., Alfaro-Cervello, C., Snider, S., et al. (2012). Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. *Brain* 135, 447–460. doi: 10.1093/brain/awr339

Darsalia, V., Allison, S. J., Cusulin, C., Monni, E., Kuzdas, D., Kallur, T., et al. (2011). Cell number and timing of transplantation determine survival of human neural stem cell grafts in stroke-damaged rat brain. *J. Cereb. Blood Flow Metab.* 31, 235–242. doi: 10.1038/jcbfm.2010.81

Doeppner, T. R., Ewert, T. A., Tonges, L., Herz, J., Zechariah, A., ElAli, A., et al. (2012). Transduction of neural precursor cells with TAT-heat shock protein 70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. *Stem Cells* 30, 1297–1310. doi: 10.1002/stem.1098

Donnan, G. A., Fisher, M., Macleod, M., and Davis, S. M. (2008). Stroke. *Lancet* 371, 1612–1623. doi: 10.1016/S0140-6736(08)60694-7

Drago, D., Cossetti, C., Iraci, N., Gaude, E., Musco, G., Bachi, A., et al. (2013). The stem cell secretome and its role in brain repair. *Biochimie* 95, 2271–2285. doi: 10.1016/j.biochi.2013.06.020

Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2009). Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* 158, 1021–1029. doi: 10.1016/j.neuroscience.2008.06.052

Fischbach, M. A., Bluestone, J. A., and Lim, W. A. (2013). Cell-based therapeutics: the next pillar of medicine. *Sci. Transl. Med.* 5, 1797. doi: 10.1126/scitranslmed.3005568

Hao, L., Zou, Z., Tian, H., Zhang, Y., Zhou, H., and Liu, L. (2014). Stem cell-based therapies for ischemic stroke. *Biomed Res. Int.* 2014, 468748. doi: 10.1155/2014/468748

Hassani, Z., O'Reilly, J., Pearse, Y., Stroemer, P., Tang, E., Sinden, J., et al. (2012). Human neural progenitor cell engraftment increases neurogenesis and microglial recruitment in the brain of rats with stroke. *PLoS ONE* 7:e50444. doi: 10.1371/journal.pone.0050444

Heldmann, U., Thored, P., Claasen, J. H., Arvidsson, A., Kokaia, Z., and Lindvall, O. (2005). TNF-alpha antibody infusion impairs survival of stroke-generated neuroblasts in adult rat brain. *Exp. Neurol.* 196, 204–208. doi: 10.1016/j.expneurol.2005.07.024

Hermann, D. M., and Chopp, M. (2012). Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurol.* 11, 369–380. doi: 10.1016/S1474-4422(12)70039-X

Hoehn, B. D., Palmer, T. D., and Steinberg, G. K. (2005). Neurogenesis in rats after focal cerebral ischemia is enhanced by indomethacin. *Stroke* 36, 2718–2724. doi: 10.1161/01.STR.0000190020.30282.cc

Jin, K., Sun, Y., Xie, L., Mao, X. O., Childs, J., Peel, A., et al. (2005). Comparison of ischemia-directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat. *Neurobiol. Dis.* 18, 366–374. doi: 10.1016/j.nbd.2004.10.010

Jin, K., Sun, Y., Xie, L., Peel, A., Mao, X. O., Batteur, S., et al. (2003). Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. *Mol. Cell. Neurosci.* 24, 171–189. doi: 10.1016/S1044-7431(03)00159-3

Jin, K., Xie, L., Mao, X., Greenberg, M. B., Moore, A., Peng, B., et al. (2011). Effect of human neural precursor cell transplantation on endogenous neurogenesis after focal cerebral ischemia in the rat. *Brain Res.* 1374, 56–62. doi: 10.1016/j.brainres.2010.12.037

Kang, S. S., Keasey, M. P., Arnold, S. A., Reid, R., Geralds, J., and Hagg, T. (2012). Endogenous CNTF mediates stroke-induced adult CNS neurogenesis in mice. *Neurobiol. Dis.* 49C, 68–78. doi: 10.1016/j.nbd.2012.08.020

Kernie, S. G., and Parent, J. M. (2010). Forebrain neurogenesis after focal Ischemic and traumatic brain injury. *Neurobiol. Dis.* 37, 267–274. doi: 10.1016/j.nbd.2009.11.002

Kitagawa, H., Sasaki, C., Zhang, W. R., Sakai, K., Shiro, Y., Warita, H., et al. (1999). Induction of glial cell line-derived neurotrophic factor receptor proteins in cerebral cortex and striatum after permanent middle cerebral artery occlusion in rats. *Brain Res.* 834, 190–195. doi: 10.1016/S0006-8993(99)01563-2

Kokaia, Z., Zhao, Q., Kokaia, M., Elmer, E., Metsis, M., Smith, M. L., et al. (1995). Regulation of brain-derived neurotrophic factor gene expression after transient middle cerebral artery occlusion with and without brain damage. *Exp. Neurol.* 136, 73–88. doi: 10.1006/exnr.1995.1085

Lappalainen, R. S., Narkilahti, S., Huhtala, T., Liimatainen, T., Suuronen, T., Narvanen, A., et al. (2008). The SPECT imaging shows the accumulation of neural progenitor cells into internal organs after systemic administration in middle cerebral artery occlusion rats. *Neurosci. Lett.* 440, 246–250. doi: 10.1016/j.neulet.2008.05.090

Lee, S. T., Chu, K., Jung, K. H., Kim, S. J., Kim, D. H., Kang, K. M., et al. (2008). Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. *Brain* 131, 616–629. doi: 10.1093/brain/awm306

Leker, R. R., Toth, Z. E., Shahar, T., Cassiani-Ingoni, R., Szalayova, I., Key, S., et al. (2009). Transforming growth factor alpha induces angiogenesis and neurogenesis following stroke. *Neuroscience* 163, 233–243. doi: 10.1016/j.neuroscience.2009.05.050

Liu, X. S., Chopp, M., Zhang, R. L., Hozeska-Solgot, A., Gregg, S. C., Buller, B., et al. (2009). Angiopoietin 2 mediates the differentiation and migration of neural progenitor cells in the subventricular zone after stroke. *J. Biol. Chem.* 284, 22680–22689. doi: 10.1074/jbc.M109.006551

Liu, Z., Fan, Y., Won, S. J., Neumann, M., Hu, D., Zhou, L., et al. (2007). Chronic treatment with minocycline preserves adult new neurons and reduces functional impairment after focal cerebral ischemia. *Stroke* 38, 146–152. doi: 10.1161/01.STR.0000251791.64910.cd

Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T. B., Flegal, K., et al. (2009). Heart disease and stroke statistics–2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 119, e21–e181. doi: 10.1161/CIRCULATIONAHA.108.191261

Martino, G., Bacigaluppi, M., and Peruzzotti-Jametti, L. (2011). Therapeutic stem cell plasticity orchestrates tissue plasticity. *Brain* 134, 1585–1587. doi: 10.1093/brain/awr115

Martino, G., and Pluchino, S. (2006). The therapeutic potential of neural stem cells. *Nat. Rev.* 7, 395–406. doi: 10.1038/nrn1908

Mine, Y., Tatarishvili, J., Oki, K., Monni, E., Kokaia, Z., and Lindvall, O. (2013). Grafted human neural stem cells enhance several steps of endogenous neurogenesis and improve behavioral recovery after middle cerebral artery occlusion in rats. *Neurobiol. Dis.* 52, 191–203. doi: 10.1016/j.nbd.2012.12.006

Minnerup, J., Kim, J. B., Schmidt, A., Diederich, K., Bauer, H., Schilling, M., et al. (2011). Effects of neural progenitor cells on sensorimotor recovery and endogenous repair mechanisms after photothrombotic stroke. *Stroke* 42, 1757–1763. doi: 10.1161/STROKEAHA.110.599282

Mirzadeh, Z., Merkle, F. T., Soriano-Navarro, M., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (2008). Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell* 3, 265–278. doi: 10.1016/j.stem.2008.07.004

Mueller, F. J., Serobyan, N., Schraufstatter, I. U., DiScipio, R., Wakeman, D., Loring, J. F., et al. (2006). Adhesive interactions between human neural stem cells and inflamed human vascular endothelium are mediated by integrins. *Stem Cells* 24, 2367–2372. doi: 10.1634/stemcells.2005-0568

Parent, J. M., Vexler, Z. S., Gong, C., Derugin, N., and Ferriero, D. M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann. Neurol.* 52, 802–813. doi: 10.1002/ana.10393

Peruzzotti-Jametti, L., Donega, M., Giusto, E., Mallucci, G., Marchetti, B., and Pluchino, S. (2014). The role of the immune system in central nervous system plasticity after acute injury. *Neuroscience* doi: 10.1016/j.neuroscience.2014.04.036 [Epub ahead of print].

Planas, A. M., Justicia, C., Soriano, M. A., and Ferrer, I. (1998). Epidermal growth factor receptor in proliferating reactive glia following transient focal ischemia in the rat brain. *Glia* 23, 120–129. doi: 10.1002/(SICI)1098-1136(199806)23:2<120::AID-GLIA3>3.0.CO;2-A

Plane, J. M., Whitney, J. T., Schallert, T., and Parent, J. M. (2008). Retinoic acid and environmental enrichment alter subventricular zone and striatal neurogenesis after stroke. *Exp. Neurol.* 214, 125–134. doi: 10.1016/j.expneurol.2008.08.006

Pluchino, S., and Cossetti, C. (2013). How stem cells speak with host immune cells in inflammatory brain diseases. *Glia* 61, 1379–1401. doi: 10.1002/glia.22500

Pluchino, S., and Peruzzotti-Jametti, L. (2013). Rewiring the ischaemic brain with human-induced pluripotent stem cell-derived cortical neurons. *Brain* 136, 3525–3527. doi: 10.1093/brain/awt330

Pluchino, S., Zanotti, L., Brambilla, E., Rovere-Querini, P., Capobianco, A., Alfaro-Cervello, C., et al. (2009). Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function. *PLoS ONE* 4:e5959. doi: 10.1371/journal.pone.0005959

Pluchino, S., Zanotti, L., Rossi, B., Brambilla, E., Ottoboni, L., Salani, G., et al. (2005). Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436, 266–271. doi: 10.1038/nature03889

Robin, A. M., Zhang, Z. G., Wang, L., Zhang, R. L., Katakowski, M., Zhang, L., et al. (2006). Stromal cell-derived factor 1alpha mediates neural progenitor cell motility after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 26, 125–134. doi: 10.1038/sj.jcbfm.9600172

Schneider, A., Kruger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., et al. (2005). The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J. Clin. Invest.* 115, 2083–2098. doi: 10.1172/JCI23559

Sims, J. R., Lee, S. W., Topalkara, K., Qiu, J., Xu, J., Zhou, Z., et al. (2009). Sonic hedgehog regulates ischemia/hypoxia-induced neural progenitor proliferation. *Stroke* 40, 3618–3626. doi: 10.1161/STROKEAHA.109.561951

Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X. O., Logvinova, A., et al. (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J. Clin. Invest.* 111, 1843–1851. doi: 10.1172/JCI200317977

Tang, Y., Wang, J., Lin, X., Wang, L., Shao, B., Jin, K., et al. (2014). Neural stem cell protects aged rat brain from ischemia-reperfusion injury through neurogenesis and angiogenesis. *J. Cereb. Blood Flow Metab.* 34, 1138–1147. doi: 10.1038/jcbfm.2014.61

Teramoto, T., Qiu, J., Plumier, J. C., and Moskowitz, M. A. (2003). EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. *J. Clin. Invest.* 111, 1125–1132. doi: 10.1172/JCI200317170

Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., et al. (2006). Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* 24, 739–747. doi: 10.1634/stemcells.2005-0281

Thored, P., Heldmann, U., Gomes-Leal, W., Gisler, R., Darsalia, V., Taneera, J., et al. (2009). Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. *Glia* 57, 835–849. doi: 10.1002/glia.20810

Thored, P., Wood, J., Arvidsson, A., Cammenga, J., Kokaia, Z., and Lindvall, O. (2007). Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke* 38, 3032–3039. doi: 10.1161/STROKEAHA.107.488445

Tornero, D., Wattananit, S., Gronning Madsen, M., Koch, P., Wood, J., Tatarishvili, J., et al. (2013). Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain* 136, 3561–3577. doi: 10.1093/brain/awt278

Tureyen, K., Vemuganti, R., Bowen, K. K., Sailor, K. A., and Dempsey, R. J. (2005). EGF and FGF-2 infusion increases post-ischemic neural progenitor cell proliferation in the adult rat brain. *Neurosurgery* 57, 1254–1263. doi: 10.1227/01.NEU.0000186040.96929.8A

Wang, L., Zhang, Z. G., Zhang, R. L., Gregg, S. R., Hozeska-Solgot, A., LeTourneau, Y., et al. (2006). Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration. *J. Neurosci.* 26, 5996–6003. doi: 10.1523/JNEUROSCI.5380-05.2006

Wang, X., Mao, X., Xie, L., Greenberg, D. A., and Jin, K. (2009). Involvement of Notch1 signaling in neurogenesis in the subventricular zone of normal and ischemic rat brain *in vivo*. *J. Cereb. Blood Flow Metab.* 29, 1644–1654. doi: 10.1038/jcbfm.2009.83

Yan, Y. P., Sailor, K. A., Lang, B. T., Park, S. W., Vemuganti, R., and Dempsey, R. J. (2007). Monocyte chemoattractant protein-1 plays a critical role in neuroblast migration after focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 27, 1213–1224. doi: 10.1038/sj.jcbfm.9600432

Zhang, P., Li, J., Liu, Y., Chen, X., Lu, H., Kang, Q., et al. (2011). Human embryonic neural stem cell transplantation increases subventricular zone cell proliferation and promotes peri-infarct angiogenesis after focal cerebral ischemia. *Neuropathology* 31, 384–391. doi: 10.1111/j.1440-1789.2010.01182.x

Zhang, R. L., Chopp, M., Gregg, S. R., Toh, Y., Roberts, C., Letourneau, Y., et al. (2009). Patterns and dynamics of subventricular zone neuroblast migration in the ischemic striatum of the adult mouse. *J. Cereb. Blood Flow Metab.* 29, 1240–1250. doi: 10.1038/jcbfm.2009.55

Zhang, R. L., Zhang, Z. G., Wang, Y., LeTourneau, Y., Liu, X. S., Zhang, X., et al. (2007). Stroke induces ependymal cell transformation into radial glia in the subventricular zone of the adult rodent brain. *J. Cereb. Blood Flow Metab.* 27, 1201–1212. doi: 10.1038/sj.jcbfm.9600430

Zhang, R., Zhang, Z., Zhang, C., Zhang, L., Robin, A., Wang, Y., et al. (2004). Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. *J. Neurosci.* 24, 5810–5815. doi: 10.1523/JNEUROSCI.1109-04.2004

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Effects of neural progenitor cells on post-stroke neurological impairment—a detailed and comprehensive analysis of behavioral tests

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Systemic transplantation of neural progenitor cells (NPCs) in rodents reduces functional impairment after cerebral ischemia. In light of upcoming stroke trials regarding safety and feasibility of NPC transplantation, experimental studies have to successfully analyze the extent of NPC-induced neurorestoration on the functional level. However, appropriate behavioral tests for analysis of post-stroke motor coordination deficits and cognitive impairment after NPC grafting are not fully established. We therefore exposed male C57BL6 mice to either 45 min (mild) or 90 min (severe) of cerebral ischemia, using the thread occlusion model followed by intravenous injection of PBS or NPCs 6 h post-stroke with an observation period of three months. Post-stroke motor coordination was assessed by means of the rota rod, tight rope, corner turn, inclined plane, grip strength, foot fault, adhesive removal, pole test and balance beam test, whereas cognitive impairment was analyzed using the water maze, the open field and the passive avoidance test. Significant motor coordination differences after both mild and severe cerebral ischemia in favor of NPC-treated mice were observed for each motor coordination test except for the inclined plane and the grip strength test, which only showed significant differences after severe cerebral ischemia. Cognitive impairment after mild cerebral ischemia was successfully assessed using the water maze test, the open field and the passive avoidance test. On the contrary, the water maze test was not suitable in the severe cerebral ischemia paradigm, as it too much depends on motor coordination capabilities of test mice. In terms of both reliability and cost-effectiveness considerations, we thus recommend the corner turn, foot fault, balance beam, and open field test, which do not depend on durations of cerebral ischemia.

Keywords: cerebral ischemia, behavioral tests, neural progenitor cells, stroke, functional outcome, stem cells, transplantation

INTRODUCTION

Stem cells or progenitor cells from various tissue sources reduce both brain injury and functional impairment after cerebral ischemia (Bliss et al., 2007, 2010; Bacigaluppi et al., 2008, 2009; Schwarting et al., 2008; Doeppner et al., 2010; Zheng et al., 2010; Leong et al., 2012). Despite recent progress using induced neural stem cells in a non-ischemic mouse model (Hemmer et al., 2014), grafted cells are generally not thought to be integrated into the residing post-ischemic neural network. Rather, the aforementioned beneficial effects due to cell transplantation are attributed to indirect effects independent of genuine cell replacement. With the therapeutic use of both embryonic stem cells and multipotent stem cells restricted due to ethical concerns and tumor formation (Blum and Benvenisty, 2008, 2009), application of adult stem/progenitor cells like neural progenitor cells (NPCs) has become a feasible therapeutic approach in experimental stroke research (Bonnamain et al., 2012).

Since endogenous neurogenesis persists within distinct regions of the adult rodent brain such as the subventricular zone of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), NPCs can be harvested from these areas and transferred to cell culture (Reynolds and Weiss, 1992). Upon *in vitro* expansion, cultivated NPCs are used for both systemic and local cell transplantation studies in various stroke models. Transplantation of NPCs using either systemic or local cell delivery routes reduces post-ischemic injury (Mochizuki et al., 2008; Bacigaluppi et al., 2009; Hicks et al., 2009; Doeppner et al., 2010, 2012a). With regard to clinical relevance, however, intravenous transplantation of NPCs is more feasible than stereotactic intracranial cell delivery.

Although original work as well as meta-analyses regarding assessment of functional outcome in both ischemic and non-ischemic rodents do exist (Gerlai et al., 2000; Li et al., 2004; Bouët et al., 2007; Brooks and Dunnett, 2009; Schaar et al., 2010; Balkaya et al., 2012), original work that systematically studies

post-stroke functional outcome after transplantation of NPCs or other stem cells on a wide variety of behavioral tests does not exist. As a matter of fact, the majority of the aforementioned experimental studies evaluating post-stroke functional outcome after cell transplantation are limited to the application of only a few behavioral tests. However, in view of clinical stroke trials applying NPC transplantation already on the way (NCT02117635 and NCT01151124) and in respect of further trials in the future, reliable and valid experimental data regarding the feasibility of NPCs in stroke treatment is vital in order to avoid negative clinical trials. Therefore, we systematically analyzed both motor coordination deficits and cognitive impairment after intravenous transplantation of NPCs for as long as three months post-stroke using a total of twelve behavioral tests. Since correlation between infarct volumes and functional outcome is certain for at least some behavioral tests such as the cylinder test and the grid walk test (Rogers et al., 1997), we used two experimental paradigms by exposing animals to either mild (45 min) or severe (90 min) cerebral ischemia followed by intravenous transplantation of NPCs 6 h post-stroke. By excluding impacts due to different laboratories and experimental modalities, our study might give some advice for studying post-stroke functional outcome after cell transplantation in rodents and thus set the path for future clinical trials.

MATERIALS AND METHODS

EXPERIMENTAL PARADIGM AND ANIMALS USED

All experimental procedures were performed according to the guidelines for the care and use of laboratory animals and were approved by local authorities, i.e., LANUV North-Rhine-Westphalia and Government of Lower Saxony. Both experiments and analyses of data were blinded to experimenters. For all experiments male C57BL6 (10–12 weeks old, 23–25 g; Charles River, Germany) mice were used that had free access to food and water. Mice were exposed to either 45 min or 90 min of cerebral ischemia as mentioned below. The animals were allowed to survive for 2 days (analysis of brain injury) or for 84 days (behavioral days and assessment of brain injury) post-stroke. Three experimental groups were defined for each condition, i.e., time point (2 days vs. 84 days) and duration of cerebral ischemia (45 min vs. 90 min). These groups consisted of mice treated with intravenous injections of NPCs or PBS at 6 h post-stroke and of sham mice. The latter underwent middle cerebral artery "occlusion" (MCAO) without actually inserting a filament, but were intravenously injected with PBS at 6 h post-stroke. The number of animals used for the 2-day survival was six for each condition with a survival rate of 100%. Since mice used for infarct volume analysis could not be used for immunohistochemistry at the same time, the total number of animals used for this time point was 72. The number of mice used for statistical analysis for the 84-day time point was as follows. For the mild cerebral ischemia paradigm, 15 sham animals (survival rate 100%), 14 NPC-treated mice (survival rate 93.3%) and 16 controls (survival rate 88.8%) were used for statistical analysis. For the severe cerebral ischemia paradigm, 15 sham animals (survival rate 100%), 16 NPC-treated mice (survival rate 84.2%), and 17 controls (survival rate 80.9%) were used. Noteworthy, some mice suffering from severe cerebral ischemia could not perform all behavioral tests due to the severity of the disease. When applicable, this is explicitly mentioned in the results part and the corresponding figure legend as well. All mice that were allowed to survive for 84 days post-stroke were used for immunohistochemistry (neuronal density) and behavioral analysis. For the latter purpose, mice were trained for each behavioral test on days 3–1 before induction of cerebral ischemia. An overview of the experimental paradigm is given in **Figure 1**.

INDUCTION OF CEREBRAL ISCHEMIA

Transient focal cerebral ischemia was performed as described before (Doeppner et al., 2012b). Stroke was induced using the MCAO model. Briefly, mice underwent anesthesia by means of isoflurane (1–1.5%, 30% O_2, remainder N_2) under constant control of body temperature using a rectal thermometer with a feedback control system. Moreover, cerebral blood flow was constantly controlled using a Laser Doppler probe (Perimed,

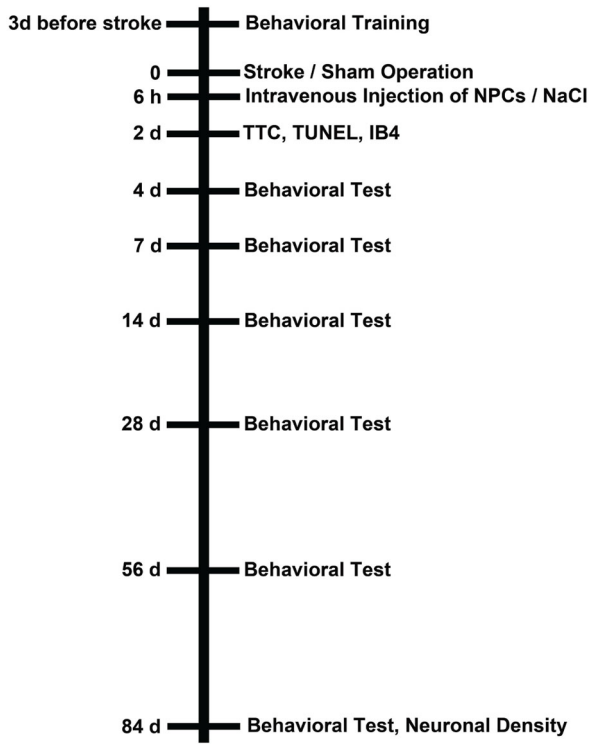

FIGURE 1 | Experimental paradigm. Male C57BL6 mice were exposed to either 45 min or 90 min of cerebral ischemia followed by survival periods of either 2 days or 84 days. Sham animals underwent the same operational technique, but without actually inserting a filament for occlusion of the left middle cerebral artery. All mice received an intravenous injection of PBS or (NPCs; 10^6 cells in 100 μl of PBS) via cannulation of the femoral vein 6 h after stroke (or sham) operation. Those animals that were sacrificed on day 2 after stroke/sham operation were used for assessment of infarct volumes and determination of $TUNEL^+$ cells as well as microglial activity using IB4 staining. Mice that were allowed to survive for up to 84 days were used for behavioral tests at the time points given as well as for analysis of neuronal density on day 84. All mice (including sham animals) were trained for behavioral tests on days 1–3 before induction of stroke or sham operation.

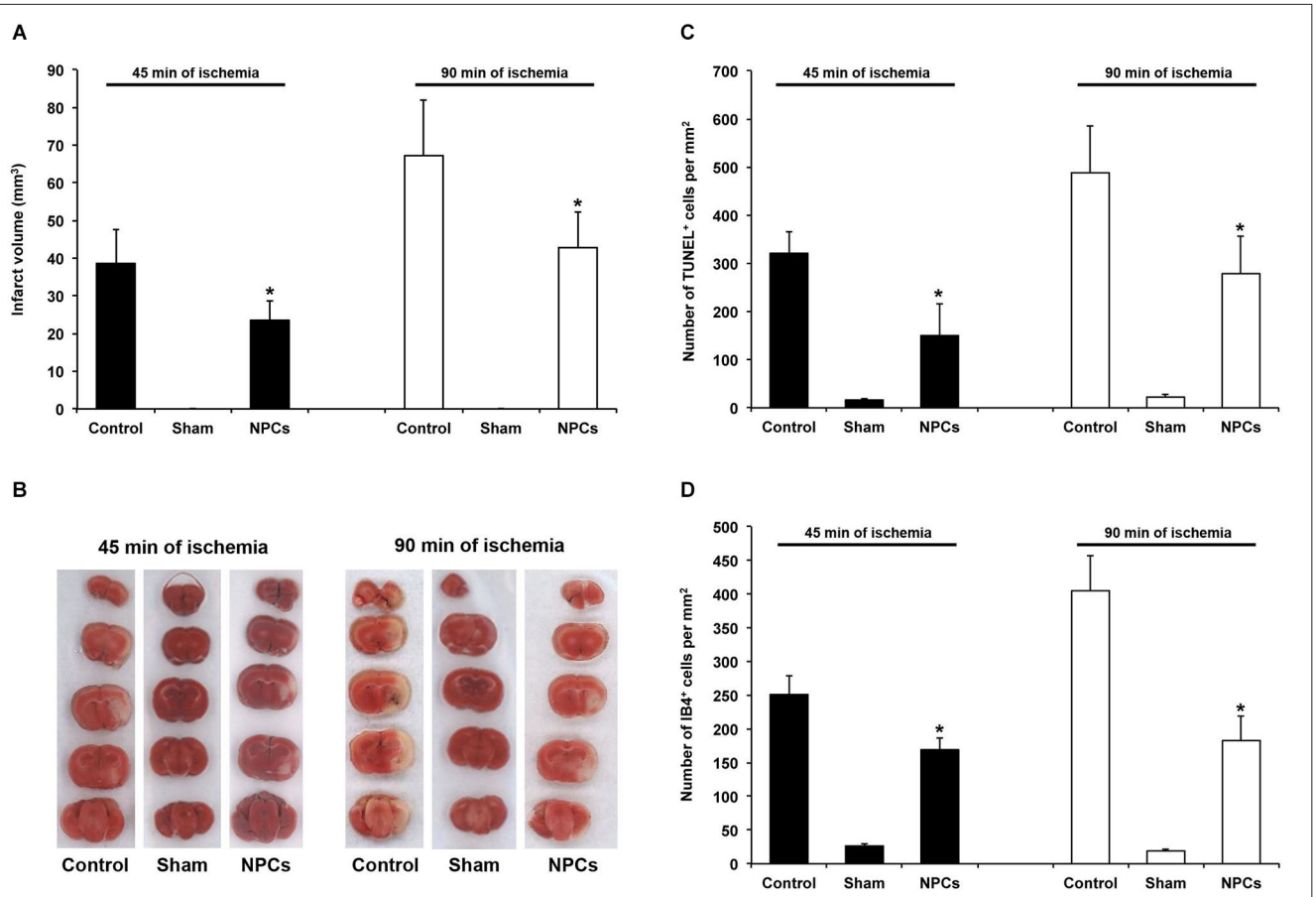

FIGURE 2 | NPCs induce acute neuroprotection after both mild and severe transient focal cerebral ischemia. PBS or neural progenitor cells (NPCs) were given 6 h after stroke induction. Sham mice underwent stroke operation without inserting the filament into the left middle cerebral artery. In order to avoid effects of anesthesia and allow proper comparison to NPC-treated animals, sham mice were kept for either 45 min or 90 min under anesthesia corresponding to stroke periods of NPC-treated mice or controls. All sham animals received additional anesthesia 6 h after "stroke" operation with cannulation of the right femoral vein and injection of PBS. **(A)** Infarct analysis on day 2 after induction of cerebral ischemia for either 45 min or 90 min using TTC staining. **(B)** Representative TTC stainings for each experimental group. **(C)** Analysis of TUNEL$^+$ cells on day 2 for each experimental group. **(D)** Quantitative analysis of IB4$^+$ cells within the ischemic striatum on day 2 post-stroke, using the same experimental paradigm as mentioned above. Number of animals used was $n = 6$ for each experimental paradigm. * Significantly different from control, $p < 0.05$.

Sweden) that was attached to the skull. MCAO itself was achieved using a 7–0 silicon rubber coated monofilament (coating length 4–5 mm, tip diameter 180 mm; Doccol, USA) that was inserted for either 45 min or 90 min in order to induce stroke. Sham operated mice received the same operation except for inserting a filament, but were kept under anesthesia for 45 min or 90 min to match corresponding periods of cerebral ischemia.

CULTIVATION AND TRANSPLANTATION OF NEURAL PROGENITOR CELLS (NPCS)

Preparation of SVZ-derived NPCs was performed as previously described (Doeppner et al., 2012a). Briefly, NPCs were isolated from the SVZ of 6–8 week old male transgenic green fluorescence protein positive animals (C57BL/6-Tg ACTB-enhanced green fluorescence protein (EGFP), 1Osb/J; JAX Laboratory, Bar Harbor, USA). EGFP expression was under control of the actin promoter, thus providing reliable and stable tracking of grafted NPCs. The prepared EGFP$^+$ NPCs were cultured in serum-free basic Dulbecco's modified Eagle's medium (DMEM)/F12 (PAA, Linz, Austria) supplemented with epidermal growth factor (EGF, 2 μg/ml), basic fibroblast growth factor (bFGF, 2 μg/ml), and penicillin-streptomycin (Invitrogen, Frankfurt, Germany). Cells were incubated with 5% CO_2 at 37°C, and growth factors were added every 2–3 days. Passaging of cells was done every 7–10 days and NPCs used for transplantation were from cell passages 3–6.

The NPCs were intravenously transplanted 6 h after stroke, a time point that has previously been shown by us to be associated with post-ischemic neuroprotection after mild cerebral ischemia (Doeppner et al., 2012a). The right femoral vein was cannulated under deep anesthesia, and 10^6 NPCs (solved in 100 μl PBS) were injected over 10 min. Controls and sham operated mice received 100 μl of PBS over 10 min.

ASSESSMENT OF BRAIN INJURY

Brain injury on day 2 post-stroke was assessed via determination of infarct volumes as well as via analysis of terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and microglial activity. Infarct volume analysis was done using a staining with 2,3,5-triphenyltetrazolium chloride (TTC; 2%). Brains were removed and cut into slices of 2 mm each followed by TTC staining and a computer-based analysis of infarct volumes using the freely available software ImageJ.

Immunohistochemical analyses were performed on days 2 and 84. Mice received i.p. injections with chloralhydrate (420 mg/kg body weight) at the time points given and were transcardially perfused with 4% paraformaldehyde. Thereafter, the brains were removed, shock-frozen in liquid nitrogen, and 16-μm thick coronal cryostat sections were prepared. For quantitative analyses, a region of interest (ROIs) within the ischemic basal ganglia was defined in which three microscopic fields were set. The size of each microscopic field was 0.25 mm^2. Quantitative analysis was performed using a 20 × magnification. The stereotactic coordinates for the definition of the ROI was 0.14 mm anterior, 2.5–3.25 mm ventral and 1.5–2.25 mm lateral from bregma. A total of three sections per condition were analyzed in the aforementioned manner.

For TUNEL staining on day 2, sections were incubated with proteinase K (7 min at 37°C) followed by exposure to the TdT enzyme reaction according to the manufacturer's manual (Roche, Switzerland). Thereafter, sections were stained with a streptavidin-Alexa-594-conjugated secondary antibody (2 h at room temperature; Invitrogen, Germany) and analyzed. Microglial activity within the aforementioned ROIs was done on day 2 using a rat biotin-conjugated anti-IB4 antibody (1:100; Vector, UK). After 18 h of incubation at 4°C and several washing steps, a Streptavidin-Alexa 488 antibody (1:500; Molecular Probes, Germany) was used as secondary antibody. Long-term post-ischemic brain injury was performed by means of analysis of neuronal density. Therefore, NeuN staining was done on day 84 post-stroke. NeuN staining was performed using a mouse monoclonal anti-NeuN antibody (1:200; Chemicon, UK; 18 h, 4°C) and a mouse anti-mouse Alexa 488 (1:400; Molecular Probes) as secondary antibody. Quantitative analysis was achieved using the above mentioned ROI.

ADHESIVE REMOVAL TEST

The test analyzes both forepaw sensitivity including presence of neglect as well as motor impairments of the forepaw. This test was performed as previously described (Bouët et al., 2007). Briefly, mice were placed in a transparent box (150 cm^2) for 1 min in order to allow habituation. Thereafter, the mouse was taken out of the box and one adhesive tape (0.3 × 0.4 cm) was attached on each mouse paw. The order of attaching the tape (right or left) was changed between different trials and different animals alike. Thereafter, the mouse was put into the box and the time to either contact and to remove the tape was analyzed for a total of 120 s. Data for analysis of the contralateral (i.e., impaired) forepaw are shown in **Figures 3**, **5**, whereas data for the ipsilateral (i.e., non-impaired) forepaw are shown in supplementary Figure S1.

BALANCE BEAM TEST

This test was performed as previously described with slight modification (Luong et al., 2011). For this test, mice were put on a 110 cm long beam with constantly reduced width, i.e., 12 mm width at the beginning and 5 mm width at the end of the beam. The wooden beam was paralleled and 60 cm elevated from the ground on which cushions were placed in order to avoid injury of the animals. A platform was located at the end of the beam and the time measured until the mouse reached that platform. Maximal testing time was 60 s. Animals that failed to reach the platform were scored 60 s. Each animal was tested twice per time point and means were calculated.

CORNER TURN TEST

Two vertical boards were attached to each other forming an angle of 30°. The mouse was put into the apparatus and tested for the side chosen to leave the corner once it made contact to the boards with its whiskers. The amount of trials performed per day was 10. Whereas healthy animals leave the corner without side preference, mice suffering from stroke preferentially leave the corner towards the non-impaired (i.e., left) body side. The laterality index was calculated as follows: (number of left turns – number of right turns)/10.

FOOT FAULT TEST

The test was performed according to Rogers et al. with slight modifications (Rogers et al., 1997). Briefly, mice were placed on an elevated steel grid. Thereafter, the total number of steps for each forelimb was counted (i.e., placement of forelimbs on the grid). In the process of moving forward, the foot fault errors (i.e., when the animal's forelimb was misplaced and thus fell through the grid) were recorded. Data is given as percentage of foot fault errors for the right impaired forelimb referring to the total amount of right forelimb steps.

GRIP STRENGTH TEST

The grip strength was performed as previously described (Kilic et al., 2010; Reitmeir et al., 2011). Briefly, the test includes a spring balance coupled with a Newton meter (Medio-Line Spring Scale, metric, 300 g, Pesola AG, Switzerland) that is attached to a triangular steel wire. Animals were pulled by the tail followed by instinctive grasping of the spring by the animal. Thus, the mouse exerts force on the steel wire, which can be measured as a read out parameter. The test was performed eight times on the right paretic forepaw of mice, whereas the left non-paretic forepaw was wrapped with an adhesive tape. The means were calculated for all eight measurements. For these data, percentage values were calculated with pre-ischemic values set as 100%.

INCLINED PLANE TEST

The test was performed as described before (Pajoohesh-Ganji et al., 2010; Popa-Wagner et al., 2010). Briefly, mice were put head down on a platform (40 cm × 60 cm) with a rubber surface followed by a successive increase of the platform angle. The experiment ended when the mouse was no longer able to hold its position for 5 s without sliding down. The last angle that the mouse was able to hold for 5 s was documented. A full experiment consisted of three trials at the time points given with an intertrial break of one minute to allow animals to recover. Means were calculated for each time point and used for statistical analysis.

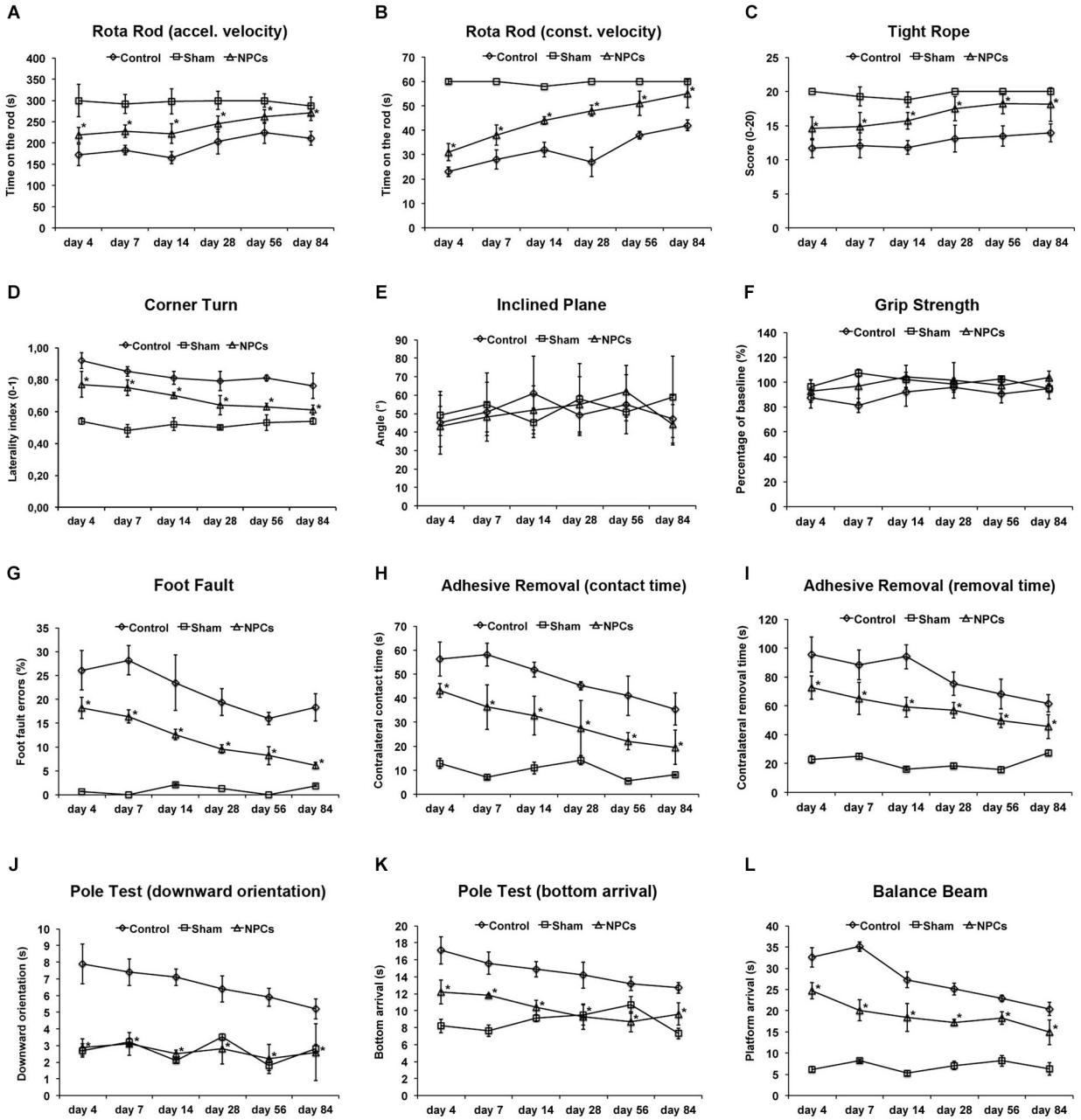

FIGURE 3 | Neural progenitor cells reduce motor coordination impairment after mild cerebral ischemia. Mice were exposed to mild cerebral ischemia (45 min) and allowed to survive for 84 days. Animals received intravenous injection of either neural progenitor cells ("NPCs") or PBS ("Control") 6 h after stroke. Sham animals ("Sham") underwent the same operational technique, but without inserting a filament followed by intravenous injection of 100 μl PBS at 6 h. Behavioral tests included the rota rod test with accelerating **(A)** and constant **(B)** velocity, for which maximal testing time was 300 s **(A)** and 60 s **(B)**. For the tight rope test **(C)**, a validated score was used from 0 (min) to 20 (max). The corner turn test **(D)** was analyzed using the laterality index (number of left turns – number of right turns)/10. For the inclined plain test **(E)**, the angle until the mice was still able to hold its ground was measured and used for statistical analysis, whereas the grip strength test **(F)** analyzed the percentage of force an animal was able to exert on a steel wire. For the foot fault test **(G)**, mice were placed on an elevated steel grid, and the relative number of foot faults of the right (impaired) forelimb was counted. The adhesive removal test included attachment of a defined tape at the paws with subsequent determination of both contact time **(H)** and removal time **(I)**. Maximal testing time of the test was 120 s. The pole test analyzed both downward orientation **(J)** and bottom arrival **(K)** when the mouse was placed head upward on the top of a vertical pole, thus predominantly testing extrapyramidal motor locomotion. Maximal testing time was 60 s for these tests. If the animal was unsuccessful for either task, it was scored 60 s. The balance beam test **(L)** consisted of an

(*Continued*)

FIGURE 3 | Continued
apparatus with a 110 cm long beam with constantly reduced width, starting with 12 mm width at the beginning and 5 mm width at the end of the beam. The time (maximal time was 60 s) until the mouse reached the platform at the end of the beam was measured. Maximal testing time was 60 s. The tests were performed at the time points given for as long as 84 days post-stroke as described in the materials and methods section of the manuscript. Number of animals used for statistical analysis was $n = 15$ for sham animals, $n = 14$ for NPC-treated mice and $n = 16$ for controls. * Significantly different from control, $p < 0.05$.

OPEN FIELD TEST

The test was used to assess both spontaneous post-ischemic locomotor activity and post-ischemic exploration behavior as previously described by our group (Kilic et al., 2008). Briefly, mice where placed into a round arena (diameter of 120 cm) surrounded by 30 cm high sidewalls. The stage consisted of three sections including an outer wall zone, a transition zone and an inner center zone. The mouse was always placed close to the wall and its behavior was analyzed for 10 min using an electronic imaging system (Ethovision XT, Noldusn The Netherlands). In order to analyze spontaneous locomotor activity of stroke animals, resting, scanning and progressing times were measured. Exploration behavior and anxiety were assessed via analysis of the percentage time spent in each of the three zones.

PASSIVE AVOIDANCE TEST

The test was performed as described before with slight modifications (Bouët et al., 2007). Briefly, mice were first introduced and conditioned to the setting apparatus using a passive avoidance cage (Harvard Apparatus, USA) that consisted of a large illuminated ("white") and a smaller non-illuminated ("dark") compartment. These two compartments were separated from each other by a guillotine gate. Animals were detected by means of high sensitivity weight transducers. According to the natural behavior of the rodent, mice initially sought to escape the white compartment through the gate, thus entering the small dark compartment. During the condition phase (twice on days 1–3 before induction of ischemia), the maximal step-through latency, i.e., time allowed for the animals to pass through the gate after having been put farthest away from the gate within the white compartment was 50 s. All mice passed the gate within this time period without significant differences between each other. After having entered the dark compartment, mice received an inevitable electric foot shock (0.4 mA, 2 s). During the testing phase (twice per time point), mice were again put into the white compartment farthest away from the guillotine gate. This time, no shock was applied and the time until the animals entered the dark compartment ("step-through latency") was monitored. Maximal testing time was 300 s. Animals that did not enter the dark compartment were scored 300 s.

POLE TEST

This test was performed with slight modifications according to a previous protocol (Bouët et al., 2007). A vertical pole (60 cm high with rough surface) was used for this test, which predominantly analyzes extrapyramidal motor locomotion. Mice were placed head upward on the top of the pole. Thereafter, both time taken to orientate the body completely downwards and to reach the floor with all four paws were recorded. Maximal testing time was 60 s. If the animal was unsuccessful for either task, it was scored 60 s. Each animal was tested four times at the time points given followed by calculation of means that were used for statistical analysis.

ROTA ROD TEST

The rota rod test was performed using a treadmill with a diameter of 3 cm (TSE Systems, Bad Homburg, Germany). This test was performed with either accelerating velocity (4–40 rpm) or at constant velocity of 40 rpm. For the accelerating test conditions, maximal velocity was achieved after 260 s with a maximal testing time of 300 s. On the contrary, maximal testing time for constant velocity (40 rpm) was 60 s. The time until the animals dropped was measured. Tests were always performed twice and means were used for statistical analysis.

TIGHT ROPE TEST

This test was performed by means of a 60 cm long rope that was attached to two opposing platforms as previously described by us (Doeppner et al., 2011). The mouse was placed with its forepaws at the middle of the rope. Two parameters were analyzed, i.e., whether or not the animal reached either of the two platforms and the time needed for reaching the platform. The maximal testing time was 60 s. Accordingly, these two parameters formed a validated score between 0 (worst performance) and 20 (best performance). The test was performed twice per time point and means were calculated.

WATER MAZE TEST

The water maze test was performed as described before by our group (Doeppner et al., 2013). Briefly, a modified water maze test was performed on days 26–28, days 54–56 and on days 82–84. Each test day consisted of four runs in the morning and four in the afternoon with a maximal time for each run of 90 s. Four runs were always grouped as one trial, thus making a total of 6 trials (I–VI) per time point. For runs 1–16 (trials I–IV) and runs 21–24 (trial VI), the transparent platform was always located in the same quadrant (i.e., upper left quadrant #1). However, for runs 17–20 (trial V) the platform was re-located within a different quadrant of the water maze pool ("task switch"). If an animal did not reach the platform, it was gently guided to the platform and scored 90 s. Both swimming speed and time needed to reach the platform were tracked using a computer-based video camera system (TSE Systems, Germany). Data are given as means per trial. Finally, relative mean times spent in quadrant #1 out of trials I–IV and trial VI was determined per time point, thus indicating spatial learning of experimental mice.

STATISTICAL ANALYSIS

The data are given as means ± standard deviation (SD). A one-way analysis of variance (ANOVA) followed by the Tukey's *post*

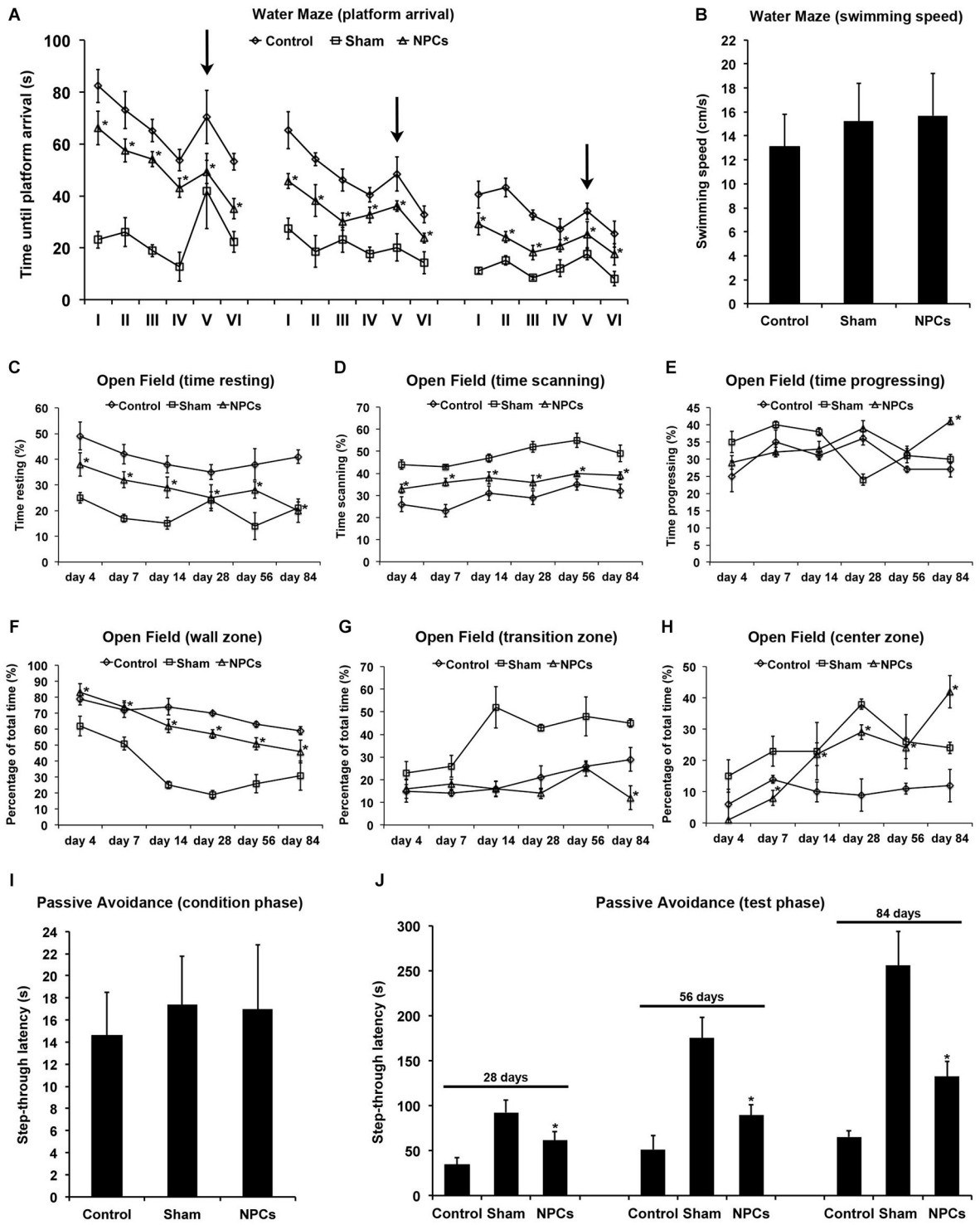

FIGURE 4 | Neural progenitor cells reduce impairment of learning and memory after mild cerebral ischemia. Cognitive impairment after 45 min of cerebral ischemia was analyzed using a modified water maze test, the open field test and the passive avoidance test. Mice were given intravenous injection of either neural progenitor cells ("NPCs"; 10^6 cells in 100 μl of PBS) or PBS ("Control") 6 h after stroke. Sham animals ("Sham") underwent the same operation, but without inserting a filament followed by intravenous injection of 100 μl PBS at 6 h. The water maze test **(A)** was performed on day 26–28, day 54–56 and on day 82–84. For each time point, four runs in the morning and four runs in the afternoon with a maximal time of 90 s were performed. Four runs were always grouped as one trial, thus making a total of 6 trials (I–VI) per time point. For runs 1–16 (trials I-IV) and runs 21–24 (trial VI), the platform was always located in the same quadrant. For runs 17–20 (trial V), the platform was re-located within a different quadrant of the water maze pool ("task switch"; indicated by arrows). Maximal testing time was 90 s, (*Continued*)

FIGURE 4 | Continued
and animals that did not reach the platform were scored 90 s. Noteworthy, swimming speed did not differ between the various groups **(B)**. The open field test was used for analysis of both spontaneous post-ischemic locomotor activity and exploration behavior. In order to analyze spontaneous locomotor activity of stroke animals, resting **(C)**, scanning **(D)** and progressing **(E)** times were measured. Exploration behavior and anxiety were assessed via analysis of the percentage of time spent in the wall zone **(F)**, the transition zone **(G)** and the center zone **(H)**. The passive avoidance test was performed using a cage consisting of a large illuminated ("white") and a smaller non-illuminated ("dark") compartment, which were separated from each other by a guillotine gate. Animals were detected by means of high sensitivity weight transducers. Natural behavior of the mouse includes escaping the white compartment through the gate, thus entering the small dark compartment. The pre-ischemic condition phase **(I)** depicts the maximal step-through latency when leaving the white compartment towards the dark compartment with a maximal testing time of 50 s. When mice entered the dark compartment, they received an inevitable electric foot shock. During the testing phase **(J)**, mice were again put into the white compartment and the time until animals reached the dark compartment was measured. No shock was applied during the testing phase, and the maximal testing time was 300 s. Animals that did not enter the dark compartment were scored 300 s. All behavioral tests were performed at the time points given as described in the materials and methods section of the manuscript. Number of animals used for statistical analysis was $n = 15$ for sham animals, $n = 14$ for NPC-treated mice and $n = 16$ for controls. * Significantly different from control, $p < 0.05$.

hoc test was performed for statistical analysis. A p value of < 0.05 was considered to be statistically significant.

RESULTS

NPCS INDUCE ACUTE NEUROPROTECTION AFTER BOTH MILD AND SEVERE CEREBRAL ISCHEMIA

Before using various behavioral tests in order to analyze post-ischemic functional recovery after treatment with NPCs, we first performed a proof of concept experiment indicating whether or not NPCs induce neuroprotection in our experimental paradigms. Using an intravenous cell delivery route with a transplantation of 10^6 NPCs at 6 h post-stroke, a time point that has been previously shown to be beneficial after a 45-min stroke (Doeppner et al., 2012a), we analyzed infarct volumes and brain injury. Noteworthy, the amount of intracerebral GFP^+ NPCs was in the same order with typical orientation within the ischemic lesion site as previously described (Doeppner et al., 2012a). Two days after stroke, we observed 89.7 ± 17.3 NPCs per mm^2 after 45 min of cerebral ischemia and 76.2 ± 25.1 NPCs per mm^2 after 90 min of cerebral ischemia. Analysis of infarct volumes revealed a significant reduction on day 2 after induction of both mild (45 min) and severe (90 min) cerebral ischemia (**Figures 2A,B**). In line with the latter, the number of $TUNEL^+$ cells (**Figure 2C**) and the amount of microglial cells (**Figure 2D**) were significantly reduced after NPC treatment when compared to PBS controls. Thus, systemic transplantation of NPCs induces significant acute neuroprotection after both mild and severe cerebral ischemia, providing the basis for enhanced functional recovery due to cell based therapy.

NPCS REDUCE MOTOR COORDINATION DEFICITS AFTER MILD CEREBRAL ISCHEMIA

Although enhanced post-stroke functional recovery due to NPC treatment has been described before, the most appropriate behavioral tests for these circumstances yet have to be defined. As a matter of fact, a great deal of behavioral tests exists for mice under various physiological and pathological circumstances, but systematic analysis of these tests in one condition is scarce. In order to systematically analyze appropriate motor coordination tests for assessment of post-stroke functional impairment after NPC treatment, we used nine well-known behavioral tests. As shown by our group before, NPC treatment after 45 min of stroke results in better test performance in the rota rod test, the tight rope test and the corner turn test (**Figures 3A–D**). Since accelerating velocity in the rota rod test may lead to exhaustion of stroke mice, we also applied a constant velocity paradigm with a testing time of only 60 s. However, there was no difference in the test results, i.e., NPC-treated mice scored better under both experimental paradigms. Noteworthy, test performance of treated mice approximated sham (non-ischemic) animals over time in the aforementioned tests. On the contrary, we did not observe any significant differences after NPC treatment using the inclined plane test and the grip strength test (**Figures 3E,F**). Further analysis of motor coordination deficits after 45 min of cerebral ischemia revealed significantly improved outcome after NPC treatment in the foot fault test, the adhesive removal test, the pole test and the balance beam test (**Figures 3G–L**). Noteworthy, contact time and removal time of the ipsilateral forepaw as assessed by the adhesive removal test were also significantly affected by NPC treatment (Figure S1). These data indicate enhanced functional recovery of stroke after NPC treatment. Nevertheless, the inclined plane and the grip strength test are not recommended for assessment of motor coordination deficits under mild cerebral ischemia.

NPCS REDUCE COGNITIVE IMPAIRMENT AFTER MILD CEREBRAL ISCHEMIA

Occlusion of the middle cerebral artery in the mouse model usually does not include injury of the hippocampal formation. Nevertheless, reduced test scores and/or cognitive impairment such as for the water maze test also do occur after injury of both basal ganglia and cortical areas (Bingham et al., 2012). We therefore analyzed post-ischemic impairment of learning and memory using the water maze test, the open field test and the passive avoidance test. Using the water maze test, NPC-treated animals significantly scored better than controls, albeit improved test performance over time was observed under all conditions (**Figure 4A**). Noteworthy, swimming speed did not significantly differ between experimental groups (**Figure 4B**). In line with this, the relative amount of time spent in quadrant #1 where the platform was located for trials I–IV and trial VI was significantly increased in mice that had been treated with NPCs. En detail, NPC-treated mice spent $48.2 \pm 6.7\%$ (day 26–28), $62.7 \pm 9.8\%$ (day 54–56) and $75.6 \pm 11.4\%$ (day 82–84) of total time in quadrant #1. On the contrary, controls spent $34.1 \pm 5.6\%$ (day 26–28), $42.7 \pm 3.9\%$ (day 54–56) and $56.2 \pm 6.3\%$ (day 82–84) of total time within the platform containing quadrant #1. These

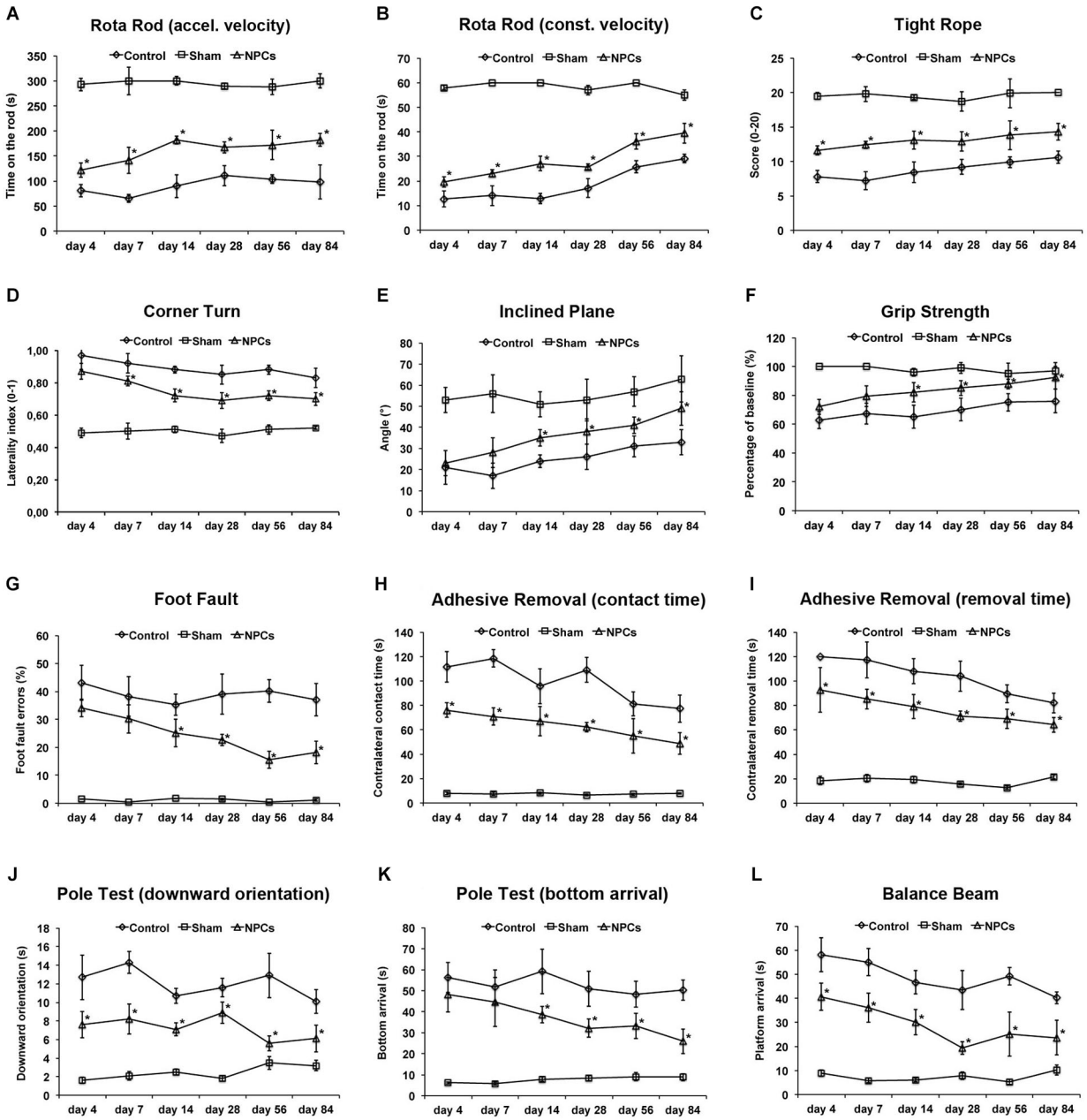

FIGURE 5 | Neural progenitor cells reduce motor coordination impairment after severe cerebral ischemia. Animals underwent 90 min of cerebral ischemia followed by a survival period of 84 days. Mice received intravenous injection of either neural progenitor cells ("NPCs"; 10^6 cells in 100 μl of PBS) or PBS ("Control") 6 h after stroke. Sham animals ("Sham") underwent the same operation, but without inserting a filament followed by intravenous injection of 100 μl PBS at 6 h. Behavioral tests included the rota rod test with accelerating **(A)** and constant **(B)** velocity, for which maximal testing time was 300 s **(A)** and 60 s **(B)**. For the tight rope test **(C)**, a validated score was used from 0 (min) to 20 (max). The corner turn test **(D)** was analyzed using the laterality index (number of left turns – number of right turns)/10. For the inclined plain test **(E)**, the angle until the mice was still able to hold its ground was measured and used for statistical analysis, whereas the grip strength **(F)** test analyzed the percentage of force an animal was able to exert on a steel wire. For the foot fault test **(G)**, mice were placed on an elevated steel grid, and the relative number of foot faults of the right (impaired) forelimb was counted. The adhesive removal test included attachment of a defined tape at the paws with subsequent determination of both contact time **(H)** and removal time **(I)**. Maximal testing time of the test was 120 s. The pole test analyzed both downward orientation **(J)** and bottom arrival **(K)** when the mouse was placed head upward on the top of a vertical pole, thus predominantly testing extrapyramidal motor locomotion. Maximal testing time was 60 s for these tests. If the animal was unsuccessful for either task, it was scored 60 s. The balance beam test **(L)** consisted of an apparatus with a 110 cm long beam with constantly reduced width, starting with 12 mm width at the beginning and 5 mm width at the end of the beam.

(*Continued*)

FIGURE 5 | Continued
The time (maximal time was 60 s) until the mouse reached the platform at the end of the beam was measured. Maximal testing time was 60 s. The tests were performed at the time points given as described in the materials and methods section of the manuscript. Number of animals used for statistical analysis was $n = 15$ for sham animals, $n = 16$ for NPC-treated mice and $n = 17$ for controls. However, one NPC-treated mouse and four control animals could not perform the tight rope test and the pole test due to the severity of the disease and were excluded from the statistical analysis in these three tests. * Significantly different from control, $p < 0.05$.

data thus suggest that the water maze test is suitable for assessing learning and memory capabilities after mild cerebral ischemia.

Not necessarily limited to post-ischemic cognitive impairment, further analysis regarding spontaneous locomotor activity and exploration behavior was done using the open field test. Locomotor activity was assessed via analysis of time stroke animals spent with resting (**Figure 4C**), scanning (**Figure 4D**) or progressing (**Figure 4E**) within the open field. As such, treatment with NPCs significantly reduced the relative amount of time mice spent with resting, suggesting an increased spontaneous locomotor activity in these animals. In line with this, NPC treatment resulted in stroke mice spending more time in the non-protected center zone of the open field when compared to controls (**Figures 4F–H**). Consequently, treatment with NPCs increased exploration behavior and reduced anxiety in these animals.

The passive avoidance test is a fear associated means to study learning and memory, using an aversive stimulus like a foot shock when mice follow their natural behavior such as entering the dark compartment. After the pre-ischemic condition phase that showed no significant difference between the experimental groups (**Figure 4I**), mice were exposed to the test setting at the time points given. Step through latencies, i.e., the latency until mice passed through the gate into the dark chamber according to their initial natural behavior, were highest in sham-operated mice. However, treatment with NPCs also resulted in significantly increased step through latencies when compared to non-treated controls (**Figure 4J**). These results suggest enhanced learning and memory patterns due to NPC-treatment after mild cerebral ischemia; all of which can be analyzed by the aforementioned three tests under these conditions.

SYSTEMIC TRANSPLANTATION OF NPCS REDUCES MOTOR COORDINATION DEFICITS AFTER SEVERE CEREBRAL ISCHEMIA

We next addressed the question whether or not systemic transplantation of NPCs also yielded long-term functional recovery as assessed by the aforementioned behavioral tests on motor coordination. As shown for mild cerebral ischemia (**Figures 3A–D**), transplantation of NPCs resulted in improved functional recovery of stroke animals when compared to controls in the rota rod, the tight rope and the corner turn test (**Figures 5A–D**). However, restrictions apply for the tight rope test that is a strenuous task, thus making it impossible for one NPC-treated mouse and four controls to actually perform the test. Instead of scoring these animals with a minimum score (i.e., 0), these mice were excluded from statistical analysis of the tight rope test. As for the inclined plane, the grip strength test, the foot fault test, the adhesive removal test, the pole test and the balance beam test (**Figures 5E–L**), NPC-treated mice always scored better than controls. Regarding the adhesive removal test, contact time and removal time of the ipsilateral forepaw were again significantly affected by NPC treatment (Figure S1). Noteworthy, the same restrictions applied for the pole test as for the aforementioned tight rope test, suggesting that both the tight rope test and the pole test are not suitable for behavioral analysis after severe cerebral ischemia.

COGNITIVE IMPAIRMENT AFTER SEVERE CEREBRAL ISCHEMIA IS REDUCED BY NPC TRANSPLANTATION

Water maze test performance including the task switch was significantly increased for as long as 84 days when mice received systemic injections of NPCs 6 h after induction of severe cerebral ischemia (**Figure 6A**). However, swimming speed of NPC-treated mice significantly differed from controls (**Figure 6B**), whereas the relative amount of time spent in the correct quadrant #1 did not differ from each other (data not shown). Thus, water maze test performance of NPC-treated mice after severe cerebral ischemia was significantly affected by reduced motor coordination deficits (**Figure 5**) of these animals. In line with this, some animals (one NPC-treated mouse and five controls) were not able to perform the water maze test due to the severity of brain injury and would have drowned, if the test had been completed. These mice were not included for final statistical analysis. Motor coordination thus significantly affected water maze test performance independent of cognitive impairment of stroke animals.

Analysis of the open field test revealed significantly increased spontaneous locomotor activity when mice were given NPCs as compared to controls (**Figures 6C–E**). Transplantation of NPCs resulted in a reduced relative amount of time that mice spent with resting, whereas scanning and progressing in these mice was increased. Likewise, NPC treatment yielded reduced anxiety as these mice significantly spent more time in the transition and center zone of the open field than controls (**Figures 6F–H**). Fear associated learning and memory impairment as assessed in the passive avoidance test (**Figures 6I–J**) was also significantly reduced in NPC-treated mice as indicated by increased step through latencies. These results suggest enhanced learning and memory capabilities due to NPC-treatment after severe cerebral ischemia, albeit the water maze test is not appropriate for this experimental paradigm.

NPCS REDUCE POST-ISCHEMIC BRAIN INJURY AFTER MILD AND SEVERE CEREBRAL ISCHEMIA

Since improved functional outcome does not necessarily reflect reduction of brain injury and vice versa, neuronal densities were assessed in the long run using NeuN staining (**Figure 7**). As such, neuronal densities were significantly increased on day 84 after both mild and severe cerebral ischemia when mice were treated with NPCs. These data suggest that the aforementioned improved motor coordination as well as learning and memory capabilities

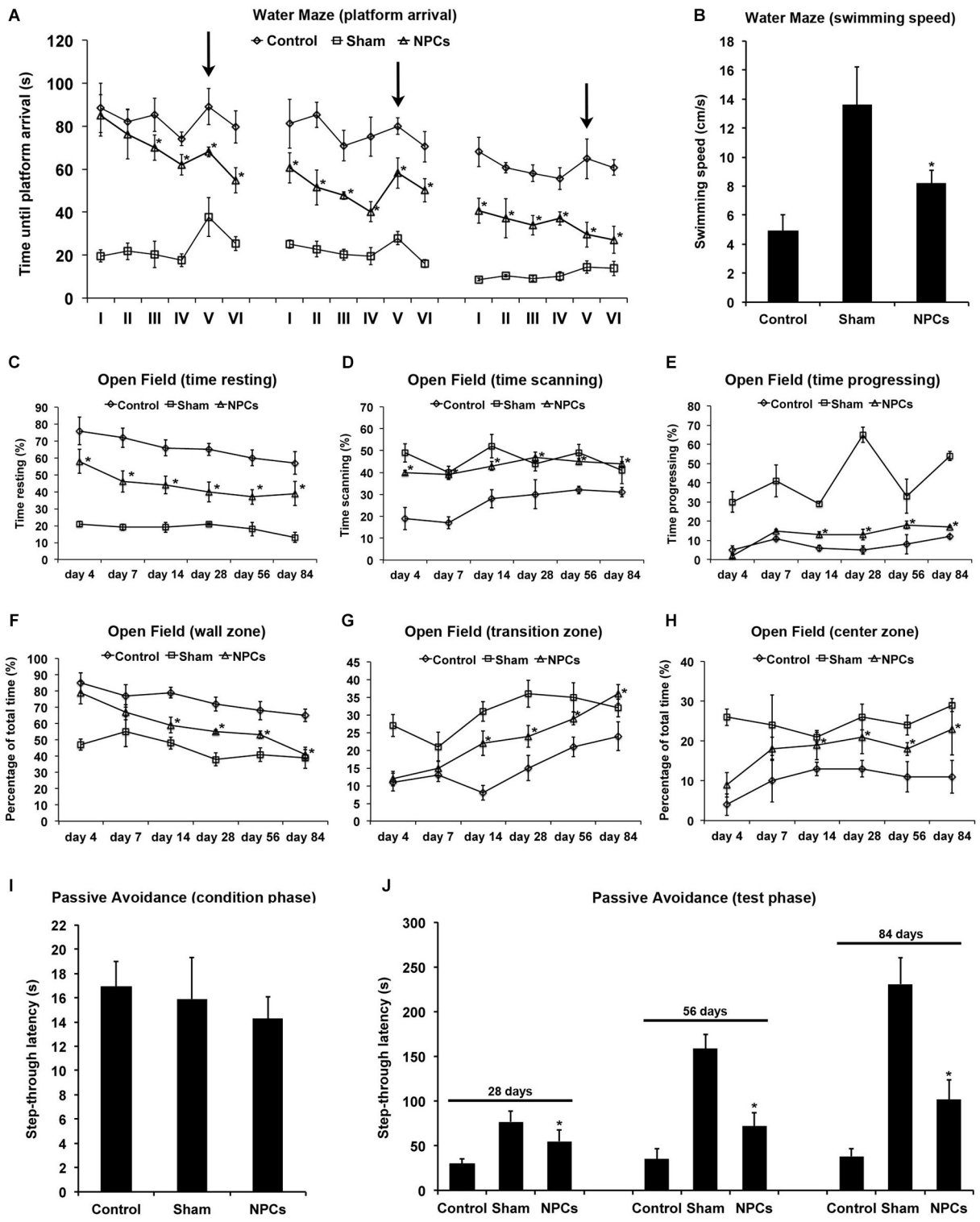

FIGURE 6 | Neural progenitor cells reduce impairment of learning and memory after severe cerebral ischemia. Cognitive impairment after 90 min of cerebral ischemia was analyzed using the water maze test, the open field test and the passive avoidance test. Intravenous injections with either neural progenitor cells ("NPCs"; 10^6 cells in 100 μl of PBS) or PBS ("Control") were performed at 6 h after stroke. Sham animals ("Sham") underwent the same operation, but without inserting a filament followed by intravenous injection of 100 μl PBS at 6 h after intervention. The water maze test **(A)** was performed on day 26–28, day 54–56 and on day 82–84 with four runs in the morning and four runs in the afternoon per test day. Maximal testing time was 90 s, and animals that did not reach the platform were scored 90 s.

(*Continued*)

FIGURE 6 | Continued
Four runs were always grouped as one trial, thus making a total of 6 trials (I–VI) per time point. For runs 1–16 (trials I–IV) and runs 21–24 (trial VI), the platform was always located in the same quadrant. For runs 17–20 (trial V), the platform was re-located within a different quadrant of the water maze pool ("task switch"; arrows). Swimming speed **(B)** is given as means out of all runs per condition. The open field test was used for analysis of both spontaneous post-ischemic locomotor activity and exploration behavior. In order to analyze spontaneous locomotor activity of stroke animals, resting **(C)**, scanning **(D)** and progressing **(E)** times were analyzed. Exploration behavior and anxiety were assessed via analysis of the percentage of time spent in the wall zone **(F)**, the transition zone **(G)** and the center zone **(H)**. The passive avoidance test was performed using a cage consisting of a large illuminated ("white") and a smaller non-illuminated ("dark") compartment, which were separated from each other by a guillotine gate. Natural behavior of the mouse includes escaping the white compartment through the gate, thus entering the small dark compartment. The pre-ischemic condition phase **(I)** depicts the maximal step-through latency when leaving the white compartment towards the dark compartment with a maximal testing time of 50 s. When mice entered the dark compartment, they received an inevitable electric foot shock. During the testing phase **(J)**, mice were again put into the white compartment and the time until animals reached the dark compartment was measured. No shock was applied during the testing phase, and the maximal testing time was 300 s. Animals that did not enter the dark compartment were scored 300 s. All behavioral tests were performed at the time points given as described in the materials and methods section of the manuscript. Number of animals used for statistical analysis was $n = 15$ for sham animals, $n = 16$ for NPC-treated mice and $n = 17$ for controls. However, one NPC-treated mouse and five control animals could not perform the water maze test due to the severity of the disease and would have drowned during the behavioral test, if the test had not been canceled. These mice were excluded from the statistical analysis of the water maze test, thus ending up with 15 sham animals, 15 NPC-treated mice and 12 control mice.
* Significantly different from control, $p < 0.05$.

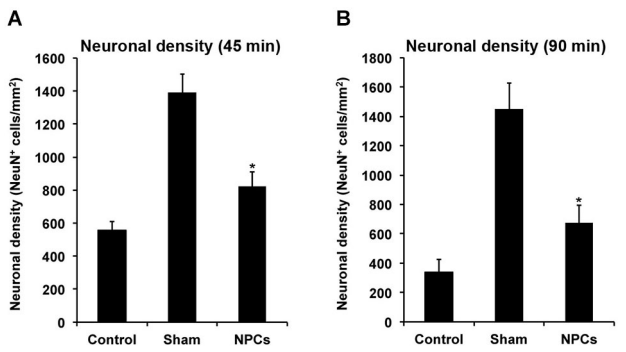

FIGURE 7 | Neural progenitor cells induce long-term neuroprotection after both mild and severe cerebral ischemia. Mice underwent 45 min **(A)** or 90 min **(B)** of cerebral ischemia followed by intravenous injection of neural progenitor cells ("NPCs") or PBS ("Control") 6 h post-stroke. Sham animals ("Sham") underwent the same operational technique, but without inserting a filament for occlusion of the middle cerebral artery. Shams received intravenous injection of PBS at 6 h after stroke. Neuronal density was assessed on day 84 via NeuN staining. The number of animals used for statistical analysis after mild cerebral ischemia was $n = 15$ (sham), $n = 14$ (NPC-treated mice) and $n = 16$ (controls), whereas for the severe cerebral ischemia paradigm $n = 15$ (sham), $n = 16$ (NPC-treated mice) and $n = 17$ (controls) was used. * Significantly different from control, $p < 0.05$.

due to treatment of NPCs are associated with reduced histological brain injury in these animals.

DISCUSSION

The present work systematically analyzed post-ischemic functional impairment after intravenous NPC transplantation with respect to motor coordination and cognitive impairment in mice. Although recent findings by our group suggest that NPC-induced functional recovery is independent of cell delivery timing (Doeppner et al., 2014), we have chosen an experimental paradigm used in previous studies (Doeppner et al., 2012a) that involves acute NPC grafting with subsequent reduction of brain injury. Emphasis during the three-month observation period was not only put on reliability and validity but also on feasibility and cost-effective considerations of the behavioral test in question.

Since motor coordination scores significantly depend on severity of focal cerebral ischemia (Rogers et al., 1997), we induced both mild and severe cerebral ischemia in mice. A great deal of motor coordination tests exists in rodents for physiological and pathological conditions (Bouët et al., 2007; Brooks and Dunnett, 2009; Balkaya et al., 2012), with some of which successfully used by our group in the past (Doeppner et al., 2009a,b, 2010, 2011, 2012a,b).

The rota rod test, which has been first described in 1957 using a drum with constant velocity (Dunham and Miya, 1957), is an excellent test for assessing motor coordination. As mentioned afore, NPCs reduced motor coordination impairment after both mild and severe cerebral ischemia. Although accelerating velocity might involve the risk of exhausting mice that suffered from severe ischemia, test results were reliable and valid using both constant and accelerating velocity paradigms (Dunham and Miya, 1957; Jones and Roberts, 1968). Despite the fact that the rota rod offers the advantage of producing automated data, one has to keep in mind that running on the drum might also involve anxiolytic behavior of the test mice independent of motor coordination deficits (Salam et al., 2009). In line with the aforementioned results of the rota rod test, assessment of sensorimotor deficits in the corner turn test showed significantly better outcome for NPC-treated mice under each experimental paradigm. Initially developed by Zhang et al. (2002), this test provides an excellent stage for analyzing post-stroke motor coordination deficits without depending on the strength of the test mice. Enhanced motor coordination recovery after treatment with NPCs was also observed after both mild and severe cerebral ischemia using the tight rope test. Originally described before as the wire hang test (Gerlai et al., 2000), this test was modified using a test score that pays attention to two parameters, i.e., reaching the platform and time spent on the rope (Doeppner et al., 2011). As such, the test proves very useful for elaborate analyses of post-stroke motor coordination deficits. However, limitations occur when animals are severely affected by stroke or other diseases, thus making them too weak to actually perform this strenuous test. This was the case after severe cerebral ischemia in the present study, resulting in some animals not able to perform the test at all.

The grip strength test and the inclined plane test are well-known tests for assessing motor coordination in rodents both after stroke and other diseases alike (Buchhold et al., 2007; Kilic et al., 2010; Popa-Wagner et al., 2010; Reitmeir et al., 2011). However, both grip strength test and inclined plane test only showed significant differences under conditions of severe cerebral ischemia; no effect of NPC treatment was observed after mild cerebral ischemia. The reason for the latter is that these tests do not only depend on motor coordination activity but also on sheer force of the test mouse, which is not necessarily reduced after mild cerebral ischemia. In this sense, both tests are less sensitive in settings of mild cerebral ischemia.

Further analysis of motor coordination deficits after both mild and cerebral ischemia revealed NPC-induced increased functional recovery using the foot fault, the adhesive removal, the pole test and the balance beam test. The foot fault test (aka as grid walking test) is a well-established and easy to use test for assessment of motor coordination deficits after stroke in rodents (Rogers et al., 1997; Chen et al., 2005; Salam et al., 2009). Noteworthy, the present data shows significant differences between control and NPC-treated mice during the complete observation period of three months, whereas former studies failed to show stroke-induced functional impairment in the foot fault test beyond a time point of 60 days (Zhang et al., 2002). Likewise, both the adhesive removal test and the balance beam test have been successfully used in stroke models (Combs and D'Alecy, 1987; Modo et al., 2000; Li et al., 2001). As for the adhesive removal test, this test even proved to be superior and more feasible than other behavioral tests used for analysis of long-term functional outcome in rodents after stroke (Freret et al., 2009). However, constant testing environments such as testing within the animal's cage are necessary in order to retrieve reliable and valid data in the adhesive removal test (Schallert and Woodlee, 2005), as should be the case for any other behavioral test as well. Similar findings were found regarding the balance beam test, which was more sensitive in assessment of motor coordination deficits than the rota rod test, albeit these tests were performed in a non-stroke model (Stanley et al., 2005). On the contrary, the pole test cannot be recommended for conditions of severe stroke since some animals are not able to perform the test at all due to the strenuous nature of the task, as has been discussed afore regarding the tight rope test.

Assessment of post-stroke cognitive impairment and therapeutic effects of NPC grafting was performed using the water maze test, the passive avoidance test and the open field test. The water maze test has long been characterized as a reliable means to analyze learning and memory under various conditions including cerebral ischemia in rodents (Morris, 1984; Olsen et al., 1994; Dahlqvist et al., 2004; Winter et al., 2004; Tsai et al., 2011). As stated afore, post-ischemic water maze test performance is also affected after cerebral infarction within the MCA territory (Bingham et al., 2012). Cognitive impairment after mild cerebral ischemia and NPC-induced effects were reliably measured in the water maze test. However, the water maze test is not suitable for detection of cognitive dysfunction after severe cerebral ischemia, since the latter too much includes significantly affected motor coordination as indicated by reduced swimming speed in the test. On the contrary, the passive avoidance test can also be performed on mice suffering from severe stroke and thus under conditions with great motor coordination impairment. In this sense, improved test performance after both mild and severe cerebral ischemia was observed after NPC transplantation. Yet, technical equipment for this test is expensive as is the video tracking system used for the water maze test, thus reducing the feasibility of both tests. The open field test is a simple test for behavioral analysis of rodents, although the test is not exclusively limited to analysis of cognitive function. In the present study, improved test performance after NPC transplantation was observed after both mild and severe cerebral ischemia. In light of its simplicity and cost-efficacy, this test is a reliable and feasible test for studying the aforementioned parameters in stroke mice.

In conclusion, the present study systematically analyzed functional impairment after both mild and severe cerebral ischemia and the effect of acute systemic transplantation of NPCs for as long as three months using a battery of twelve behavioral tests. In terms of reliability and cost-effectiveness considerations, results from our study recommend the use of the corner turn, foot fault, balance beam, and open field test, which do not depend on durations of cerebral ischemia. The study thus provides suggestions for the use of further behavioral tests, which aim to assess functional recovery after stem cell or progenitor cell grafting in stroke models.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Alvarez-Buylla, A., and Garcia-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. *J. Neurosci.* 22, 629–634.

Bacigaluppi, M., Pluchino, S., Martino, G., Kilic, E., and Hermann, D. M. (2008). Neural stem/precursor cells for the treatment of ischemic stroke. *J. Neurol. Sci.* 265, 73–77. doi: 10.1016/j.jns.2007.06.012

Bacigaluppi, M., Pluchino, S., Peruzzotti-Jametti, L., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251. doi: 10.1093/brain/awp174

Balkaya, M., Kröber, J. M., Rex, A., and Endres, M. (2012). Assessing behavior in mouse models of focal ischemia. *J. Cereb. Blood Flow Metab.* 33, 330–338. doi: 10.1038/jcbfm.2012.185

Bingham, D., Martin, S. J., Macrae, I. M., and Carswell, H. V. (2012). Watermaze performance after middle cerebral artery occlusion in the rat: the role of sensorimotor versus memory impairments. *J. Cereb. Blood Flow Metab.* 32, 989–999. doi: 10.1038/jcbfm.2012.16

Bliss, T. M., Andres, R. H., and Steinberg, G. K. (2010). Optimizing the success of cell transplantation therapy for stroke. *Neurobiol. Dis.* 37, 275–283. doi: 10.1016/j.nbd.2009.10.003

Bliss, T., Guzman, R., Daadi, M., and Steinberg, G. K. (2007). Cell transplantation therapy for stroke. *Stroke* 38, 817–826. doi: 10.1161/01.str.0000247888.25985.62

Blum, B., and Benvenisty, N. (2008). The tumorigenicity of human embryonic stem cells. *Adv. Cancer Res.* 100, 133–158. doi: 10.1016/s0065-230x(08)00005-5

Blum, B., and Benvenisty, N. (2009). The tumorigenicity of diploid and aneuploid human pluripotent stem cells. *Cell cycle.* 8, 3822–3830. doi: 10.4161/cc.8.23.10067

Bonnamain, V., Neveu, I., and Naveilhan, P. (2012). Neural stem/progenitor cells as a promising candidate for regenerative therapy of the central nervous system. *Front. Cell. Neurosci.* 6:17. doi: 10.3389/fncel.2012.00017

Bouët, V., Freret, T., Toutain, J., Divoux, D., Boulouard, M., and Schumann-Bard, P. (2007). Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. *Exp. Neurol.* 203, 555–567. doi: 10.1016/j.expneurol.2006.09.006

Brooks, S. P., and Dunnett, S. B. (2009). Tests to assess motor phenotype in mice: a user's guide. *Nat. Rev. Neurosci.* 10, 519–529. doi: 10.1038/nrn2652

Buchhold, B., Mogoanta, L., Suofu, Y., Hamm, A., Walker, L., Kessler, C., et al. (2007). Environmental enrichment improves functional and neuropathological indices following stroke in young and aged rats. *Restor. Neurol. Neurosci.* 25, 467–484.

Chen, J., Zhang, C., Jiang, H., Li, Y., Zhang, L., Robin, A., et al. (2005). Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J. Cereb. Blood Flow Metab.* 25, 281–290. doi: 10.1038/sj.jcbfm.9600034

Combs, D. J., and D'Alecy, L. G. (1987). Motor performance in rats exposed to severe forebrain ischemia: effect of fasting and 1,3-butanediol. *Stroke* 18, 503–511. doi: 10.1161/01.str.18.2.503

Dahlqvist, P., Rönnbäck, A., Bergström, S. A., Söderström, I., and Olsson, T. (2004). Environmental enrichment reverses learning impairment in the Morris water maze after focal cerebral ischemia in rats. *Eur. J. Neurosci.* 19, 2288–2298. doi: 10.1111/j.0953-816x.2004.03248.x

Doeppner, T. R., Bretschneider, E., Doehring, M., Segura, I., Senturk, A., Acker-Palmer, A., et al. (2011). Enhancement of endogenous neurogenesis in ephrin-B3 deficient mice after transient focal cerebral ischemia. *Acta Neuropathol.* 122, 429–442. doi: 10.1007/s00401-011-0856-5

Doeppner, T. R., Dietz, G. P., El Aanbouri, M., Gerber, J., Witte, O. W., Bähr, M., et al. (2009a). TAT-Bcl-x(L) improves survival of neuronal precursor cells in the lesioned striatum after focal cerebral ischemia. *Neurobiol. Dis.* 34, 87–94. doi: 10.1016/j.nbd.2008.12.013

Doeppner, T. R., El Aanbouri, M., Dietz, G. P., Weise, J., Schwarting, S., and Bähr, M. (2010). Transplantation of TAT-Bcl-xL-transduced neural precursor cells: long-term neuroprotection after stroke. *Neurobiol. Dis.* 40, 265–276. doi: 10.1016/j.nbd.2010.05.033

Doeppner, T. R., Ewert, T. A., Tönges, L., Herz, J., Zechariah, A., Elali, A., et al. (2012a). Transduction of neural precursor cells with TAT-heat shock protein 70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. *Stem Cells* 30, 1297–1310. doi: 10.1002/stem.1098

Doeppner, T. R., Kaltwasser, B., Fengyan, J., Hermann, D. M., and Bähr, M. (2013). TAT-Hsp70 induces neuroprotection against stroke via anti-inflammatory actions providing appropriate cellular microenvironment for transplantation of neural precursor cells. *J. Cereb. Blood Flow Metab.* 33, 1778–1788. doi: 10.1038/jcbfm.2013.126

Doeppner, T. R., Kaltwasser, B., Teli, M. K., Bretschneider, E., Bähr, M., and Hermann, D. M. (2014). Effects of acute versus post-acute systemic delivery of neural progenitor cells on neurological recovery and brain remodeling after focal cerebral ischemia in mice. *Cell Death Dis.* 5:e1386. doi: 10.1038/cddis.2014.359

Doeppner, T. R., Mlynarczuk-Bialy, I., Kuckelkorn, U., Kaltwasser, B., Herz, J., Hasan, M. R., et al. (2012b). The novel proteasome inhibitor BSc2118 protects against cerebral ischaemia through HIF1A accumulation and enhanced angioneurogenesis. *Brain* 135, 3282–3297. doi: 10.1093/brain/aws269

Doeppner, T. R., Nagel, F., Dietz, G. P., Weise, J., Tonges, L., Schwarting, S., et al. (2009b). TAT-Hsp70-mediated neuroprotection and increased survival of neuronal precursor cells after focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* 29, 1187–1196. doi: 10.1038/jcbfm.2009.44

Dunham, N. W., and Miya, T. S. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J. Am. Pharm. Assoc. Am. Pharm. Assoc. (Baltim)* 46, 208–209. doi: 10.1002/jps.3030460322

Freret, T., Bouet, V., Leconte, C., Roussel, S., Chazalviel, L., Divoux, D., et al. (2009). Behavioral deficits after distal focal cerebral ischemia in mice: usefulness of adhesive removal test. *Behav. Neurosci.* 123, 224–230. doi: 10.1037/a0014157

Gerlai, R., Thibodeaux, H., Palmer, J. T., van Lookeren Campagne, M., and Van Bruggen, N. (2000). Transient focal cerebral ischemia induces sensorimotor deficits in mice. *Behav. Brain Res.* 108, 63–71. doi: 10.1016/s0166-4328(99)00130-8

Hemmer, K., Zhang, M., van Wülen, T., Sakalem, M., Tapia, N., Baumuratov, A., et al. (2014). Induced neural stem cells achieve long-term survival and functional integration in the adult mouse brain. *Stem Cell Reports* 3, 423-431. doi: 10.1016/j.stemcr.2014.06.017

Hicks, A. U., Lappalainen, R. S., Narkilahti, S., Suuronen, R., Corbett, D., Sivenius, J., et al. (2009). Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. *Eur. J. Neurosci.* 29, 562–574. doi: 10.1111/j.1460-9568.2008.06599.x

Jones, B. J., and Roberts, D. J. (1968). The quantiative measurement of motor incoordination in naive mice using an acelerating rotarod. *J. Pharm. Pharmacol.* 20, 302–304. doi: 10.1111/j.2042-7158.1968.tb09743.x

Kilic, E., ElAli, A., Kilic, U., Guo, Z., Ugur, M., Uslu, U., et al. (2010). Role of Nogo-A in neuronal survival in the reperfused ischemic brain. *J. Cereb. Blood Flow Metab.* 30, 969–984. doi: 10.1038/jcbfm.2009.268

Kilic, E., Kilic, U., Bacigaluppi, M., Guo, Z., Abdallah, N. B., Wolfer, D. P., et al. (2008). Delayed melatonin administration promotes neuronal survival, neurogenesis and motor recovery and attenuates hyperactivity and anxiety after mild focal cerebral ischemia in mice. *J. Pineal Res.* 45, 142–148. doi: 10.1111/j.1600-079x.2008.00568.x

Leong, W. K., Henshall, T. L., Arthur, A., Kremer, K. L., Lewis, M. D., Helps, S. C., et al. (2012). Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. *Stem Cells Transl. Med.* 1, 177–187. doi: 10.5966/sctm.2011-0039

Li, X., Blizzard, K. K., Zeng, Z., DeVries, A. C., Hurn, P. D., and McCullough, L. D. (2004). Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. *Exp. Neurol.* 187, 94–104. doi: 10.1016/j.expneurol.2004.01.004

Li, Y., Chen, J., Wang, L., Lu, M., and Chopp, M. (2001). Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* 56, 1666–1672. doi: 10.1212/wnl.56.12.1666

Luong, T. N., Carlisle, H. J., Southwell, A., and Patterson, P. H. (2011). Assessment of motor balance and coordination in mice using the balance beam. *J. Vis. Exp.* 49:2376. doi: 10.3791/2376

Mochizuki, N., Takagi, N., Kurokawa, K., Onozato, C., Moriyama, Y., Tanonaka, K., et al. (2008). Injection of neural progenitor cells improved learning and memory dysfunction after cerebral ischemia. *Exp. Neurol.* 211, 194–202. doi: 10.1016/j.expneurol.2008.01.027

Modo, M., Stroemer, R. P., Tang, E., Veizovic, T., Sowniski, P., and Hodges, H. (2000). Neurological sequelae and long-term behavioural assessment of rats with transient middle cerebral artery occlusion. *J. Neurosci. Methods* 104, 99–109. doi: 10.1016/s0165-0270(00)00329-0

Morris, R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* 11, 47–60. doi: 10.1016/0165-0270(84)90007-4

Olsen, G. M., Scheel-Krüger, J., Møller, A., and Jensen, L. H. (1994). Relation of spatial learning of rats in the Morris water maze task to the number of viable CA1 neurons following four-vessel occlusion. *Behav. Neurosci.* 108, 681–690. doi: 10.1037//0735-7044.108.4.681

Pajoohesh-Ganji, A., Byrnes, K. R., Fatemi, G., and Faden, A. I. (2010). A combined scoring method to assess behavioral recovery after mouse spinal cord injury. *Neurosci. Res.* 67, 117–125. doi: 10.1016/j.neures.2010.02.009

Popa-Wagner, A., Stöcker, K., Balseanu, A. T., Rogalewski, A., Diederich, K., Minnerup, J., et al. (2010). Effects of granulocyte-colony stimulating factor after stroke in aged rats. *Stroke* 41, 1027–1031. doi: 10.1161/STROKEAHA.109.575621

Reitmeir, R., Kilic, E., Kilic, U., Bacigaluppi, M., ElAli, A., Salani, G., et al. (2011). Post-acute delivery of erythropoietin induces stroke recovery by promoting perilesional tissue remodelling and contralesional pyramidal tract plasticity. *Brain* 134, 84–99. doi: 10.1093/brain/awq344

Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707–1710. doi: 10.1126/science.1553558

Rogers, D. C., Campbell, C. A., Stretton, J. L., and Mackay, K. B. (1997). Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. *Stroke* 28, 2060–2066. doi: 10.1161/01.str.28.10.2060

Salam, J. N., Fox, J. H., Detroy, E. M., Guignon, M. H., Wohl, D. F., and Falls, W. A. (2009). Voluntary exercise in C57 mice is anxiolytic across several measures of anxiety. *Behav. Brain Res.* 197, 31–40. doi: 10.1016/j.bbr.2008.07.036

Schaar, K. L., Brenneman, M. M., and Savitz, S. I. (2010). Functional assessments in the rodent stroke model. *Exp. Transl. Stroke Med.* 2:13. doi: 10.1186/2040-7378-2-13

Schallert, T., and Woodlee, M. T. (2005). "Orienting and placing," in *The Behavior of the Laboratory Rat: A Handbook with Tests*, eds I. Whishaw and B. Kolb (Oxford: Oxford University Press), 129–140.

Schwarting, S., Litwak, S., Hao, W., Bahr, M., Weise, J., and Neumann, H. (2008). Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. *Stroke* 39, 2867–2875. doi: 10.1161/strokeaha.108.513978

Stanley, J. L., Lincoln, R. J., Brown, T. A., McDonald, L. M., Dawson, G. R., and Reynolds, D. S. (2005). The mouse beam walking assay offers improved sensitivity over the mouse rotarod in determining motor coordination deficits induced by benzodiazepines. *J. Psychopharmacol.* 19, 221–227. doi: 10.1177/0269881105051524

Tsai, Y. W., Yang, Y. R., Wang, P. S., and Wang, R. Y. (2011). Intermittent hypoxia after transient focal ischemia induces hippocampal neurogenesis and c-Fos expression and reverses spatial memory deficits in rats. *PLoS One* 6:e24001. doi: 10.1371/journal.pone.0024001

Winter, B., Bert, B., Fink, H., Dirnagl, U., and Endres, M. (2004). Dysexecutive syndrome after mild cerebral ischemia? Mice learn normally but have deficits in strategy switching. *Stroke* 35, 191–195. doi: 10.1161/01.str.0000107188.29688.2c

Zhang, L., Schallert, T., Zhang, Z. G., Jiang, Q., Arniego, P., Li, Q., et al. (2002). A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J. Neurosci. Methods* 117, 207–214. doi: 10.1016/s0165-0270(02)00114-0

Zheng, W., Honmou, O., Miyata, K., Harada, K., Suzuki, J., Liu, H., et al. (2010). Therapeutic benefits of human mesenchymal stem cells derived from bone marrow after global cerebral ischemia. *Brain Res.* 1310, 8–16. doi: 10.1016/j.brainres.2009.11.012

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Stem cell therapies in preclinical models of stroke associated with aging

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Stroke has limited treatment options, demanding a vigorous search for new therapeutic strategies. Initial enthusiasm to stimulate restorative processes in the ischemic brain by means of cell-based therapies has meanwhile converted into a more balanced view recognizing impediments related to unfavorable environments that are in part related to aging processes. Since stroke afflicts mostly the elderly, it is highly desirable and clinically important to test the efficacy of cell therapies in aged brain microenvironments. Although widely believed to be refractory to regeneration, recent studies using both neural precursor cells and bone marrow-derived mesenchymal stem cells for stroke therapy suggest that the aged rat brain is not refractory to cell-based therapy, and that it also supports plasticity and remodeling. Yet, important differences exist in the aged compared with young brain, i.e., the accelerated progression of ischemic injury to brain infarction, the reduced rate of endogenous neurogenesis and the delayed initiation of neurological recovery. Pitfalls in the development of cell-based therapies may also be related to age-associated comorbidities, e.g., diabetes or hyperlipidemia, which may result in maladaptive or compromised brain remodeling, respectively. These age-related aspects should be carefully considered in the clinical translation of restorative therapies.

Keywords: aging, stroke, cell therapy, comorbidities, neurogenesis

AGE AS MAJOR RISK FACTOR FOR STROKE

Stroke is a highly prevalent disease, which represents the second most common cause of death in Europe, and the third most common cause of death in the United States and Canada (Lloyd-Jones et al., 2010; Roger et al., 2012). Age is the principal non-modifiable risk factor for stroke. The incidence of stroke increases significantly with age in both men and women, with half of all strokes occurring in subjects over 75 years of age, and one-third in subjects over 85 years of age (Roger et al., 2012). Although the entire older population is at risk of stroke, there are important sex differences in stroke incidence. The incidence of stroke is higher in men than women up to 75 years old, equilibrates in subjects aged 75–84 years, and gets higher in women than men aged 85 years or older (Roger et al., 2012). This may be attributed to sex-related differences in risk factor profiles, resulting in a higher life expectancy in women than men. Importantly, age-associated changes show great variability among individuals, which are modified by genetic and long-term lifestyle factors (Tacutu et al., 2010, 2011).

STROKE MODELS USING AGED ANIMALS ARE CLINICALLY MORE RELEVANT

Effects of age and sex on stroke incidence, functional neurological recovery and stroke mortality have been shown both in humans and in animal models (Badan et al., 2003; Buchhold et al., 2007; Gokcay et al., 2011). Specifically, the age-dependent acceleration in the progression of ischemic tissue into infarction strongly suggests that age is a biological marker for the variability in stroke outcome (Ay et al., 2005).

Over the past 10 years, a variety of models of middle cerebral artery occlusion (MCAO) have been established in rodents (Bacigaluppi et al., 2010). MCAO in aged rodents has been produced with permanent occlusion or transient occlusion for 30–120 min using (i) non-localized photothrombosis achieved by irradiation of the right carotid artery (Futrell et al., 1991); (ii) thermocoagulation after microcraniotomy (Davis et al., 1995); (iii) intraluminal using a silicon-coated suture (Sutherland et al., 1996); (iv) a hook attached to a micromanipulator (Popa-Wagner et al., 1998); (v) cauterization (Katsman et al., 2003); (vi) photothrombosis (Zhao et al., 2005); (vii) injection of a thrombus via the external carotid artery (Zhang et al., 2005, 2013; Dinapoli et al., 2006); or (viii) endothelin-1 injection (Soleman et al., 2010). These models differ in the localization and size of the ischemic lesions, as well as in mortality rates following surgery, as summarized in **Table 1**.

Since focal cerebral ischemia is technically difficult to perform in very old rodents and since in humans stroke is highly prevalent in late middle aged (60–70 years old) subjects (Feigin et al., 2003), middle aged rodents may represent a reasonable choice for stroke studies (Popa-Wagner et al., 2007). In the following, specific responses of the aged brain to ischemic stroke and cell-based therapy are outlined. Results from cell transplantation studies in aged rodents are summarized in **Table 2**.

SPONTANEOUS STROKE RECOVERY IN AGED PATIENTS AND ANIMALS

Neurological recovery is thought to occur via recruitment of neighboring neuronal circuitries (Hallett, 2001; Hermann and

Table 1 | Experimental stroke models and mortality rates in aged rats.

Strain	MCA occlusion	Age (mo)	Ischemia	Mortality (%)	Localization	Anesthetic	Reference
Wistar	Thermocoagulation	32	Permanent	NA	Neocortical	Halothane	Davis et al. (1995)
Wistar	Intraluminal nylon monofilament	27	Permanent or reversible	10	Necortical & striatal	Halothane	Sutherland et al. (1996)
Wistar	Photothrombosis	24	Permanent	4	Neocortical	Halothane	Zhao et al. (2005)
Fischer	Photothrombosis non-localized	24	Reversible	16	Neocortical	Chloral hydrate	Futrell et al. (1991)
Wistar	Photothrombosis	24	Permanent	4	Neocortical	Halothane	Zhao et al. (2005)
Wistar	Endothelin-1	20–23	Reversible	13.3	Neocortical	Isoflurane	Soleman et al. (2010)
Wistar	ECA embolization	18–20	Spontaneous reperfusion	33	Necortical & striatal	Isoflurane	Zhang et al. (2013)
Sprague-Dawley (m)	Vascular clip/hook	18–20	Reversible	25	Neocortical	Halothane	Popa-Wagner et al. (1998)
Sprague-Dawley (f)	ECA embolization	18	rt-PA reperfusion	33	Neocortical	Ketamine	Dinapoli et al. (2006)
Wistar	ECA embolization	18	Spontaneous reperfusion	47	Necortical & striatal	Halothane	Zhang et al. (2005)

NA, not assessed; ECA, external carotid artery; f, female; m, male.

Chopp, 2012; Zhang et al., 2014). In clinical practice, physical therapy is used for stimulating post stroke brain remodeling (Liepert et al., 2004; Honmou et al., 2012).

Stroke patients regain some of their lost neurological functions during the first weeks or months after the stroke, most likely due to functional reorganization of the lesioned area (Zhang et al., 2014). In animal models of stroke, complete spontaneous recovery may occur in young–adult rats, depending on the size and location of the ischemic lesion. However, stroke recovery is delayed and often incomplete in aged rats. While young–adult rats typically begin to show improvements of neurological function starting by day 2 post stroke, neurological recovery is hardly detectable in aged rats before days 4 or 5, and achieve about 75% of the functional improvement observed in young–adult rats by day 14 (Buchhold et al., 2007). Housing experimental animals in an enriched environment enhances the recovery from brain damage both in young–adult and aged animals (Buchhold et al., 2007). When aged rats were allowed to recover in an enriched environment, the delay period was shortened and behavioral performance was significantly improved. The improvement in task performance positively correlated with slower infarct development, fewer proliferating astrocytes and smaller glial scars (Buchhold et al., 2007). Even more effective rehabilitation of the contralateral forelimb could be achieved by combining enriched environment with physical training (Hicks et al., 2007).

STRATEGIES TO IMPROVE FUNCTIONAL NEUROLOGICAL RECOVERY AND TISSUE REPAIR AFTER STROKE BY CELL THERAPY

Although rehabilitation is important for improving functional recovery in the early stages after stroke, it does not provide a replacement of lost tissue. With this understanding, cell therapies have initially been implemented with the aim of replacing lost tissue in human stroke patients (Kondziolka et al., 2000; Bang et al., 2005).

Most clinical studies to date have used neural cells derived from human fetal donors. The techniques to achieve effective survival and growth of neural tissues transplanted into the CNS are meanwhile well established (Dunnett, 2013). Even though effective, neural grafting has not become a standard treatment for several reasons including the limited supply of fetal tissue of human origin and controversies about the beneficial effects (Morizane et al., 2008). Of the various options, the transplantation of adult stem and precursor cells, propagated in cell culture, or the use of inducible pluripotent stem cells (iPSCs) derived from human patients and trans-differentiated into neural cells, are reasonable alternatives (Haas et al., 2005; Stoll, 2014). iPSCs hold great promise for stroke treatment, since they lack ethical concerns and the risk of graft rejection. However, if and how the aged brain responds to grafted cells is still vaguely known unclear.

NEURAL PRECURSOR AND STEM CELLS IN SUBCORTICAL STROKE

Spontaneous recovery is commonly observed if the infarct is located in the striatum, a subcortical structure that exhibits a natural activity-dependent plasticity. In animal models, neurological recovery is associated with structural dendritic and synaptic plasticity in the contralesional striatum (Qin et al., 2014) and with axonal plasticity in contralesional motor cortex (Reitmeir et al., 2012). This suggests that spontaneous recovery after a striatal stroke may also be augmented by inputs from contralateral striatum and could explain why patients with subcortical stroke are more likely to exhibit spontaneous functional neurological recovery (Rothrock et al., 1995; Bejot et al., 2008).

Table 2 | Cell therapy studies in aged animals.

Cell type	Donor	Host	Age (mo)	Sex	Infarct	MCAO	Dose	Time after stroke	Route of administration	Reference
BM MSC	Rat, male	Wistar	11	Female	Neocortical & subcortical	Intraluminal suture occlusion	2×10^6	24 h	Intra-carotid	Shen et al. (2007)
BM MSC	Rat	Long evans rat	12	Male	Neocortical	Cranioectomy, clipping	$4–10 \times 10^6$	24 h	Intra-carotid	Brenneman et al. (2010)
NPC	Human	Fischer 344 rat	24	Male	Neocortical	Cranioectomy, coagulation	1×10^5	3 weeks	Infarct cavity	Jin et al. (2010a)
NPC	Human	Fischer 344 rat	24	Male	Neocortical	Cranioectomy, coagulation	1.2×10^5	2 weeks	Infarct cavity	Jin et al. (2011)
BM MSC	Young SHR-SP rat	Old SHR-SP rat	15	Female	Neocortical	Cranioectomy, coagulation	0.5×10^5	30 days before stroke	Intravenous	Taguchi et al. (2011)
UCTC	Human	Wistar rat	19	Male	Neocortical & subcortical	Embolization	1×10^7	24 h	Intravenous	Zhang et al. (2013)
BM MSC	Rat	Sprague Dawley rat	19	Male	Neocortical	Cranioectomy, hook occlusion	1×10^6	6 h	Intravenous	Balseanu et al. (2014)
iPSC	Human	Sprague Dawley rat	24	Male	Neocortical & subcortical	Intraluminal suture occlusion	0.3×10^6	2 days	Intracortical	Tatarishvili et al. (2014)

BM MSC, bone marrow-derived mesenchymal stem cells; NPC, neural precursor cells; UCTC, umbilical cord tissue cells; iPSC, inducible pluripotent stem cells.

Cell-based therapy augments this endogenous response (Kokaia and Darsalia, 2011). Thus, human iPSCs implanted into striatum of young–adult animals at 1 week after MCAO, protected substantia nigra from atrophy, probably through a trophic effect via release of survival-promoting growth factors (Oki et al., 2012; Polentes et al., 2012; Tornero et al., 2013; Yuan et al., 2013).

How the cells are transplanted and where they are delivered are important issues in stem cell therapy. Data from many groups have shown that stroke increases the proliferation of endogenous neural precursor cells (NPCs) in the ipsilesional subventricular zone (SVZ) of young–adult rodents with a maximum at 1–2 weeks. These endogenous NPCs migrated along a scaffold of blood vessels to the peri-infarct striatum over a period of several months (Darsalia et al., 2007; Kojima et al., 2010). Some NPCs differentiated into medium size spiny neurons and may become part of the neuronal network (Arvidsson et al., 2002; Jin et al., 2006; Thored et al., 2006; Hou et al., 2008; Bacigaluppi et al., 2009; Doeppner et al., 2012; Mine et al., 2013). Noteworthy, the transplanted NPCs also stimulated neurogenesis in the ipsilateral SVZ and subgranular zone of the dentate gyrus (DG; Jin et al., 2011; Zhang et al., 2011; Mine et al., 2013).

In subcortical stroke, the location of the ischemic lesion in relation to the SVZ is thought to play a major role in stroke recovery. Unfortunately, the proportion of surviving neurons is discouragingly low (Arvidsson et al., 2002; Parent et al., 2002). Yet, the formation of neurons in the striatum is preserved in aged animals. Thus, the number of new striatal neurons in aged rodents after stroke was similar to that in young–adult rodents (Darsalia et al., 2005; Ahlenius et al., 2009), despite 50% decline in neurogenesis in the SVZ of elderly compared with young–adult animals (Enwere et al., 2004).

The subventricular cavity is lined up by ependymal cells that are quiescent and do not contribute to neurogenesis under normal conditions. However, in response to stroke ependymal cells enter the cell cycle and generate astrocytes and neuroblasts (Carlén et al., 2009). Signaling through the Notch pathway is required to maintain ependymal cell quiescence and suppresses neuronal differentiation and forced Notch signaling blocked the ependymal cell response to stroke (Carlén et al., 2009). However, in a more recent study ischemia-induced cell proliferation in the SVZ in aged rodents was enhanced by Notch1 activation and was associated with a reduced infarct volume and improved motor deficits (Wang et al., 2009; Sun et al., 2013). Conversely, ablation of doublecortin (DCX)-expressing cells with ganciclovir before MCAO in DCX-TK transgenic mice resulted in an increased infarct size and an adverse effect on functional outcome from cerebral ischemia (Jin et al., 2010b). Likewise, disruption of neurogenesis by low dose of irradiation, which, in part, inhibits DG neurogenesis, is associated with more severe functional impairments after cerebral global ischemia in gerbils (Raber et al., 2004).

Studies on post-mortem brains provided evidence for enhanced SVZ cell proliferation and neuroblast formation after stroke even in aged humans (Jin et al., 2006; Macas et al., 2006; Minger et al., 2007; Martí-Fàbregas et al., 2010). In line with the observation that new neurons are continuously formed in the adult human striatum (Ernst et al., 2014), an increased number of putative neuroblasts was noted in the human striatum in response to stroke (Macas et al., 2006).

However, whether endogenous neurogenesis contributes to spontaneous stroke recovery is still to be established. Human iPSC-derived long-term neuroepithelial-like stem cells (hiPSC-lt-NES) derived from a young adult had the potential to survive, differentiate into immature and mature neurons, and migrate to the peri-infarct tissue, when transplanted into the stroke-injured striatum and cortex in young–adult rats (Oki et al., 2012; Tornero et al., 2013). Young–adult rats treated with these cells showed improved neurological recovery as compared with animals not receiving such grafts (Oki et al., 2012; Tornero et al., 2013). Whether iPSC-lt-NES cells similarly promote neurological recovery in aged rats, when transplanted into subcortical brain areas, still remains to be shown.

NEURAL PRECURSOR AND STEM CELLS IN CORTICAL STROKE

Cortical strokes lack the vicinity of a neurogenic niche like the SVZ, which impedes brain remodeling processes. Indeed, the post-acute delivery of adult mouse NPCs does not prevent secondary degeneration in the young–adult mouse cerebral cortex as much as the striatum, when animals are submitted to intraluminal MCAO (Bacigaluppi et al., 2009). Despite the less potent effects on neuronal survival, NPC delivery reduced glial scar formation in the surrounding of neocortical strokes (Bacigaluppi et al., 2009) and increased axonal plasticity in the contralesional motor cortex (Andres et al., 2011). These data convincingly show that the cerebral cortex does respond to restorative therapies.

If and how the aged brain responds to exogenous NPCs is poorly understood. The DG is one of the few brain regions to support neurogenesis in the adult by the recruitment of new granule cells into the hippocampal circuitry (van Praag et al., 2002). However, the extent of new granule cell recruitment is dramatically reduced in middle-aged (12 month-old) and aged (24 month-old) compared with young–adult (6 week-old) rats (Heine et al., 2004). The reduced precursor cell proliferation was not only caused by a general decline in total precursor cell numbers, but also by a reduced proliferation of the NPCs (Walter et al., 2011).

In animal models, focal neocortical infarcts induced proliferative changes that have been associated with an enhanced number of newborn neurons in the DG of young–adult but not aged mice (Walter et al., 2010). Therefore, later studies examined if exogenous NPC delivery might restore neurogenesis in the DG of old rodents. To test this hypothesis, NPCs isolated from embryonic caudal neural tubes of Sox-2:EGFP transgenic mice were expanded *in vitro* and bilaterally injected into the hippocampus of middle-aged (12 month-old) Fisher-344 rats (Hattiangady et al., 2007). Grafted NPCs migrated to all layers of the hippocampus and enhanced the number of new dentate granule cells. Increased dentate neurogenesis, measured by DCX staining in the aging hippocampus following grafting likely reflected a more conducive microenvironment for proliferation of endogenous NPCs in the presence of grafts of exogenous NPCs (Hattiangady et al., 2007). In another study, intracerebroventricular administration

of conditionally immortalized human fetal brain (CTX0E03) cells in 22 month-old rats, stimulated the proliferation of NPCs in the subgranular zone of the DG as demonstrated by immunohistochemical staining for the immature neuronal marker DCX (Park et al., 2010).

Most previous studies of NPC transplantation in rodents have employed post-ischemic intervals of 1 week or less. However, the option for delayed treatment is clinically important. In aged (24 month-old) Sprague-Dawley rats, transplantation of human embryonic stem cell (hESC)-derived NPCs integrated into Matrigel scaffolds reduced ischemic infarct volume and improved neurological recovery even when implanted into the cavity of neocortical infarcts as late as 3 weeks after stroke (Jin et al., 2010a). In a subsequent study, the transplantation of human NPCs together with Matrigel into the lesioned neocortex of young adult (3 month-old) and aged (24 month-old) male Fisher-344 rats at 2 weeks after stroke stimulated neurogenesis in the SVZ ipsilateral to stroke, as demonstrated by increased numbers of cells expressing the early neuronal lineage marker Dcx at 60 days post-transplantation (Jin et al., 2011).

In a recent study, human iPSC transplanted directly into the damaged neocortex of aged rats survived, differentiated into neurons and improved functional recovery in cylinder test at 4 and 7 weeks (Tatarishvili et al., 2014). The grafted hiPSC suppressed microglia/macrophage activation in the stroke-injured cortex as evidenced by differential morphological changes of these cells in the cell-grafted and vehicle-injected animals (Tatarishvili et al., 2014). Although it is not clear how microglia/macrophages were affected at earlier time-points after stroke, it seems possible that the observed immunomodulatory action of the grafts could contribute to both neuroprotective and plasticity responses. Consistent with our findings, previous studies have shown that human fetal NPCs transplanted into cortex or striatum reduced the number of microglia/macrophages in the peri-infarct tissue 1, 6, and 14 weeks after stroke (Horie et al., 2011; Mine et al., 2013). Similarly, inhibition of microglial activation has also been reported in ischemic mice following systemic delivery of mouse NPCs (Bacigaluppi et al., 2009).

Inducible pluripotent cells generated from human fibroblasts of aged humans may be differentiated into specific cell types, namely into functional motor cortical neurons (Mohamad et al., 2013; Phanthong et al., 2013). Interestingly, the differentiation capacity into motor cortical neurons was the same for iPSCs obtained from 29 and 82 year-old individuals (Boulting et al., 2011).

MESENCHYMAL STEM CELLS IN STRIATAL AND NEOCORTICAL STROKE

Patients with cerebrovascular diseases have decreased numbers of circulating bone marrow-derived CD34+ precursor cells, which have been suggested to have prognostic value for neurologic function in patients with history of brain infarction (Taguchi et al., 2009). These findings have prompted experiments aiming at the restoration of circulating bone marrow-derived precursor cells in stroke models by transplantation of autologous hematopoietic progenitor cells.

Mesenchymal stem cells (MSC) derived either from bone marrow or adipose tissue have repeatedly been shown to ameliorate neurological recovery in experimental stroke models (Schwarting et al., 2008; Honmou et al., 2012; Kocsis and Honmou, 2012). When administered in the acute stroke phase, MSCs decreased infarct volume, improved neurological function, enhanced neurogenesis, and exerted anti-inflammatory actions. MSCs enhance recovery processes in the injured brain by promoting angiogenesis, neurogenesis, and neural reorganization (Hayase et al., 2009; Bao et al., 2011; Lim et al., 2011; Hsieh et al., 2013).

Improved neurological recovery associated with preservation of pyramidal tract axons ipsilateral to the stroke have also been described in 12-month-old ischemic rats systemically treated with bone marrow-derived MSCs (Shen et al., 2007). Neurological improvements persisted for at least 1 year (Shen et al., 2007). When delivered to 12-month-old ischemic rats, the improvement of neurological recovery and reduction of infarct volume induced by autologous bone marrow-derived MSC transplantation resembled that in 2–3-month-old rats (Brenneman et al., 2010), indicating that aging does not impair the responsiveness to MSC therapy. This was particularly noteworthy as the number of transplanted cells in the peri-infarct tissue decreased within hours and were almost undetectable after 7 days (Brenneman et al., 2010).

Similar to bone marrow-derived cells, umbilical cord-derived blood cells (UCBCs) are widely available, representing an attractive source for cell-based therapies. Human UCBCs are rich in mesenchymal and endothelial precursor cells and can be collected without the ethical concerns associated with embryonic or fetal cells. Intravenous injection of human UCBCs in aged (20-month-old) rats restored the age-related decrease of endogenous neurogenesis and reduced brain inflammation (Bachstetter et al., 2008). In young–adult rats exposed to focal cerebral ischemia, intravenous delivery of CD34+ UCBCs enhanced endogenous angiogenesis, neurogenesis and functional neurological recovery in some (Chen et al., 2001; Taguchi et al., 2004a,b), but not other (Mäkinen et al., 2006) studies. In aged (18–20-month-old) rats exposed to focal cerebral ischemia, intravenous delivery of human umbilical tissue-derived cells improved functional neurological recovery and increased angiogenesis and synaptogenesis (Zhang et al., 2013). This suggests that umbilical cells may be used for stroke treatment in the aged ischemic brain.

Based on these observations, further clinical studies using intravenously delivered MSCs have been initiated in human stroke subjects (Lee et al., 2010; Moniche et al., 2012). Transplantation of autologous bone marrow-derived MSCs in pilot studies has been proven safe in human patients with acute middle cerebral artery infarcts (Bang et al., 2005; Battistella et al., 2011; Savitz et al., 2011; Friedrich et al., 2012; Moniche et al., 2012; Banerjee et al., 2014). Moreover, improvements of neurological recovery were noted (Bang et al., 2005; Battistella et al., 2011; Savitz et al., 2011; Friedrich et al., 2012; Moniche et al., 2012; Banerjee et al., 2014). Unfortunately, these studies lacked appropriate control groups, which limits conclusions around the efficacy of cell therapy.

In the translation of studies from bench to bedside, care should be taken that not only aging, but also age-related co-morbidities may affect brain remodeling and responses to cell-based therapies. Thus, the delivery of bone marrow-derived MSCs did not improve

neurological recovery in rats exhibiting streptozotocin-induced type I diabetes, but increased mortality, blood–brain barrier leakage and brain hemorrhage (Chen et al., 2011). Besides, excessive angiogenesis was noted in diabetic rats receiving MSCs that was associated with cerebral arteriole narrowing and neointima formation inside the internal carotid artery (Chen et al., 2011). In histochemical studies, increased macrophage accumulation was noted in blood vessels of diabetic MSC treated rats. The authors suggested that MSC treatment should not be considered in diabetic patients.

Not only type I diabetes, but also hyperlipidemia may affect post-ischemic brain remodeling, as shown in studies showing the attenuation of VEGF-induced angiogenesis in ischemic ApoE−/− mice receiving Western diet (Zechariah et al., 2013), presumably via internalization and degradation of VEGF receptor-2 (Jin et al., 2013). Further studies identifying the conditions of efficacy and safety of cell-based therapy under conditions of vascular risk factors and diseases are warranted.

CO-TRANSPLANTATION STRATEGIES AND COMBINATION THERAPIES

Poor survival and differentiation of both the transplanted cells and their progenies in the inhospitable environment of the infarcted cortex has prompted the search for alternatives and new concepts like the neurovascular unit to limit the loss of transplanted cells. To this end cotransplantation of endothelial cells with NPCs in a mouse model of stroke enhanced the survival, proliferation, and differentiation of transplanted cells and improved functional neurological recovery (Nakagomi et al., 2009). Even more challenging, cotransplantation of mouse embryonic stem cell-derived vascular progenitor cells (VPCs) with NPCs after ischemic stroke produced not only neural cells but also endothelial cells and pericytes, thus providing nearly all important components for recovery of the neurovascular unit, i.e., neurons, astroglia, endothelial cells, and mural vascular cells (Li et al., 2014).

Granulocyte colony stimulating factor (G-CSF) has been particularly successful when used to improve neurological function in various types of focal cerebral ischemia in young–adult animals (Shyu et al., 2004; Schäbitz and Schneider, 2007). We have recently been able to show that G-CSF treatment in 20-month-old aged rats enhances animal survival, improved functional neurological recovery, and induced neurogenesis in the ipsilesional SVZ (Popa-Wagner et al., 2010). Next, we reasoned that the efficiency of the bone marrow-derived-cell therapy may be increased by simultaneous application of G-CSF. In particular, we tested the hypothesis that grafting of pre-differentiated bone marrow-derived MSCs in G-CSF-treated animals would improve long term functional outcome in aged rodents (Balseanu et al., 2014). Although the combination therapy significantly improved neurological recovery and increased microvessel density in the former infarct core, neither G-CSF nor the combination decreased animal mortality, reduced infarct volume or increased neurogenesis (Balseanu et al., 2014).

The mechanisms by which MSCs may ameliorate infarcted brain tissue seem related more to the capacity of MSCs to release neuroprotective factors (paracrine action) than to their capacity to replace damaged neural cells. Thus, around the former infarct core, several groups noted vigorous angiogenesis as evidenced by BrdU-positive endothelial cells (reviewed in Bronckaers et al., 2014). In our study, the density of the newly formed blood vessels was significantly higher in the brains of aged animals treated with the combination of G-CSF and MSCs as compared to controls and G-CSF, but not MSCs alone (Balseanu et al., 2014; **Figure 1**).

CONCLUSION

Taken together,

(i) the restorative potential of the brain is preserved in aged, ischemic animals, although specific age-related aspects appear to exist related to the accelerated progression of infarction, the decreased proliferation of endogenous NPCs and the delayed initiation of neurological recovery (Zhang et al., 2013; Balseanu et al., 2014),

(ii) the environment of the aged brain does not preclude effects of grafted NPCs, MSCs, or iPSCs on brain remodeling, endogenous neurogenesis and functional neurological recovery,

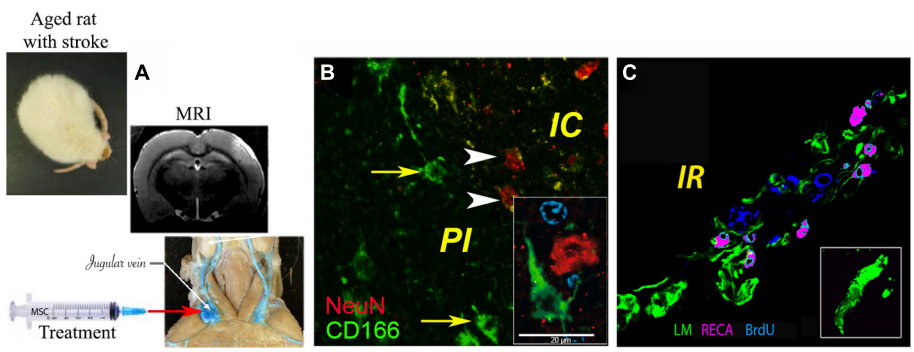

FIGURE 1 | Delivery of bone marrow-derived MSCs in aged rats exposed to focal cerebral ischemia. (A) Administration of MSCs in the acute stroke phase via the jugular vein, brain infarcts being detected by MRI; **(B)** histochemical analysis of CD166+ cells (green) in the infarcted area, where ∼1% of injected MSCs were found intermingled with NeuN+ neurons (green); **(C)** angiogenesis as revealed by rat endothelial cell antigen (RECA; purple)/BrdU (blue) immunostaining. Basal laminae were counterstained with antibody against laminin (LM; green; modified based on Balseanu et al., 2014). Scale bar, 20 μm.

(iii) detrimental consequences may result from age-related vascular co-morbidities, namely from diabetes or hyperlipidemia, in which maladaptive or impaired brain remodeling has been described,

(iv) although endogenous neurogenesis has been observed, even in aged humans, questions remain regarding the functional relevance of newly formed neurons for stroke recovery in human patients, where distances between endogenous stem cell niches and stroke injuries are so much larger due to the bigger size of the brain. Further research must explore optimal transplantation protocols (Dailey et al., 2013). Fascinating possibilities have recently been identified in the transdifferentiation capacity of iPSCs to specific neuronal subtypes, which might open new perspectives for cell replacement therapy. Whether such strategies may at all contribute to functional neurological recovery, remains unclear.

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REFERENCES

Ahlenius, H., Visan, V., Kokaia, M., Lindvall, O., and Kokaia, Z. (2009). Neural stem and progenitor cells retain their potential for proliferation and differentiation into functional neurons despite lower number in aged brain. *J. Neurosci.* 29, 4408–4419. doi: 10.1523/JNEUROSCI.6003-08.2009

Andres, R. H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., et al. (2011). Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain* 134, 1777–1789. doi: 10.1093/brain/awr094

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Ay, H., Koroshetz, W. J., Vangel, M., Benner, T., Melinosky, C., Zhu, M., et al. (2005). Conversion of ischemic brain tissue into infarction increases with age. *Stroke* 36, 2632–2636. doi: 10.1161/01.STR.0000189991.23918.01

Bachstetter, A. D., Pabon, M. M., Cole, M. J., Hudson, C. E., Sanberg, P. R., Willing, A. E., et al. (2008). Peripheral injection of human umbilical cord blood stimulates neurogenesis in the aged rat brain. *BMC Neurosci.* 9:22. doi: 10.1186/1471-2202-9-22

Bacigaluppi, M., Comi, G., and Hermann, D. M. (2010). Animal models of ischemic stroke. Part two: modeling cerebral ischemia. *Open Neurol. J.* 4, 34–38. doi: 10.2174/1874205X01004020026

Bacigaluppi, M., Pluchino, S., Jametti, L. P., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251. doi: 10.1093/brain/awp174

Badan, I., Buchhold, B., Hamm, A., Gratz, M., Walker, L. C., Platt, D., et al. (2003). Accelerated glial reactivity to stroke in aged rats correlates with reduced functional recovery. *J Cereb. Blood Flow Metab.* 23, 845–854. doi: 10.1097/01.WCB.0000071883.63724.A7

Balseanu, A. T., Buga, A. M., Catalin, B., Wagner, D. C., Boltze, J., Zagrean, A. M., et al. (2014). Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. *Front. Aging Neurosci.* 6:130. doi: 10.3389/fnagi.2014.00130

Banerjee, S., Bentley, P., Hamady, M., Marley, S., Davis, J., and Shlebak, A. (2014). Intra-Arterial immunoselected CD34+ stem sells for acute ischemic stroke. *Stem Cells Transl. Med.* doi: 10.5966/sctm.2013-0178 [Epub ahead of print].

Bang, O. Y., Lee, J. S., Lee, P. H., and Lee, G. (2005). Autologous mesenchymal stem cell transplantation in stroke patients. *Ann. Neurol.* 57, 874–882. doi: 10.1002/ana.20501

Bao, X., Wei, J., Feng, M., Lu, S., Li, G., Dou, W., et al. (2011). Transplantation of human bone marrow-derived mesenchymal stem cells promotes behavioral recovery and endogenous neurogenesis after cerebral ischemia in rats. *Brain Res.* 1367, 103–113. doi: 10.1016/j.brainres.2010.10.063

Battistella, V., de Freitas, G. R., da Fonseca, L. M., Mercante, D., Gutfilen, B., Goldenberg, R. C., et al. (2011). Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke. *Regen. Med.* 6, 45–52. doi: 10.2217/rme.10.97

Bejot, Y., Catteau, A., Caillier, M., Rouaud, O., Durier, J., Marie, C., et al. (2008). Trends in incidence, risk factors, and survival in symptomatic lacunar stroke in Dijon, France, from 1989 to 2006: a population-based study. *Stroke* 39, 1945–1951. doi: 10.1161/STROKEAHA.107.510933

Boulting, G. L., Kiskinis, E., Croft, G. F., Amoroso, M. W., Oakley, D. H., Wainger, B. J., et al. (2011). A functionally characterized test set of human induced pluripotent stem cells. *Nat. Biotechnol.* 29, 279–286. doi: 10.1038/nbt.1783

Brenneman, M., Sharma, S., Harting, M., Strong, R., Cox, C. S. Jr., Aronowski, J., et al. (2010). Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J. Cereb. Blood Flow Metab.* 30, 140–149. doi: 10.1038/jcbfm.2009.198

Bronckaers, A., Hilkens, P., Martens, W., Gervois, P., Ratajczak, J., Struys, T., et al. (2014). Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol. Ther.* 143, 181–196. doi: 10.1016/j.pharmthera.2014.02.013

Buchhold, B., Mogoanta, L., Suofu, Y., Hamm, A., Walker, L., Kessler, Ch., et al. (2007). Environmental enrichment improves functional and neuropathological indices following stroke in young and aged rats. *Restor. Neurol. Neurosci.* 25, 467–484.

Carlén, M., Meletis, K., Göritz, C., Darsalia, V., Evergren, E., Tanigaki, K., et al. (2009). Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. *Nat. Neurosci.* 12, 259–267. doi: 10.1038/nn.2268

Chen, J., Sanberg, P. R., Li, Y., Wang, L., Lu, M., Willing, A. E., et al. (2001). Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 32, 2682–2688. doi: 10.1161/hs1101.098367

Chen, J., Ye, X., Yan, T., Zhang, C., Yang, X. P., Cui, X., et al. (2011). Adverse effects of bone marrow stromal cell treatment of stroke in diabetic rats. *Stroke* 42, 3551–3558. doi: 10.1161/STROKEAHA.111.627174

Dailey, T., Metcalf, C., Mosley, Y. I., Sullivan, R., Shinozuka, K., Tajiri, N., et al. (2013). An update on translating stem cell therapy for stroke from bench to bedside. *J. Clin. Med.* 2, 220–241. doi: 10.3390/jcm2040220

Darsalia, V., Heldmann, U., Lindvall, O., and Kokaia, Z. (2005). Stroke-induced neurogenesis in aged brain. *Stroke* 36, 1790–1795. doi: 10.1161/01.STR.0000173151.36031.be

Darsalia, V., Kallur, T., and Kokaia, Z. (2007). Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. 1193903071 *Eur. J. Neurosci.* 26, 605–614. doi: 10.1111/j.1460-9568.2007.05702.x

Davis, M., Mendelw, A. D., Perry, R. H., Chambers, I. R., and James, O. F. (1995). Experimental stroke and neuroprotection in the aging rat brain. *Stroke* 26, 1072–1078. doi: 10.1161/01.STR.26.6.1072

Dinapoli, V. A., Rosen, C. L., Nagamine, T., and Crocco, T. (2006). Selective MCA occlusion: a precise embolic stroke model. *J. Neurosci. Methods* 154, 233–238. doi: 10.1016/j.jneumeth.2005.12.026

Doeppner, T. R., Ewert, T. A., Tönges, L., Herz, J., Zechariah, A., ElAli, A., et al. (2012). Transduction of neural precursor cells with TAT-heat shock protein 70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. *Stem Cells* 30, 1297–1310. doi: 10.1002/stem.1098

Dunnett, S. B. (2013). Neural tissue transplantation, repair, and rehabilitation. *Handb. Clin. Neurol.* 110, 43–59. doi: 10.1016/B978-0-444-52901-5.00004-6

Enwere, E., Shingo, T., Gregg, C., Fujikawa, H., Ohta, S., and Weiss, S. (2004). Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis. *J. Neurosci.* 24, 8354–8365. doi: 10.1523/JNEUROSCI.2751-04.2004

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., et al. (2014). Neurogenesis in the striatum of the adult human brain. *Cell* 156, 1072–1083. doi: 10.1016/j.cell.2014.01.044

Feigin, V. L., Lawes, C. M., Bennett, D. A., and Anderson, C. S. (2003). Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol.* 2, 43–53. doi: 10.1016/S1474-4422(03)00266-7

Friedrich, M. A., Martins, M. P., Arau´jo, M. D., Klamt, C., Vedolin, L., Garicochea, B., et al. (2012). Intra-arterial infusion of autologous bone-marrow mononuclear cells in patients with moderate to severe middle-cerebral-artery acute ischemic stroke. *Cell Transplant.* 21(Suppl. 1), S13–S21. doi: 10.3727/096368912X612512

Futrell, N., Garcia, J. H., Peterson, E., and Millikan, C. (1991). Embolic stroke in aged rats. *Stroke* 22, 1582–1591. doi: 10.1161/01.STR.22.12.1582

Gokcay, F., Arsava, E. M., Baykaner, T., Vangel, M., Garg, P., Wu, O., et al. (2011). Age-dependent susceptibility to infarct growth in women. *Stroke* 42, 947–951. doi: 10.1161/STROKEAHA.110.603902

Haas, S., Weidner, N., and Winkler, J. (2005). Adult stem cell therapy in stroke. *Curr. Opin. Neurol.* 18, 59–64. doi: 10.1097/00019052-200502000-00012

Hallett, M. (2001). Plasticity of the human motor cortex and recovery from stroke. *Brain Res. Rev.* 36, 169–174. doi: 10.1016/S0165-0173(01)00092-3

Hattiangady, B., Shuai, B., Cai, J., Coksaygan, T., Rao, M. S., and Shetty, A. K. (2007). Increased dentate neurogenesis after grafting of glial restricted progenitors or neural stem cells in the aging hippocampus. *Stem Cells* 25, 2104–2117. doi: 10.1634/stemcells.2006-0726

Hayase, M., Kitada, M., Wakao, S., Itokazu, Y., Nozaki, K., Hashimoto, N., et al. (2009). Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke. *J. Cereb. Blood Flow Metab.* 29, 1409–1420. doi: 10.1038/jcbfm.2009.62

Heine, V. M., Maslam, S., Joëls, M., and Lucassen, P. J. (2004). Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. *Neurobiol. Aging* 25, 361–375. doi: 10.1016/S0197-4580(03)00090-3

Hermann, D. M., and Chopp, M. (2012). Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurol.* 11, 369–380. doi: 10.1016/S1474-4422(12)70039-X

Hicks, A. U., Hewlett, K., Windle, V., Chernenko, G., Ploughman, M., Jolkkonen, J., et al. (2007). Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. *Neuroscience* 146, 31–40. doi: 10.1016/j.neuroscience.2007.01.020

Honmou, O., Onodera, R., Sasaki, M., Waxman, S. G., and Kocsis, J. D. (2012). Mesenchymal stem cells: therapeutic outlook for stroke. *Trends Mol. Med.* 18, 292–297. doi: 10.1016/j.molmed.2012.02.003

Horie, N., Pereira, M. P., Niizuma, K., Sun, G., Keren-Gill, H., Encarnacion, A., et al. (2011). Transplanted stem cell-secreted vascular endothelial growth factor effects poststroke recovery, inflammation, and vascular repair. *Stem Cells* 29, 274–285. doi: 10.1002/stem.584

Hou, S. W., Wang, Y. Q., Xu, M., Shen, D. H., Wang, J. J., Huang, F., et al. (2008). Functional integration of newly generated neurons into striatum after cerebral ischemia in the adult rat brain. *Stroke* 39, 2837–2844. doi: 10.1161/STROKEAHA.107.510982

Hsieh, J.-Y., Wang, H.-W., Chang, S.-J., Liao, K.-H., Lee, I.-H., Lin, W. S., et al. (2013). Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. *PLoS ONE* 8:e72604. doi: 10.1371/journal.pone.0072604

Jin, F., Hagemann, N., Brockmeier, U., Schäfer, S. T., Zechariah, A., and Hermann, D. M. (2013). LDL attenuates VEGF-induced angiogenesis via mechanisms involving VEGFR2 internalization and degradation following endosome-trans-Golgi network trafficking. *Angiogenesis* 16, 625–637. doi: 10.1007/s10456-013-9340-2

Jin, K., Mao, X., Xie, L., Greenberg, R. B., Peng, B., Moore, A., et al. (2010a). Delayed transplantation of human neural precursor cells improves outcome from focal cerebral ischemia in aged rats. *Aging Cell* 9, 1076–1083. doi: 10.1111/j.1474-9726.2010.00638.x

Jin, K., Wang, X., Xie, L., Mao, X. O., and Greenberg, D. A. (2010b). Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. *Proc. Natl. Acad. Sci. U.S.A.* 27, 7993–7998. doi: 10.1073/pnas.1000154107

Jin, K., Wang, X., Xie, L., Mao, X. O., Zhu, W., Wang, Y., et al. (2006). Evidence for stroke-induced neurogenesis in the human brain. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13198–13202. doi: 10.1073/pnas.0603512103

Jin, K., Xie, L., Mao, X., Greenberg, M. B., Moore, A., Peng, B., et al. (2011). Effect of human neural precursor cell transplantation on endogenous neurogenesis after focal cerebral ischemia in the rat. *Brain Res.* 1374, 56–62. doi: 10.1016/j.brainres.2010.12.037

Katsman, D., Zheng, J., Spinelli, K., and Carmichael, S. T. (2003). Tissue microenvironments within functional cortical subdivisions adjacent to focal stroke. *J. Cereb. Blood Flow Metab.* 23, 997–1009. doi: 10.1097/01.WCB.0000084252.20114.BE

Kocsis, J. D., and Honmou, O. (2012). Bone marrow stem cells in experimental stroke. *Prog. Brain Res.* 201, 79–98. doi: 10.1016/B978-0-444-59544-7.00005-6

Kojima, T., Hirota, Y., Ema, M., Takahashi, S., Miyoshi, I., Okano, H., et al. (2010). Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells* 28, 545–554. doi: 10.1002/stem.306

Kokaia, Z., and Darsalia, V. (2011). Neural stem cell-based therapy for ischemic stroke. *Transl. Stroke Res.* 2, 272–278. doi: 10.1007/s12975-011-0100-6

Kondziolka, D., Wechsler, L., Goldstein, S., Meltzer, C., Thulborn, K. R., Gebel, J., et al. (2000). Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* 55, 565–569. doi: 10.1212/WNL.55.4.565

Lee, J. S., Hong, J. M., Moon, G. J., Lee, P. H., Ahn, Y. H., and Bang, O. Y. (2010). A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 28, 1099–1106. doi: 10.1002/stem.430

Li, J., Tang, Y., Wang, Y., Tang, R., Jiang, W., Yang, G. Y., et al. (2014). Neurovascular recovery via cotransplanted neural and vascular progenitors leads to improved functional restoration after ischemic stroke in rats. *Stem Cell Rep.* 3, 101–114. doi: 10.1016/j.stemcr.2014.05.012

Liepert, J., Hamzei, F., and Weiller, C. (2004). Lesion-induced and training-induced brain reorganization. *Restor. Neurol. Neurosci.* 22, 269–277.

Lim, J. Y., Jeong, C. H., Jun, J. A., Kim, S. M., Ryu, C. H., Hou, Y., et al. (2011). Therapeutic effects of human umbilical cord blood-derived mesenchymal stem cells after intrathecal administration by lumbar puncture in a rat model of cerebral ischemia. *Stem Cell Res. Ther.* 2, 38. doi: 10.1186/scrt79

Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., De Simone, G., et al. (2010). Executive summary: heart disease and stroke statistics—2010 update: a report from the American Heart Association. *Circulation* 121, 948–954. doi: 10.1161/CIRCULATIONAHA.109.192666

Macas, J., Nern, C., Plate, K. H., and Momma, S. (2006). Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J. Neurosci.* 26, 13114–13119. doi: 10.1523/JNEUROSCI.4667-06.2006

Mäkinen, S., Kekarainen, T., Nystedt, J., Liimatainen, T., Huhtala, T., Närvänen, A., et al. (2006). Human umbilical cord blood cells do not improve sensorimotor or cognitive outcome following transient middle cerebral artery occlusion in rats. *Brain Res.* 1123, 207–215. doi: 10.1016/j.brainres.2006.09.056

Martí-Fàbregas, J., Romaguera-Ros, M., Gómez-Pinedo, U., Martínez-Ramírez, S., Jiménez-Xarrié, E., Marín, R., et al. (2010). Proliferation in the human ipsilateral subventricular zone after ischemic stroke. *Neurology* 74, 357–365. doi: 10.1212/WNL.0b013e3181cbccec

Mine, Y., Tatarishvili, J., Oki, K., Monni, E., Kokaia, Z., and Lindvall, O. (2013). Grafted human neural stem cells enhance several steps of endogenous neurogenesis and improve behavioral recovery after middle cerebral artery occlusion in rats. *Neurobiol. Dis.* 52, 191–203. doi: 10.1016/j.nbd.2012.12.006

Minger, S. L., Ekonomou, A., Carta, E. M., Chinoy, A., Perry, R. H., and Ballard, C. G. (2007). Endogenous neurogenesis in the human brain following cerebral infarction. *Regen. Med.* 2, 69–74. doi: 10.2217/17460751.2.1.69

Mohamad, O., Drury-Stewart, D., Song, M., Faulkner, B., Chen, D., Yu, S. P., et al. (2013). Vector-free and transgene-free human iPS cells differentiate into functional neurons and enhance functional recovery after ischemic stroke in mice. *PLoS ONE* 23:8. doi: 10.1371/journal.pone.0064160

Moniche, F., Gonzalez, A., Gonzalez-Marcos, J. R., Carmona, M., Piñero, P., Espigado, I., et al. (2012). Intra-arterial bone marrow mononuclear cells in ischemic stroke: a pilot clinical trial. *Stroke* 43, 2242–2244. doi: 10.1161/STROKEAHA.112.659409

Morizane, A., Li, J. Y., and Brundin, P. (2008). From bench to bed: the potential of stem cells for the treatment of Parkinson's disease. *Cell Tissue Res.* 331, 323–336. doi: 10.1007/s00441-007-0541-0

Nakagomi, N., Nakagomi, T., Kubo, S., Nakano-Doi, A., Saino, O., Takata, M., et al. (2009). Endothelial cells support survival, proliferation, and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor

cells after cerebral infarction. *Stem Cells* 27, 2185–2195. doi: 10.1002/stem.161

Oki, K., Tatarishvili, J., Wood, J., Koch, P., Wattananit, S., Mine, Y., et al. (2012). Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells* 30, 1120–1133. doi: 10.1002/stem.1104

Parent, J. M., Vexler, Z. S., Gong, C., Derugin, N., and Ferriero, D. M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann. Neurol.* 52, 802–813. doi: 10.1002/ana.10393

Park, D. H., Eve, D. J., Sanberg, P. R., Musso, J. III, Bachstetter, A. D., Wolfson, A., et al. (2010). Increased neuronal proliferation in the dentate gyrus of aged rats following neural stem cell implantation. *Stem Cells Dev.* 19, 175–180. doi: 10.1089/scd.2009.0172

Phanthong, P., Raveh-Amit, H., Li, T., Kitiyanant, Y., and Dinnyes, A. (2013). Is aging a barrier to reprogramming? Lessons from induced pluripotent stem cells. *Biogerontology* 14, 591–602. doi: 10.1007/s10522-013-9455-2

Polentes, J., Jendelova, P., Cailleret, M., Braun, H., Romanyuk, N., Tropel, P., et al. (2012). Human induced pluripotent stem cells improve stroke outcome and reduce secondary degeneration in the recipient brain. *Cell Transplant.* 21, 2587–2602. doi: 10.3727/096368912X653228

Popa-Wagner, A., Carmichael, S. T., Kokaia, Z., Kessler, C., and Walker, L. C. (2007). The response of the aged brain to stroke: too much, too soon? *Curr. Neurovasc. Res.* 4, 216–227. doi: 10.2174/156720207781387213

Popa-Wagner, A., Schroder, E., Walker, L. C., and Kessler, C. (1998). β-Amyloid precursor protein and β-amyloid peptide immunoreactivity in the rat brain after middle cerebral artery occlusion: effect of age. *Stroke* 29, 2196–2202. doi: 10.1161/01.STR.29.10.2196

Popa-Wagner, A., Stocker, K., Balseanu, A. T., Rogalewski, A., Diederich, K., Minnerup, J., et al. (2010). Effects of granulocyte-colony stimulating factor after stroke in aged rats. *Stroke* 41, 1027–1031. doi: 10.1161/STROKEAHA.109.575621

Qin, L., Jing, D., Parauda, S., Carmel, J., Ratan, R. R., Lee, F. S., et al. (2014). An adaptive role for BDNF Val66Met polymorphism in motor recovery in chronic stroke. *J. Neurosci.* 34, 2493–2502. doi: 10.1523/JNEUROSCI.4140-13.2014

Raber, J., Fan, Y., Matsumori, Y., Liu, Z., Weinstein, P. R., Fike, J. R., et al. (2004). Irradiation attenuates neurogenesis and exacerbates ischemia-induced deficits. *Ann. Neurol.* 55, 381–389. doi: 10.1002/ana.10853

Reitmeir, R., Kilic, E., Reinboth, B. S., Guo, Z., ElAli, A., Zechariah, A., et al. (2012). Vascular endothelial growth factor induces contralesional corticobulbar plasticity and functional neurological recovery in the ischemic brain. *Acta Neuropathol.* 123, 273–284. doi: 10.1007/s00401-011-0914-z

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., et al. (2012). Heart disease and stroke statistics-2012 update: a report from the American Heart Association. *Circulation* 125, e2–e220. doi: 10.1161/CIR.0b013e31823ac046

Rothrock, J. F., Clark, W. M., and Lyden, P. D. (1995). Spontaneous early improvement following ischemic stroke. *Stroke* 26, 1358–1360. doi: 10.1161/01.STR.26.8.1358

Savitz, S. I., Misra, V., Kasam, M., Juneja, H., Cox, C. S. Jr., Alderman, S., et al. (2011). Intravenous autologous bone marrow mononuclear cells for ischemic stroke. *Ann. Neurol.* 70, 59–69. doi: 10.1002/ana.22458

Schäbitz, W. R., and Schneider, A. (2007). New targets for established protein: exploring G-CSF for the treatment of stroke. *Trends Pharmacol. Sci.* 28, 157–161. doi: 10.1016/j.tips.2007.02.007

Schwarting, S, Litwak, S., Hao, W., Baehr, M., Weise, J., and Neumann, H. (2008). Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. *Stroke* 39, 2867–2875. doi: 10.1161/STROKEAHA.108.513978

Shen, L. H., Li, Y., Chen, J., Cui, Y., Zhang, C., Kapke, A., et al. (2007). One-year follow-up after bone marrow stromal cell treatment in middle-aged female rats with stroke. *Stroke* 38, 2150–2156. doi: 10.1161/STROKEAHA.106.481218

Shyu, W. C., Lin, S. Z., Yang, H. I., Tzeng, Y. S., Pang, C. Y., Yen, P. S., et al. (2004). Functional recovery of stroke rats induced by granulocyte colony-stimulating factor-stimulated stem cells. *Circulation* 110, 1847–1854. doi: 10.1161/01.CIR.0000142616.07367.66

Soleman, S., Yip, P., Leasure, J. L., and Moon, L. (2010). Sustained sensorimotor impairments after endothelin-1 induced focal cerebral ischemia (stroke) in aged rats. *Exp. Neurol.* 222, 13–24. doi: 10.1016/j.expneurol.2009.11.007

Stoll, E. A. (2014). Advances toward regenerative medicine in the central nervous system: challenges in making stem cell therapy a viable clinical strategy. *Mol. Cell. Ther.* 2, 12. doi: 10.1186/2052-8426-2-12

Sun, F., Mao, X., Xie, L., Ding, M., Shao, B., and Jin, K. (2013). Notch1 signaling modulates neuronal progenitor activity in the subventricular zone in response to aging and focal ischemia. *Aging Cell* 12, 978–987. doi: 10.1111/acel.12134

Sutherland, G. R., Dix, G. A., and Auer, R. N. (1996). Effect of age in rodent models of focal and forebrain ischemia. *Stroke* 27, 1663–1667. doi: 10.1161/01.STR.27.9.1663

Tacutu, R., Budovsky, A., and Fraifeld, V. E. (2010). The NetAge database: a compendium of networks for longevity, age-related diseases and associated processes. *Biogerontology* 11, 513–522. doi: 10.1007/s10522-010-9265-8

Tacutu, R., Budovsky, A., Yanai, H., and Fraifeld, V. E. (2011). Molecular links between cellular senescence, longevity and age-related diseases-a systems biology perspective. *Aging (Albany N.Y.)* 3, 1178–1191.

Taguchi, A., Matsuyama, T., Moriwaki, H., Hayashi, T., Hayashida, K., Nagatsuka, K., et al. (2004a). Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation* 109, 2972–2975. doi: 10.1161/01.CIR.0000133311.25587.DE

Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., et al. (2004b). Administration of $CD34^+$ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J. Clin. Invest.* 114, 330–338. doi: 10.1172/JCI200420622

Taguchi, A., Nakagomi, N., Matsuyama, T., Kikuchi-Taura, A., Yoshikawa, H., Kasahara, Y., et al. (2009). Circulating CD34-positive cells have prognostic value for neurologic function in patients with past cerebral infarction. *J. Cereb. Blood Flow Metab.* 29, 34–38. doi: 10.1038/jcbfm.2008.92

Taguchi, A., Zhu, P., Cao, F., Kikuchi-Taura, A., Kasahara, Y., Stern, D. M., et al. (2011). Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. *J. Cereb. Blood. Flow. Metab.* 31, 855–867. doi: 10.1038/jcbfm.2010.165

Tatarishvili, J., Oki, K., Monni, E., Koch, P., Memanishvili, T., Buga, A. M., et al. (2014). Human induced pluripotent stem cells improve recovery in stroke-injured aged rats. *Restor. Neurol. Neurosci.* 32, 547–558. doi: 10.3233/RNN-140404

Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Ekdahl, C. T., et al. (2006). Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* 24, 739–747. doi: 10.1634/stemcells.2005-0281

Tornero, D., Wattananit, S., Gronning-Madsen, M., Koch, P., Wood, J., Tatarishvili, J., et al. (2013). Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain* 136, 3561–3577. doi: 10.1093/brain/awt278

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D., and Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature* 415, 1030–1034. doi: 10.1038/4151030a

Walter, J., Keiner, S., Witte, O. W., and Redecker, C. (2010). Differential stroke-induced proliferative response of distinct precursor cell subpopulations in the young and aged dentate gyrus. *Neuroscience* 169, 1279–1286. doi: 10.1016/j.neuroscience.2010.05.035

Walter, J., Keiner, S., Witte, O. W., and Redecker, C. (2011). Age-related effects on hippocampal precursor cell subpopulations and neurogenesis. *Neurobiol. Aging* 32, 1906–1914. doi: 10.1016/j.neurobiolaging.2009.11.011

Wang, X., Mao, X., Xie, L., Greenberg, D. A., and Jin, K. (2009). Involvement of Notch1 signaling in neurogenesis in the subventricular zone of normal and ischemic rat brain *in vivo*. *J. Cereb. Blood Flow Metab.* 29, 1644–1654. doi: 10.1038/jcbfm.2009.83

Yuan, T., Liao, W., Feng, N. H., Lou, Y. L., Niu, X., Zhang, A. J., et al. (2013). Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurologic function in a rat model of middle cerebral artery occlusion. *Stem Cell Res. Ther.* 4, 73. doi: 10.1186/scrt224

Zechariah, A., ElAli, A., Hagemann, N., Jin, F., Doeppner, T. R., Helfrich, I., et al. (2013). Hyperlipidemia attenuates vascular endothelial growth factor-induced angiogenesis, impairs cerebral blood flow, and disturbs stroke recovery via decreased pericyte coverage of brain endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 33, 1561–1567. doi: 10.1161/ATVBAHA.112.300749

Zhang, J., Meng, L., Qin, W., Liu, N., Shi, F. D., and Yu, C. (2014). Structural damage and functional reorganization in ipsilesional m1 in well-recovered patients with subcortical stroke. *Stroke* 45, 788–793. doi: 10.1161/STROKEAHA.113.003425

Zhang, L., Yi, L., Chopp, M., Kramer, B. C., Romanko, M., Gosiewska, A., et al. (2013). Intravenous administration of human umbilical tissue-derived cells improves neurological function in aged rats after embolic stroke. *Cell Transplant.* 22, 1569–1576. doi: 10.3727/096368912X658674

Zhang, L., Zhang, R. L., Wang, Y., Zhang, C., Zhang, Z. G., Chopp, M., et al. (2005). Functional recovery in aged and young rats after embolic stroke: treatment with a phosphodiesterase type 5 inhibitor. *Stroke* 36, 847–852. doi: 10.1161/01.STR.0000158923.19956.73

Zhang, R. L., Chopp, M., Roberts, C., Jia, L., Wei, M., Lu, M., et al. (2011). Ascl1 lineage cells contribute to ischemia-induced neurogenesis and oligodendrogenesis. *J. Cereb. Blood Flow Metab.* 31, 614–625. doi: 10.1038/jcbfm.2010.134

Zhao, C.-S., Puurunen, K., Schallert, T., Sivenius, J., and Jolkkonen, J. (2005). Behavioral effects of photothrombotic ischemic cortical injury in aged rats treated with the sedative-hypnotic GABAergic drug zopiclone. *Behav. Brain Res.* 160, 260–266. doi: 10.1016/j.bbr.2004.12.007

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Stem cells for neonatal stroke- the future is here

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STEM CELLS

In recent years stem cell therapy has emerged as a potential treatment for neonatal ischemic brain injury. The efficacy of cell- based therapies in restoring damaged brain tissue has been tested in a multitude of models for different CNS diseases. Several different stem and progenitor cell populations have been utilized as cell-based therapy, including neural stem cells, embryonic stem cells, human umbilical cord blood cells (HUBCs), hematopoietic stem and progenitor cells, and mesenchymal stem cells (MSCs). Most stem cell types appear to enhance recovery to some extent (Pimentel-Coelho and Mendez-Otero, 2010). However, because of their low immunogenicity, availability and positive results obtained from preclinical studies, MSCs are a particularly promising candidate to repair the devastating effects that are associated with neonatal stroke. MSCs were first isolated and identified in bone marrow, but can now be isolated from many tissues, including adipose tissue, muscle, skin and extraembryonic tissues like the placenta, umbilical cord and Wharton's jelly. The latter sources are of particular interest for neonates that experience an ischemic event around the time of birth, at which time cells can be harvested and transplanted from an autologous source. MSCs derived from different sources have slightly different characteristics, but as of yet it is unknown whether this influences their therapeutic potential.

Our group and others have shown that administration of MSCs reduces lesion volume, provides positive effects on the white matter and improves motor function (van Velthoven et al., 2012). Numerous studies have been done under the premise that transplanted stem cells contribute to brain repair by directly replacing damaged or lost tissue. While there is evidence that transplanted cells undergo differentiation toward neuronal lineages, improved outcomes have been observed even when survival of transplanted cells is low and engrafted cells are absent. This suggests that rather than replacing damaged cells, transplanted cells may improve outcome via indirect mechanisms. For example, MSCs have been shown to secrete many factors that can influence important processes like apoptosis, neurogenesis, angiogenesis and synaptogenesis.

MECHANISMS OF STEM CELL MEDIATED BRAIN REPAIR—CELLULAR ASPECTS

Although results from experimental animal models using cell-based therapies to treat ischemic brain injury are very promising, there are several issues that need to be addressed. For example, the therapeutic time window for stem cell therapy is not well-defined. This has to do with the fact that several models for ischemic brain injury are being used and several different stem cell types are being tested. Beneficial effects of stem cells have been observed when they are transplanted anywhere from 3 h to 10 days after onset of injury. In a study where MSCs were administered at 17 days post-injury, no beneficial effects were observed. These data indicate that stem cells might serve both a neuroprotective and a neuroregenerative role. This dual role is further underlined by results showing that apoptosis is reduced after transplantation of stem cells, while on the other hand, endogenous neurogenesis is enhanced. In a recent study, multiple injections of MSCs were shown to be more beneficial than a single injection. Moreover, the timing of injection may influence different repair processes. A first injection with MSCs at 3 days after injury stimulated cell proliferation and differentiation, while a second injection at 10 days stimulated axonal remodeling. Combined these results show that it is imperative to investigate via which mechanism MSCs and other stem cells exert their beneficial effects, since this will help identify more precise targets to improve both neuroprotection and regeneration.

The distribution of cells within injured brain after intravenous injection or other modes of delivery, and potential adverse effects of exogenous administration, have not been thoroughly explored. The fact that MSCs administered via intranasal delivery migrate toward injured regions provides a promising practical avenue for such treatments.

The temporal-spatial effects of stem cells often depend on the animal model of brain injury and the source or manipulation of these cells. Stem cells often home to different regions of the brain, with emphasis on injured or inflamed areas. Once in the circulation, these cells can also differentiate into a wide variety of cell types, including immune cells, endothelial progenitors, and neuronal or glial cell types (Liu et al., 2014). In other organs, MSCs persist for as long as several months. While growth factors play a role in the efficacy of exogenous stem cells and endogenous precursor cells, it is likely that stem cells act as a trophic factor factory enhancing neuroprotective

and endogenous neurogenic capacity. In this sense a therapeutic strategy in which application of stem cells is combined with growth factor administration to target a specific event may be more beneficial in combatting the events that cause progression of brain injury after stroke but, again, the timing of administration would ultimately determine the added repair efficacy in the brain. Mobilization of circulating endothelial progenitor cells (EPC) may be another key mechanism via which stem cells exert beneficial effects, as adequate blood supply is essential for effective repair of damaged tissue.

MECHANISMS OF STEM CELL MEDIATED BRAIN REPAIR—MOLECULAR ASPECTS

The evidence for the role of growth factors on cell proliferation and tissue repair after stem cell transplantation includes increased expression of a number of factors that lead to proliferation and integration of endogenous cells into neural networks, while also enhancing angiogenesis. As mentioned, HUBCs have demonstrated an ability to express neuronal, astrocytic, and oligodendrocyte markers *in vitro*, but a very small percentage of engrafted HUCBs or MSCs survive and express neuronal phenotypes. Many grafted cells remain undifferentiated far from the lesion, where these undifferentiated stem/progenitor cells can directly release growth and trophic factors, or promote the release of such factors from host brain cells. For example, MSCs have been shown to produce trophic factors that induce survival and regeneration of host neurons, while also producing extracellular matrix molecules, resulting in functional improvement at both early and late time points after brain injury. Some of these factors include brain-derived neurotrophic factor (BDNF), glial - derived neurotrophic factor (GDNF), nerve growth factor (NGF), hepatocyte growth factor (HGF), vascular endothelial growth factors (VEGF), angiopoietin-1 (Ang-1) (Liu et al., 2014).

Thus, improvements in histological injury and neurological function in animals after stem cell transplantation are poorly understood but appear to be secondary to effects on the microenvironment in the injured brain. This could enhance host cell connections by improving synaptic plasticity and axonal connections, while also increasing neurogenesis and angiogenesis. For example, VEGF and its receptor are shown to rebound after middle cerebral artery occlusion (MCAO) in animals treated with MSCs. Similarly, cytokines and growth factors such as macrophage inflammatory protein 1 (MIP-1), matrix metalloproteinases (MMPs), erythropoietin receptor and tumor necrosis factor receptor are increased after MSC injection (Yang et al., 2010). This involves rapid change, occurring by 1 week after stroke, with continued elevation of levels at 4 weeks. Immune modulation may also play a significant role in this beneficial response. There is still much to be learned about how stem cell therapies may regulate innate and acquired immunity. Grafted cells often remain in the spleen and other organs and attenuate inflammatory mediators. This may result in increased bioavailability of soluble factors such as GDNF and BDNF.

Effects of stem cell therapy on angiogenesis and blood vessel remodeling may be particularly important following stroke. Transplantation of HUCB-derived MSCs increased the formation of new blood vessels and increased cortical blood flow in a rat model of MCAO. *In vitro* studies of MSCs have demonstrated production of a number of angiogenic factors, including VEGF, Ang-1, IGF-1, and G-CSF. This leads to enhancement of endothelial cell proliferation and recruitment of endogenous progenitor cells, promoting the growth of new vessels. Angiogenesis is mainly regulated by the VEGF/VEGF receptor and the angiopoietin/Tie-2 signaling pathway, and the expression of Tie-2 was increased after HUCB transplantation.

All of these data have led to the alternative strategy of combining cell-based therapy and gene delivery. In a hind limb ischemia model, the combination of intravenous infusion of EPCs over-expressing VEGF with local SDF-1 application was more efficient in improving local blood supply than either alone. Transplantation of EPCs over-expressing IGF-1 has led to inhibition of cardiac apoptosis, enhancement of cardiomyocyte proliferation, and increased capillary numbers in the peri-infarct area. Interestingly, VEGF over-expression in EPCs could increase the expression of CXCR4, leading to further enhancement of EPC homing. However, VEGF is also known to enhance vascular permeability in the brain early after an ischemic insult, and genetically modified MSCs expressing VEGF may actually increase functional deficits. On the other hand, hypoxia preconditioning enhances VEGFR2 expression on EPCs, and accordingly, augments the neovascularization efficacy of EPCs after administration. In addition, pre-incubating EPCs with SDF-1 enhances their pro-angiogenic potential in hind limb ischemia.

Studies using stem cells that over-express other neurotrophic factors, such as BDNF, have also shown promise for treating ischemic brain injury (Chen et al., 2013). BDNF is secreted by brain cells and induces neuroprotection, while promoting the synaptic and axonal plasticity associated with learning, memory, and sensorimotor recovery, and increasing newly born neurons in several regions of the brain. Administration of BDNF modified MSCs has produced therapeutic benefits in a rat model of transient MCAO. Transplantation of BDNF gene-modified human MSCs results in increased BDNF levels in the ischemic lesion and stronger therapeutic effects than MSCs alone. BDNF secreted by MSCs may protect against hyperexcitability and modulate neuronal excitability via downregulation of the potassium-chloride co-transporter KCC2. BDNF may also modulate vascular permeability and BBB breakdown, and early effects on the brain may be secondary to changes in cerebral edema. Benefit has also been demonstrated in a spinal cord injury model with improved functional outcome and enhanced sprouting of raphé–spinal axons. In addition, reduction in ischemic lesion volume and improved functional outcome after stroke in the rat has been seen following treatment with human MSCs genetically modified to express GDNF. FGF-2-modified MSCs also significantly reduced infarct volume 14 days after MCAO.

Overall, stem cell therapy for brain repair after stroke and other neurological conditions has shown benefit and may be closer than we think.

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REFERENCES

Chen, C., Wang, Y., and Yang, G. Y. (2013). Stem cell-mediated gene delivering for the treatment of cerebral ischemia: progress and prospectives. *Curr. Drug Targets* 14, 81–89. doi: 10.2174/138945013804806497

Liu, X., Ye, R., Yan, T., Yu, S. P., Wei, L., Xu, G., et al. (2014). Cell based therapies for ischemic stroke: from basic science to bedside. *Prog. Neurobiol.* 115, 92–115. doi: 10.1016/j.pneurobio.2013.11.007

Pimentel-Coelho, P. M., and Mendez-Otero, R. (2010). Cell Therapy for neonatal hypoxic-ischemic encephalopathy. *Stem Cells Dev.* 19, 299–310. doi: 10.1089/scd.2009.0403

van Velthoven, C. T. J., Kavelaars, A., and Heijnen, C. J. (2012). Mesenchymal stem cells as a treatment for neonatal ischemic brain damage. *Pediatr. Res.* 71, 474–481. doi: 10.1038/pr.2011.64

Yang, M., Wei, X., Li, J., Heine, L. A., Rosenwasser, R., and Iacovitti, L. (2010). Changes in host blood factors and brain glia accompanying the functional recovery after systemic administration of bone marrow stem cells in ischemic stroke rats. *Cell Transplant.* 19, 1073–184. doi: 10.3727/096368910X503415

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Impurity of stem cell graft by murine embryonic fibroblasts – implications for cell-based therapy of the central nervous system

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Stem cells have been demonstrated to possess a therapeutic potential in experimental models of various central nervous system disorders, including stroke. The types of implanted cells appear to play a crucial role. Previously, groups of the stem cell network NRW implemented a feeder-based cell line within the scope of their projects, examining the implantation of stem cells after ischemic stroke and traumatic brain injury. Retrospective evaluation indicated the presence of spindle-shaped cells in several grafts implanted in injured animals, which indicated potential contamination by co-cultured feeder cells (murine embryonic fibroblasts – MEFs). Because feeder-based cell lines have been previously exposed to a justified criticism with regard to contamination by animal glycans, we aimed to evaluate the effects of stem cell/MEF co-transplantation. MEFs accounted for $5.3 \pm 2.8\%$ of all cells in the primary FACS-evaluated co-culture. Depending on the culture conditions and subsequent purification procedure, the MEF-fraction ranged from 0.9 to 9.9% of the cell suspensions *in vitro*. MEF survival and related formation of extracellular substances *in vivo* were observed after implantation into the uninjured rat brain. Impurity of the stem cell graft by MEFs interferes with translational strategies, which represents a threat to the potential recipient and may affect the graft microenvironment. The implications of these findings are critically discussed.

Keywords: stem cell transplantation, feeder-based cell line, murine embryonic fibroblasts, stroke, brain injury, cell graft contamination

INTRODUCTION

Cell replacement strategies have been proposed to be a promising therapeutic approach for various disorders of the central nervous system. Conditions predominantly associated with a loss of one specific cell population, such as amyotrophic lateral sclerosis, Parkinson's disease, or subarachnoid hemorrhage, may be targeted using specifically pre-differentiated cell grafts. In the case of traumatic or ischemic brain injury, a whole tissue segment (including neurons, glia, and vascular cells) has to be replaced by cells, which are able to differentiate into all lost cell types; alternatively, a heterogeneous graft containing different cell populations can also be used (Schouten et al., 2004; Molcanyi et al., 2007; Riess et al., 2007; Lohr et al., 2008; Burns et al., 2009; Richardson et al., 2010; Benchoua and Onteniente, 2011).

Previously, groups of the Stem Cell Network North-Rhine Westphalia evaluated the use of GFP-positive pluripotent embryonic stem cells (ESCs) after experimental ischemia and traumatic brain injury (TBI). ESC migration and differentiation was reported in ischemic animals (Hoehn et al., 2002; Erdo et al., 2003). In contrast, neurological improvement followed by gradual loss of implanted cells, came to the forefront in TBI-injured rats receiving ESC grafts (Molcanyi et al., 2007, 2013; Riess et al., 2007). Nestin was, *inter alia*, utilized to examine early differentiation along the neural pathway in injured animals. Nestin was co-expressed by only a few GFP-positive ESCs. However, nestin staining was abundant at trauma and implantation sites and was predominantly expressed by cells lacking any co-localization with GFP (e.g., activated resident glia). Additional presence of nestin-expressing,

GFP-negative spindle-shaped cellular elements localized inside the implanted graft was observed during confocal analysis of the implanted grafts; however, it was not systematically evaluated in our previous studies (see Supplementary Material) (Molcanyi et al., 2007, 2013). Current re-assessment of previous observations (Molcanyi et al., 2007, 2009) revealed the presence of such cells in several grafted animals. Because implemented ESCs were grown on a feeder layer consisting of inactivated murine embryonic fibroblasts (MEFs), these histological findings raised concerns regarding potential cell graft contamination by co-cultured feeder cells.

In this study, we quantified the amount of MEFs in proportion to co-cultured ESCs *in vitro* under standard conditions and after re-plating procedure. Furthermore, MEF survival was observed *in vivo* after transplantation into healthy rat brain and was evaluated with respect to survival and interaction with the surrounding brain microenvironment. Feeder-based cell lines have been subject to criticism regarding the contamination of ESCs by feeder-derived animal proteins. Our findings revealed the potential of additional graft impurity during the transplantation procedures. The effect of these findings on previously established stem cell protocols is discussed.

MATERIALS AND METHODS

CELL CULTURES

Murine embryonic fibroblasts cells were prepared from day 13 to 14 embryos (decapitated body, removed inner organs). MEF cells were G418-resistant (selection drug used in isolating homologous recombinants) and thus, prepared from mice harboring the neo gene. We used a CD1 neo mouse, which harbors pSC2neo. MEFs were inactivated using 10-μg/ml mitomycin for 2–3 h prior to culture. For transplantation, the MEF monoculture was trypsinized and resuspended in PBS to achieve a final concentration of 10^3 cells/μl. For immunohistochemistry, MEFs were cultured on gelatinized coverslips and alternatively on plates in Dulbecco modified Eagle medium (DMEM), containing 10% fetal calf serum (FCS), 1% non-essential amino acids (NEAA), and 50 μM β-mercaptoethanol (all from Thermo Scientific, USA) for further co-culturing with ES cells. The CGR8 feeder-free cell line, which was used as a control cell line for immunohistochemistry, was cultured in GMEM with stable glutamine und sodium pyruvate (Thermo Scientific, Germany) supplemented with 10% FCS, 1000 U/ml leukemia-inhibiting factor (Millipore, Germany), and 50 μM β-mercaptoethanol on coverslips.

Murine ESCs of the D3 cell line stably transfected with the pCX-(-act)-enhanced-GFP expression vector as previously described (Arnhold et al., 2000) were cultured on a feeder-layer in DMEM containing 15% FCS, 1% NEAA, 1% penicillin-streptomycin, 50 μM 2-mercaptoethanol, and 1000 U/ml LIF (Millipore, Germany). ESCs were cultured on plastic dishes in the presence of leukemia-inhibitory factor on a layer of mitotically inactivated MEFs.

IMMUNOCYTOCHEMISTRY AND FACS

Murine embryonic fibroblasts cultured on coverslips were fixed for 5 min in 2% paraformaldehyde, washed twice with PBS, and stained with standard hematoxylin-eosin for morphological evaluation. For immunocytochemistry, the cells were fixed, washed, permeabilized for 15 min in PBS-0.2% Triton X-100, and blocked with 5% normal goat serum (NGS). Incubation with primary antibodies (1:100 dilution in PBS-NGS-Triton solution) was performed for 2 h at room temperature. Rinsing in PBS was followed by incubation with secondary antibodies (1:100, at room temperature for 2 h.) and DAPI-counterstaining. The following primary antibodies were used: anti-mouse nestin (Millipore, Germany) and anti-mouse vimentin (Sigma, USA), anti-mouse-feeder-PE (Miltenyi Biotec, Germany). The following secondary antibody was used: anti-mouse IgG Alexa 555 (Life Technologies, Germany) for nestin und vimentin, and the PE-conjugated anti-feeder antibody signal was amplified using anti-rat IgG Alexa 555 (Life Technologies, Germany). Labeled cells were mounted upside-down onto glass slides with DAKO fluorescent mounting medium (Dako, Denmark) and evaluated using conventional/fluorescent microscopy. Primary antibody was omitted in negative controls. CGR8 was implemented as an additional negative control for anti-mouse-feeder staining to exclude an unspecific binding of the primary antibody.

For FACS analysis, 0.5×10^6 D3-βactin-GFP(P8) ESCs were plated on 0.8×10^6 mitomycin inactivated MEFs. After 2 days, the ESCs were trypsinized or alternatively purified on 0.1% gelatin-coated dishes (Sigma, Germany) for 1 h (re-plating procedure). Cell quantification was assessed using trypan-blue, followed by FACS analysis of unstained cell suspensions to determine the GFP-positive fraction. Alternatively, 0.5×10^6 purified (replated) and unpurified cells were fixed using 0.1% PFA, stained using anti-MEF-PE (Miltenyi Biotec, Germany) (1:11) in 0.5% BSA buffer and normal mouse IgG-PE (Santa Cruz Biotechnology, USA) as a isotype control for 10 min in 2–8°C in darkness. Enhanced GFP-fluorescence and anti-feeder-PE staining were confirmed using fluorescence microscopy immediately prior to FACS analysis (see **Figures 3A,B**). FACS analysis was performed using FACS ARIA (Becton Dickinson, USA) and analyzed with WinMDI2.8 (Scripps Research Institute, USA).

MEF IMPLANTATION

All experiments were performed according to the animal protection guidelines and were approved and registered by the local governmental authorities of North-Rhine Westphalia, Germany. The animals included in this study primarily served as a control group in the previous study (Molcanyi et al., 2009). Adult male Sprague-Dawley rats (250–300 g, age 12–14 weeks, supplied by Harlan, Germany) were intraperitoneally anesthetized with 60 mg/kg body weight pentobarbital. The animals were placed in a stereotactic frame (David Kopf Instruments, USA). The cranial soft tissue was opened and a small craniotomy was drilled, using a 2-mm trephine at calculated coordinates. Five microliters of a cell suspension containing 5×10^3 MEF cells in PBS were injected using a Hamilton needle under stereotactic conditions at the following coordinates: AP −3.4, ML 5.0, and DV −3.2 relative to bregma. The decision, regarding the amount of MEFs to implant (5×10^3) was initially met based on the fluorescent cell-counting assessment of MEF portion, which contaminated the co-culture/cell graft. FACS re-evaluation of MEF contamination under standardized co-culturing conditions of D3-ESC/MEFs in our facility (ranging from approximately 1 to 10% of a standard

graft containing 10^5 cells) approved the MEF amount implemented in the *in vivo* part of the study. MEF grafts were placed into the cortex of eight animals. The control group, which consisted of six animals, received an analogical injection of PBS. Twenty-four hours prior to implantation, the animals received an intraperitoneal injection of cyclosporin A (CsA, 10 mg/kg body weight, Sandimun, Novartis, Germany) as previously described (Molcanyi et al., 2007, 2009). Subsequently, the immunosuppressive drug was administered daily for up to 14 days after implantation. Animals were sacrificed 14 days post-implantation by lethal dose of sodium pentobarbital and transcardially perfused with 200 ml of heparinized PBS followed by 250 ml of 4% paraformaldehyde solution (Merck, Germany). Brains were removed from the skull, post-fixed in 2% paraformaldehyde for 2 days, processed, and embedded in paraffin blocks.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Paraffin-embedded brains were cut using a microtome (6 µm coronal sections) and mounted on poly-L-lysine coated glass slides (Biochrom, Germany). Dewaxing and rehydration were performed using subsequent xylene, alcohol, and distilled-water baths. Hematoxylin-eosin (HE) and Nissl stainings were performed according to standard protocols. Alcian-blue staining, which visualizes extracellular substances, such as acidic polysaccharides (e.g., glycosaminoglycans and mucopolysaccharides), was performed according to a standard protocol under pH-controlled conditions. Van Gieson staining, which detects collagen, was performed according to a standard protocol.

Conventional immunohistochemistry was started by blocking of endogenous peroxidase using 1% H_2O_2 (Merck, Germany) in methanol (Merck, Germany) for 20 min. The sections were shortly microwaved (1200 W, 1 min.) in a pH6 antigen-retrieval solution (DAKO, Denmark). Non-specific bindings were blocked using 5% NGS in PBS/Triton solution (analogous to immunocytochemistry). Monoclonal anti-CD-68/ED-1 (1:100, Serotec, Germany) in NGS-PBS-Triton solution was applied to sections for 2 h at room temperature. After two PBS-wash steps, the sections were incubated with biotinylated goat anti-mouse antibody (1:100, DAKO, Denmark) for 2 h at room temperature and visualized using streptavidin-horseradish peroxidase/chromogen 3,3′-diaminobenzidine (DAB) systems as recommended by the manufacturer: VECTASTAIN Elite ABC (Vector Laboratories, USA) and (DAKO, Denmark). For fluorescent immunohistochemistry, the sections were blocked with 5% NGS in PBS-Triton, incubated with anti-feeder-PE antibody (1:100 for 2 h at room temperature), and additionally incubated with anti-rat-Alexa555 antibody (1:100 for 2 h at room temperature) to amplify the signal of PE-conjugated antibody (primarily developed for flow-cytometry), followed by DAPI-counterstaining. Adjacent sections, which were incubated with a secondary antibody (omitting the primary antibody) served as negative controls. All specimens were viewed using a conventional/fluorescent Leica DMRB microscope (Leica, Germany) equipped with a 3CCD JVC live-camera (JVC, Japan). Images were captured using Diskus imaging software (Königswinter, Germany). Supplementary data (see Introduction) shows a histological section, which neighbored the section that was previously published but not the identical one. The animal demonstrating post-implantation MEF survival was briefly mentioned but not further analyzed in our previous publication (Molcanyi et al., 2007, 2009).

STATISTICS

Immunocytochemistry was performed on $n = 12$ culture dishes of MEF cells and $n = 4$ culture dishes of CGR8 cells (grown on coverslips). Immunohistochemistry was performed in 14 ($n = 8$ grafted, $n = 6$ control) animals. Cell loss during the re-plating procedure and further FACS analysis were assessed using 14 ESC/MEF culture dishes ($n = 7$ untreated and $n = 7$ gelatin-replated). The Kolmogonov–Smirnov-test was performed to evaluate the data distribution. All data sets exhibited a normal distribution. The maxima and minima were designated as individual %-values; all other results are shown as the mean ± standard deviation, if not otherwise stated. We used t-tests for group comparison. $P < 0.05$ was considered to be significant. Statistical analyses were performed using IBM SPSS (IBM, USA).

RESULTS

To confirm our initial presumption that previously observed nestin+ spindle-shaped cells might have been co-transplanted MEFs, the expression profile of implemented feeder-cell line was characterized. Sub-confluent MEFs manifested both spindle-like and planar phenotypes, as observed using HE staining (**Figure 1A**). Spindle-shaped cells stained positive for nestin, which supported our initial hypothesis regarding co-transplanted feeders (**Figure 1B**). Phenotypic characterization of the MEF culture showed vimentin staining of the entire population, including both spindle-shaped and flat cells, indicating a pure fibroblast monoculture (**Figure 1C**). Furthermore, we successfully tested a novel anti-feeder-PE antibody, which was initially developed for FACS and depletion procedures (Knoebel et al., 2010). Specific antibody binding was determined from granular staining, which covered all cell phenotypes (**Figure 1D**). Controls, which were counterstained using DAPI (lacking primary antibodies), showed no specific signals in the red emission spectrum and no non-specific autofluorescence in the green channel (**Figures 1E,F**). An additional negative control, the CGR8 cell line, showed no specific labeling using the anti-feeder antibody (**Figures 1G,H**).

Because the anti-feeder-antibody was demonstrated to bind specifically to established MEF cultures, we also used this antibody in FACS analysis of the MEF/ESC co-culture. GFP-positive ESCs grown on a MEF monolayer were detached from the dish and incubated using the anti-feeder antibody. Specific signals of the cell suspension (native GFP-fluorescence of ESCs and red-fluorescence of anti-feeder-PE stained MEFs) were examined and confirmed using fluorescent microscopy prior to FACS (**Figures 2A,B**). Anti-feeder-PE staining exhibited a typical granular pattern, as previously observed (compare with **Figure 1D**). Analysis of detached cell suspensions showed that the feeder cells accounted for 5.33 ± 2.81% (full range 2.2–9.9%) of the entire cell suspension (**Figures 2C,F**). Alternatively, cell suspensions were replated on a gelatin-coated dish for 1 h; this is an extra step that allows the feeder-cell fraction to attach. Free-floating cell populations were harvested and assessed by FACS, which

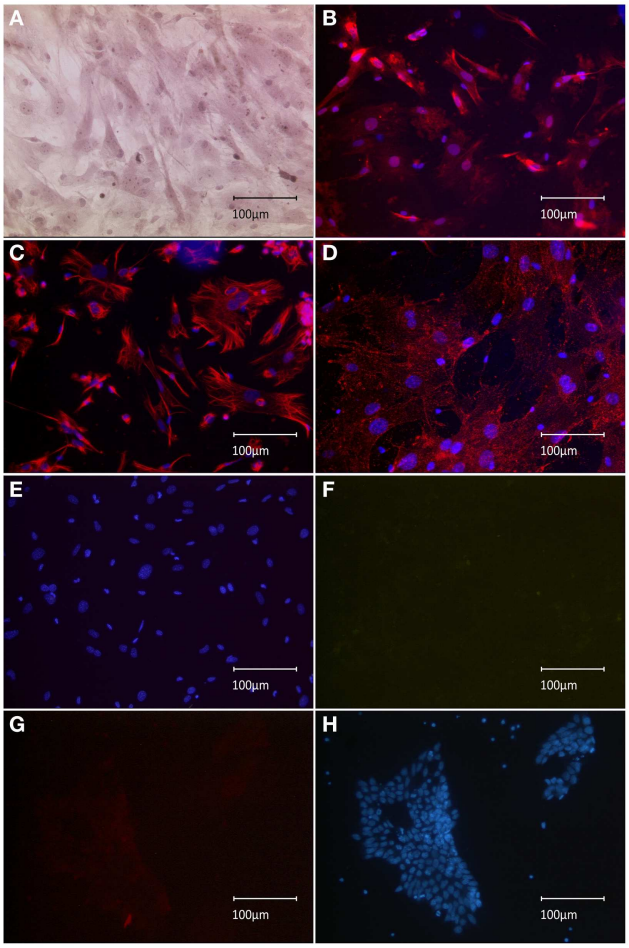

FIGURE 1 | Immunocytochemical characterization of MEF monolayer *in vitro*. **(A)** HE staining revealed the presence of both spindle-form and planar-shaped cells. **(B)** Anti-nestin-Alexa555 staining is strongly exhibited by spindle-shaped fibroblasts; much weaker signal is associated with planar phenotypes. **(C)** Uniform anti-vimentin-Alexa555 staining of all cells indicates a pure fibroblast monoculture. **(D)** Labeling by anti-feeder-PE antibody, amplified by anti-rat-Alexa555 yielded a uniform staining of all cells exhibiting a granular pattern. **(E)** Control dishes (omitted primary antibody) incubated with secondary antibodies and counterstained by DAPI showed no specific signal in the red emission spectrum and **(F)** no non-specific background autofluorescence when inspected in the green emission channel. **(G)** No specific staining of the CGR8 cell line using the anti-feeder-PE antibody in the red channel, **(F)** the same CGR8 culture area counterstained with DAPI.

yielded a significant reduction in the feeder-cell fraction down to $1.45 \pm 0.27\%$ (full range 0.9–1.7%) ($p = 0.011$, $n = 7$, respectively; **Figures 2D,F**). The re-plating step also led to a reduction in the entire cell suspension (overall decrease in cell counts, compared to untreated dishes), as apparently both MEFs and also ESCs attached to gelatine coating to some extent. The total cell loss and the concomitant loss of GFP+ cell fraction were both significant and was as high as approximately 10% of the initial cell suspension ($p < 0.001$, $n = 7$, respectively; **Figure 2E**). The effect of single re-plating was examined in this study, as some authors previously implemented this procedure to reduce the number of contaminating MEFs, prior to the transplantation (see Discussion). Currently assessed FACS-values of %-MEF contamination under standardized co-culturing conditions of D3-ESC/MEFs in our facility highlighted the presence of a considerable MEF-fraction in both untreated/gelatin-treated cultures and justified the cell amount used in the *in vivo* part of this study (see Materials and Methods).

To examine the translational effect of inactivated MEFs, we transplanted 5×10^3 MEFs into the cortices of healthy rat brains and evaluated their survival and interaction with surrounding microenvironment *in vivo*. The transplantation procedure was successfully performed in all animals; eight rats receiving MEFs and six control animals receiving a PBS injection. All animals survived the observation period of 2 weeks. Next, the animals were sacrificed and histologically examined. The site of the former graft implantation could easily be identified in all HE- and Nissl-stained brains (see below and description of **Figure 3**). In seven grafted animals, no fibroblast-resembling cells could morphologically be discerned, most likely due to macrophage-mediated clearance. In one animal, spindle-shaped cells were found in close proximity to the transplantation site, which was clearly delineated from the neighboring cortex (**Figures 3A,B**). The transplantation site exhibited a cortical discontinuity, which was invaded by round cells of variable sizes, many of which carried hemosiderin deposits. Considerable invasion could also be observed in the adjacent cortex. Anti-CD68 staining demonstrated that these cells were macrophages, which were most likely responsible for scavenging of the implanted graft (**Figure 3C**). As the texture of transplantation site differed from healthy cortical tissue, we tested for the presence of extracellular matrix as a potential by-product of the implanted feeder cells. Alcian-blue staining confirmed the presence of acidic polysaccharides inside and at the margin of the transplantation site (no blue signal detected in the healthy cortex) (**Figures 3D,E**). An abundant presence of collagen was confirmed using van Gieson staining at the site of transplantation (no red staining detected in the healthy cortex) (**Figures 3F,G**). Both stainings embodied an indirect proof of survival and metabolic activity of the implanted feeder cells. Labeling of the sections of this animal using an anti-feeder-PE antibody resulted in specific signals that morphologically resembled spindle-shaped cells within the red emission spectrum with no specific red signal in the control slides and no interfering autofluorescence (as additionally inspected in the green emission channel) (**Figures 3H–K**). The implantation sites were identified in all HE and Nissl-stained brains, based on the presence of cortical discontinuity and hypercellularity (in grafted brains) and needle-track (in control animals) (**Figures 3L,M**). The labeling of these brains, primarily exhibiting no morphological characteristics of MEF survival, using anti-feeder-PE antibody showed no specific signaling (data not shown). These findings represent the mid-term survival and metabolic activity of feeder cells after transplantation into a healthy rat brain, as a proof of principle.

DISCUSSION

The results of the present study demonstrate a feeder-cell layer consisting of MEFs to be a source of impurity, which may interfere

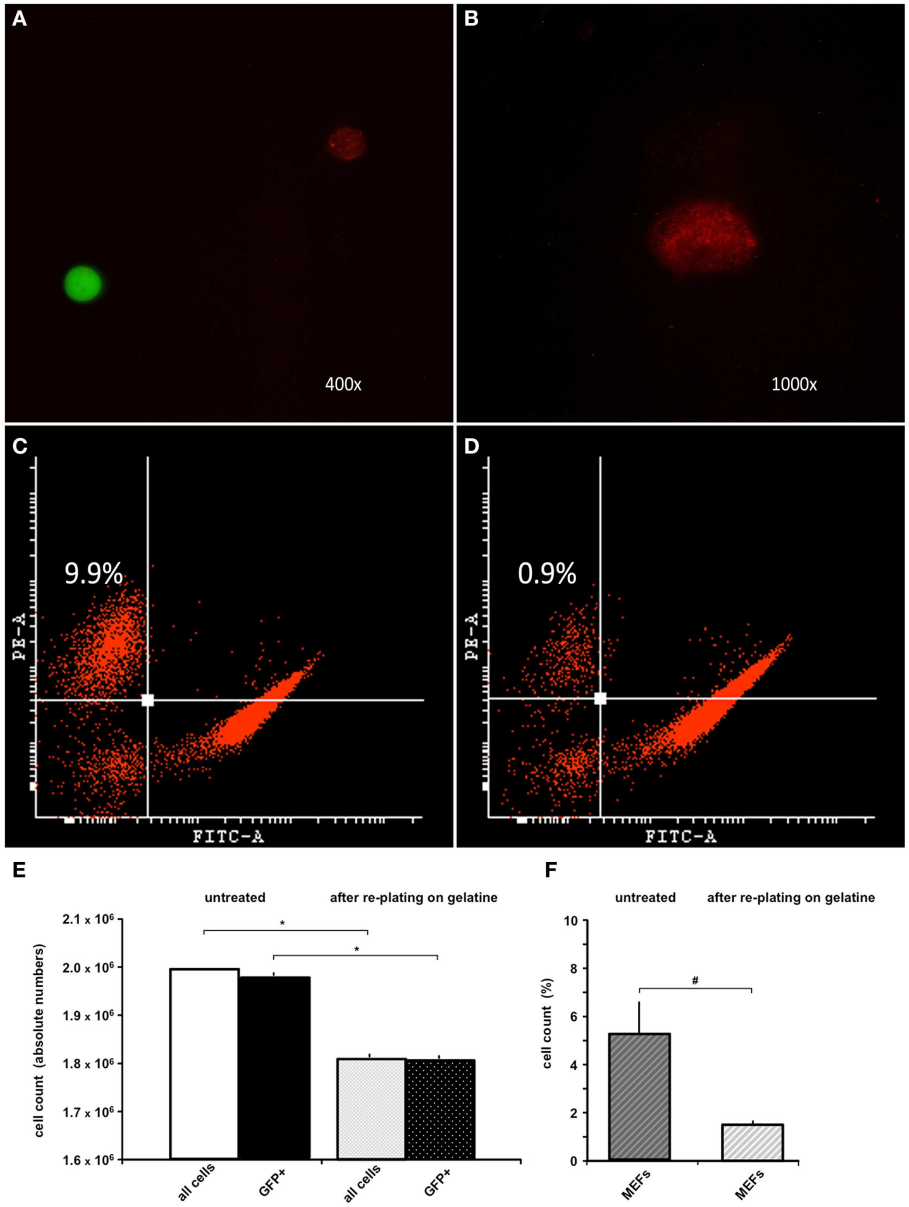

FIGURE 2 | FACS analysis of trypsinized ESC-MEF co-culture with/without re-plating on a gelatin-coated dish. **(A)** Confirmation of specific signaling using fluorescent microscopy prior to FACS: GFP-positive ESCs exhibited high-intensity signal in the green emission spectrum, MEFs labeled using the anti-feeder-PE antibody emitted a specific red signal. **(B)** Grainy pattern of the specific anti-feeder-PE labeling, as previously observed in the immunocytochemistry data. **(C)** Trypsinized ESC-MEF cell suspension showed a well-delineated GFP+ cell population depicted on the right side of the plot, with the anti-feeder-PE + MEF-fraction situated in the left upper corner; this particular measurement showed the highest measured value of 9.9% of the overall cell-count. **(D)** Amount of MEFs contaminating the cell suspension decreased to a minimum of 0.9% after re-plating step (Note: both plots show representative maximum and minimum values acquired by FACS assessment of two individual cell-culture dishes, which was further followed by repeated measurements of additional dishes – see next) **(E)** Bar diagram presenting the absolute values (mean ± SD) of $n = 7$ untreated cell suspensions and $n = 7$ after re-plating on gelatin-coated dish, with a total cell loss accounting for approximately 10% of the primary cell suspension. The cell loss was statistically significant, when examined for both the entire cell suspension and GFP+ fraction – marked by $^{*}p < 0.001$. **(F)** Additional diagram presenting %-mean and ±SD values of FACS-acquired MEF contaminations in untreated versus gelatin-treated dishes, showing a statistically significant reduction of MEF amount – marked as $^{\#}p = 0.011$. Despite the reduction, MEF contamination still accounted for 1.4 ± 0.2% of the entire cell suspension.

with translational downstream ESC applications. Characterization of MEFs *in vitro* and the degree of ESC-graft contamination were evaluated using immunocytochemistry and FACS analysis. Implantation of feeder cells into healthy rat brains was performed to evaluate the mid-term survival and metabolic activity of grafted MEFs *in vivo*.

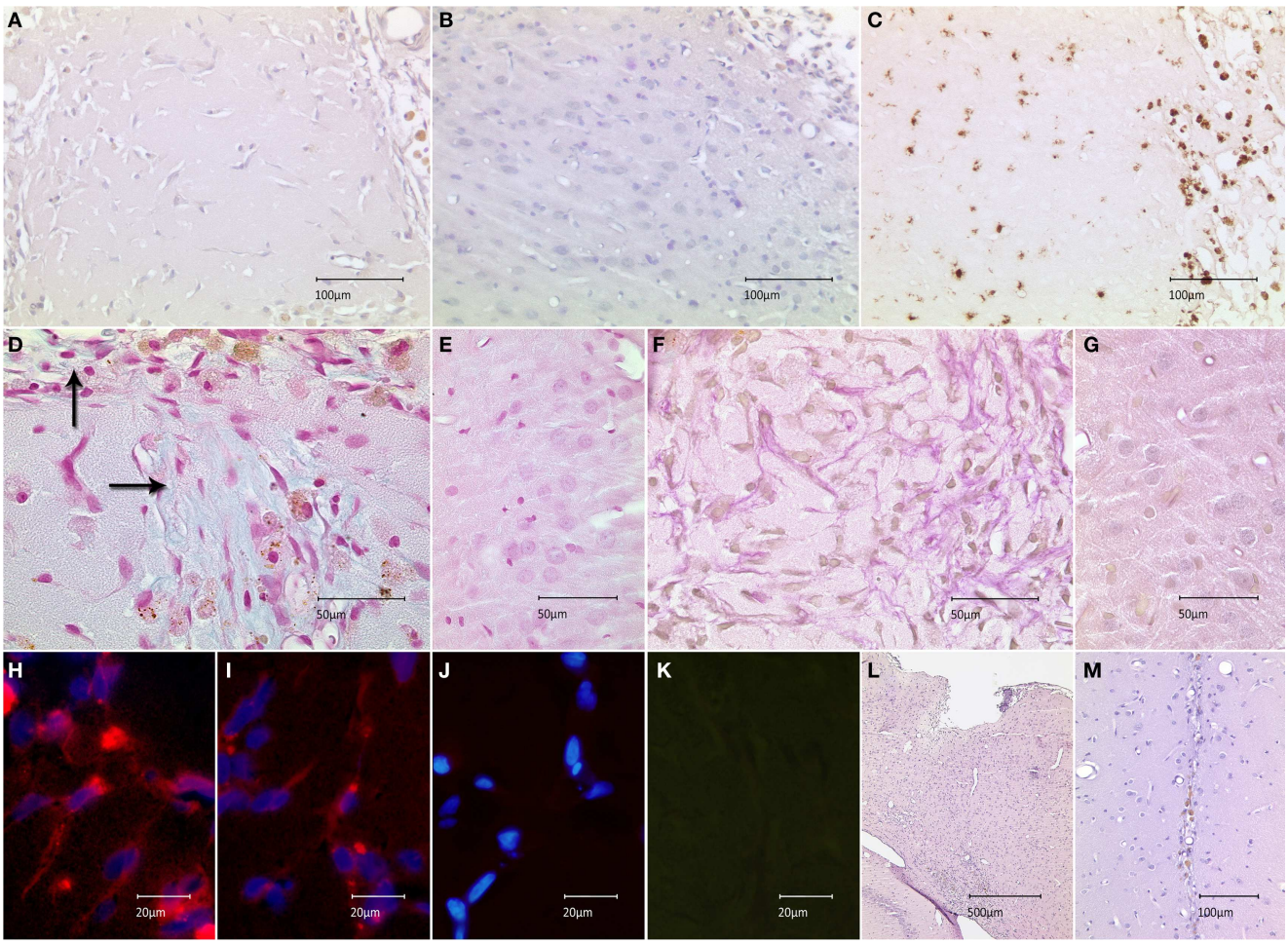

FIGURE 3 | Survival of feeder cells and their metabolic activity after implantation into healthy rat brain is shown. **(A)** Spindle-shape cells at close proximity of implantation site in HE-stained section with marginal infiltration of hemosiderin-laden cells compared to **(B)** adjacent cortex. **(C)** Infiltrating cells stained positive for the macrophage marker CD68-DAB; the staining shows abundant populations at the implantation site and minor invasion of the adjacent cortex. **(D)** Alcian-blue staining at the implantation site, indicating the presence of acidic polysaccharides (extracellular matrix) with **(E)** no signs of specific blue signal in the healthy cortex (control). **(F)** Abundant red staining, secundum Van Gieson, indicates the presence of collagenous extracellular matrix with **(G)** missing collagen expression in the healthy cortex. **(H,I)** Anti-feeder-PE staining demonstrating spindle-shaped cells to be implanted MEFs; with no specific red signal in **(J)** control section (omitted first antibody, counterstained by DAPI) and **(K)** no interfering autofluorescence as examined in the green emission channel. **(L)** Cortical discontinuity and hypercellularity at the site of former implantation in Nissl-stained animals lacking surviving fibroblasts. **(M)** Needle-track with some hemosiderin-laden cells in control animals receiving PBS injection.

In the past two decades, various cell lines have been used in a number of translational studies, which focus on the experimental therapy of stroke and other central nervous system disorders. Many of the ESCs or precursor cells utilized in these studies required an initial co-culturing with feeder cells for non-differentiated growth, self-renewal, and/or expression of some particular characteristics (Bjorklund et al., 2002; Barberi et al., 2003; Schouten et al., 2004; Burns et al., 2009; Locatelli et al., 2009; Kawai et al., 2010; Richardson et al., 2010; Benchoua and Onteniente, 2011; Rhee et al., 2011; Oki et al., 2012; Polentes et al., 2012; Jensen et al., 2013; Cattaneo and Bonfanti, 2014). However, for the use of ESCs in translational applications or tissue engineering, feeder cells have to be considered as contaminations that might interfere not just with the analysis of experimental data but also with the integration and function of transplanted cells *in vivo* (Schneider et al., 2008). Potential adverse effects of the contaminating feeder cells have been proposed to account for the discrepant results in pre/clinical studies observed by different research groups (Pereira et al., 2011). The depletion of feeder cells from stem cells prior to implantation has rarely been discussed in experimental cell replacement studies, although different methods of reducing the feeder-cell content have previously been described as early as in 1980s. Most of these feeder-reducing methods were based on the different adhesive characteristics of stem and feeder cells, i.e., the preferential adhesion of MEFs to uncoated culture plates (alternatively coupled to solid-phase immunoadsorption) or the weaning off feeders over several passages (Edwards et al., 1980; Halaban and Alfano, 1984;

Paraskeva et al., 1985; Linge et al., 1989; Knoebel et al., 2010; Jensen et al., 2013).

However, these methods do not achieve a complete removal of MEFs in one single step and are associated with a concomitant loss of ESC populations. Li et al. reported MEF contaminations as high as 17.5–25% despite two rounds of re-plating on uncoated dishes (Li et al., 2008). In contrast, other authors considered a single re-plating or even a simple trypsinization to be appropriate to eliminate MEF contamination prior to the transplantation procedure (Shintani et al., 2008; Kawai et al., 2010). Generally, MEF contamination is thought to account for approximately 10% of the primary cell suspension, which is consistent with our findings (Knoebel et al., 2010). The degree of contamination is variable, depending on the cell line used and facility-based culturing protocols. Considerable advances were achieved after the re-plating technique was amended using additional gradient separation. Utilizing this method, ESCs were enriched to purity >99% with a recovery rate higher than 90% (Li et al., 2008). Our purification step, implementing a gelatin-coated culture dish, also resulted in a high MEF adhesion and reduction of contamination to $1.45 \pm 0.27\%$, in contrast to higher contaminations, which were reported when using uncoated dishes (Knoebel et al., 2010). However, in the case of *in vivo* applications, even a minor contamination of large batch preparations can subsequently translate into a substantial cell count and associated complications (Li et al., 2008; Pereira et al., 2011).

Until recently, cell-sorting technologies (such as MACS Cell Separation) could not be utilized for feeder depletion due to the lack of a pan-fibroblast surface marker, which is common to all feeder strains. However, a novel mEF-SK4 antibody, which specifically docks to all tested fibroblast types, was newly developed and coupled to paramagnetic particles (Feeder Removal MicroBeads) for subsequent MACS cell separation of feeders from ESCs. This technology was shown to be a superior system for the efficient selection of highly purified stem cell populations, which contain <0.15% remaining MEFs (Knoebel et al., 2010). PE-conjugated mEF-SK4 antibody (alternatively amplified by Alexa555 for immunocyto- and histochemistry) was also successfully used to label the feeder cells in our study (**Figures 1D**, **2A,B**, **3H,I**). Alternatively, a complete feeder-free purification of stem cells was also achieved using an automated cell selection system, which aimed at the aspiration of distinct stem cell colonies. In this previously established method, we showed that the early complete "freeing" of stem cell colonies from feeder cells did not interfere with subsequent differentiation processes *in vitro* (Schneider et al., 2008). Thus, the complete withdrawal of feeder cells should be considered for all downstream translational approaches, because of potentially detrimental effects of contaminating feeder cells (Li et al., 2008; Schneider et al., 2008; Pereira et al., 2011).

It is widely accepted that detrimental effects may occur due to the release of a variety of humoral factors, cell–cell interactions at transplantation site (with both grafted ESCs and surrounding host cells), or via activation of the immune response of the host–environment. Various interactions between different cell types and the brain microenvironment were reported in several studies (Bentz et al., 2006, 2007, 2010; Molcanyi et al., 2007). We previously showed that incubation of ESCs with brain extract *in vitro* resulted in the release of neurotrophic factors, which was accompanied by the considerable co-production of these neurotrophins by inactivated co-cultured MEFs (Bentz et al., 2007). The metabolic potential of inactivated feeder cells is not surprising as MEFs are expected to produce humoral factors, which maintain the characteristics of co-cultured ESCs. Contaminating MEFs are similarly suspected to continually secrete anti-differentiation factors *in vivo*, which exert an effect on the local microenvironment after co-transplantation (Li et al., 2008). The effect of this phenomenon on tumorigenesis after cell grafting in experimental models of stroke and other cerebral disorders has remained unresolved (Molcanyi et al., 2009). Moreover, our current study demonstrated the release of MEF-associated extracellular matrix (otherwise, not present in healthy brain tissue), which may negatively affect the local microenvironment, as well (**Figures 3D,F**).

In this study, we observed a pronounced immune reaction at the site of MEF implantation despite administered immunosuppression (**Figure 3C**). Previously, the cellular immune response was shown to be responsible for scavenging the stem cell grafts implanted into the central nervous system (Li et al., 2005; Molcanyi et al., 2007). Immune system activation has been observed and attributed to different mechanisms, such as rejection, the removal of necrotic and/or apoptotic cells, or the combination of trauma and transplantation stimulus (Olanow et al., 2003; Li et al., 2005; Molcanyi et al., 2007, 2013; Pereira et al., 2011). Immune cell infiltration observed in the current study was very likely due to macrophage activation (first line defense) as a response to a local stimulus (needle injury and grafting of heterotopic cell suspension), rather than a specific host-versus-graft rejection. However, this issue is not completely resolved yet and the authors are planning to examine the immune response in all previously grafted brains (injured and healthy ones) in contrast to the brains receiving different control media (PBS versus feeder cells). In the light of current findings, additional amplification of immune response by co-transplanted feeder cells appears to be likely. This assumption is consistent with the observations of other authors (Pereira et al., 2011) who proposed that implanted fibroblasts activate immune cascades, resulting in detrimental effects. Pereira et al. showed that umbilical cord-derived mesenchymal stem cells induced potent neuroprotection in a rat model of Parkinson's disease. However, transplantation of fibroblast-contaminated grafts reversed the therapeutic efficacy and caused harmful effects, such as exacerbation of neurodegeneration and motor deficits. Surviving fibroblasts were observed as late as 3 weeks after engraftment into the rat striatum in their study (Pereira et al., 2011), which confirmed our observations.

In the beginning of the cell-therapy era, pluripotent cells maintained on feeder layers were thought to engraft, differentiate, and replace lost cells in the damaged target tissue. Negative effects, such as tumor formation resulted in a paradigm shift toward the use of precursor cells, as the pre-differentiation was shown to circumvent the threat of tumorigenesis (Benchoua and Onteniente, 2011). In addition, feeder-based cell lines were shown to be contaminated by animal proteins, which interfered with implementation

in clinical use (Bardor et al., 2005; Klimanskaya et al., 2005; Lanctot et al., 2007). This resulted in the establishment of feeder-free and subsequently entirely xeno-free culture conditions (Klimanskaya et al., 2005; Marinho et al., 2013). However, many cell lines (both embryonic and induced pluripotent stem cells) still required the presence of feeder cells such as MEFs, MSCs, or HDFs, at least in a specific phase of the culture protocol, e.g., for initial propagation and expansion (Klimanskaya et al., 2005; Willmann et al., 2013). Subsequent differentiation into neural phenotypes was temporarily performed using different feeder or stromal cells (e.g., PA6, MS5, MS5SHH, S2, Sertoli cells) (Perrier et al., 2004; Saporta et al., 2004; Benchoua and Onteniente, 2011; Kim and Park, 2011; Rhee et al., 2011). Shintani et al., who were aware of potential contamination, developed a differentiation protocol using bone marrow stromal cells (BMSC), resulting in the generation of functional dopaminergic neurons. Thus, the contamination of the neural graft by co-cultured autologous BMSCs (particularly, if implemented in a clinical testing) presents a risk, which was considerably lower than that of xenogeneic feeders (Shintani et al., 2008). Contemporary differentiation protocols translocate once feeder-initiated stem cells onto coated dishes for terminal differentiation in the absence of a feeder layer (Barberi et al., 2003; Perrier et al., 2004; Dubois-Dauphin et al., 2010; Kim and Park, 2011; Rhee et al., 2011). Further advancements have moved toward the culturing and differentiation of stem cells in completely feeder-free conditions (Cooper et al., 2010). The conclusions of our study highlight the necessity of this trend; conversely, the trend also presents a major limitation of our study, as we currently do not expect pluripotent stem cells or feeder-layer-based precursors to be considered for translational applications. Another limitation of our study is the observation of MEF-survival, which is restricted to one animal. However, the presence of MEFs has been proven by directly using a specific anti-feeder-antibody and indirectly by demonstrating the formation of MEF-associated extracellular substances. Consistent with our observations, analogous data were obtained by Perreira et al., who detected fibroblasts (using a species-specific antibody) surviving up to 3 weeks after transplantation in rat brains (Pereira et al., 2011).

CONCLUSION

The majority of previously implemented embryonic and induced pluripotent cell lines required the presence of an additional feeder-cell layer at a specific phase of the culturing protocol. It is known that feeder cells present a potential source of impurity, e.g., in the form of feeder-derived xeno-proteins. In this study, we analyzed the level of direct MEF contamination in ESC preparations and their engraftment *in vivo*. Despite the re-plating procedure, the residual impurity *in vitro* was still evident. Our observations confirm MEFs to impede the transplantation strategies, as they are able to survive a mid-term period after grafting and to produce extracellular substance *in vivo*. Presented data clearly support the current trend aiming for feeder-free technologies and provides critical insight into MEF effects with respect to graft fate, cell commitment, and graft–host interaction. These observations should be considered when interpreting a broad spectrum of previously published studies of this field.

AUTHOR CONTRIBUTIONS

Marek Molcanyi, Ute Schäfer, Peter Riess, Jürgen Hescheler, and Bert Bosche conceived the study. Marek Molcanyi, Narges Zare Mehrjardi, Nadia Nabil Haj-Yasein, Michael Brockmann, Marina Penner, Peter Riess, Bernhard Rieger, Tobias Hannes, and Bert Bosche performed the experiments and data analyses. Marek Molcanyi, Ute Schäfer, Michael Brockmann, Peter Riess, Clemens Reinshagen, Jürgen Hescheler, and Bert Bosche wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fncel.2014.00257/abstract

REFERENCES

Arnhold, S., Lenartz, D., Kruttwig, K., Klinz, F. J., Kolossov, E., Hescheler, J., et al. (2000). Differentiation of green fluorescent protein-labeled embryonic stem cell-derived neural precursor cells into Thy-1-positive neurons and glia after transplantation into adult rat striatum. *J. Neurosurg.* 93, 1026–1032. doi:10.3171/jns.2000.93.6.1026

Barberi, T., Klivenyi, P., Calingasan, N. Y., Lee, H., Kawamata, H., Loonam, K., et al. (2003). Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat. Biotechnol.* 21, 1200–1207. doi:10.1038/nbt870

Bardor, M., Nguyen, D. H., Diaz, S., and Varki, A. (2005). Mechanism of uptake and incorporation of the non-human sialic acid N-glycolylneuraminic acid into human cells. *J. Biol. Chem.* 280, 4228–4237. doi:10.1074/jbc.M412040200

Benchoua, A., and Onteniente, B. (2011). Intracerebral transplantation for neurological disorders. Lessons from developmental, experimental, and clinical studies. *Front. Cell. Neurosci.* 6:2. doi:10.3389/fncel.2012.00002

Bentz, K., Molcanyi, M., Hess, S., Schneider, A., Hescheler, J., Neugebauer, E., et al. (2006). Neural differentiation of embryonic stem cells is induced by signalling from non-neural niche cells. *Cell. Physiol. Biochem.* 18, 275–286. doi:10.1159/000097674

Bentz, K., Molcanyi, M., Riess, P., Elbers, A., Pohl, E., Sachinidis, A., et al. (2007). Embryonic stem cells produce neurotrophins in response to cerebral tissue extract: cell line-dependent differences. *J. Neurosci. Res.* 85, 1057–1064. doi:10.1002/jnr.21219

Bentz, K., Molcanyi, M., Schneider, A., Riess, P., Maegele, M., Bosche, B., et al. (2010). Extract derived from rat brains in the acute phase following traumatic brain injury impairs survival of undifferentiated stem cells and induces rapid differentiation of surviving cells. *Cell. Physiol. Biochem.* 26, 821–830. doi:10.1159/000323991

Bjorklund, L. M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., Mcnaught, K. S., et al. (2002). Embryonic stem cells develop into functional

dopaminergic neurons after transplantation in a Parkinson rat model. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2344–2349. doi:10.1073/pnas.022438099

Burns, T. C., Verfaillie, C. M., and Low, W. C. (2009). Stem cells for ischemic brain injury: a critical review. *J. Comp. Neurol.* 515, 125–144. doi:10.1002/cne.22038

Cattaneo, E., and Bonfanti, L. (2014). Therapeutic potential of neural stem cells: greater in people's perception than in their brains? *Front. Neurosci.* 8:79. doi:10.3389/fnins.2014.00079

Cooper, O., Hargus, G., Deleidi, M., Blak, A., Osborn, T., Marlow, E., et al. (2010). Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol. Cell. Neurosci.* 45, 258–266. doi:10.1016/j.mcn.2010.06.017

Dubois-Dauphin, M. L., Toni, N., Julien, S. D., Charvet, I., Sundstrom, L. E., and Stoppini, L. (2010). The long-term survival of in vitro engineered nervous tissue derived from the specific neural differentiation of mouse embryonic stem cells. *Biomaterials* 31, 7032–7042. doi:10.1016/j.biomaterials.2010.06.017

Edwards, P. A., Easty, D. M., and Foster, C. S. (1980). Selective culture of epithelioid cells from a human squamous carcinoma using a monoclonal antibody to kill fibroblasts. *Cell Biol. Int. Rep.* 4, 917–922.

Erdo, F., Buhrle, C., Blunk, J., Hoehn, M., Xia, Y., Fleischmann, B., et al. (2003). Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *J. Cereb. Blood Flow Metab.* 23, 780–785. doi:10.1097/01.WCB.0000071886.63724.FB

Halaban, R., and Alfano, F. D. (1984). Selective elimination of fibroblasts from cultures of normal human melanocytes. *In vitro* 20, 447–450. doi:10.1007/BF02619590

Hoehn, M., Kustermann, E., Blunk, J., Wiedermann, D., Trapp, T., Wecker, S., et al. (2002). Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16267–16272. doi:10.1073/pnas.242435499

Jensen, M. B., Yan, H., Krishnaney-Davison, R., Al Sawaf, A., and Zhang, S. C. (2013). Survival and differentiation of transplanted neural stem cells derived from human induced pluripotent stem cells in a rat stroke model. *J. Stroke Cerebrovasc. Dis.* 22, 304–308. doi:10.1016/j.jstrokecerebrovasdis.2011.09.008

Kawai, H., Yamashita, T., Ohta, Y., Deguchi, K., Nagotani, S., Zhang, X., et al. (2010). Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. *J. Cereb. Blood Flow Metab.* 30, 1487–1493. doi:10.1038/jcbfm.2010.32

Kim, Y. S., and Park, C. H. (2011). Dopamine neuron generation from human embryonic stem cells. *Int. J. Stem Cells* 4, 85–87. doi:10.15283/ijsc.2011.4.2.85

Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M. D., and Lanza, R. (2005). Human embryonic stem cells derived without feeder cells. *Lancet* 365, 1636–1641. doi:10.1016/S0140-6736(05)66473-2

Knoebel, S., Kurtz, A., Schwarz, C, Eckardt, D., Barral, S., and Bossio, A. (2010). "Novel fibroblast-specific marker for rapid, efficient removal of mEFs from stem cell cultures by magnetic separation," in *ISSCR 2010 Poster Abstract Book, P1256.* San Francisco, CA.

Lanctot, P. M., Gage, F. H., and Varki, A. P. (2007). The glycans of stem cells. *Curr. Opin. Chem. Biol.* 11, 373–380. doi:10.1016/j.cbpa.2007.05.032

Li, P., Tessler, A., Han, S. S., Fischer, I., Rao, M. S., and Selzer, M. E. (2005). Fate of immortalized human neuronal progenitor cells transplanted in rat spinal cord. *Arch. Neurol.* 62, 223–229. doi:10.1001/archneur.62.2.223

Li, Z., Barron, M. R., Lough, J., and Zhao, M. (2008). Rapid single-step separation of pluripotent mouse embryonic stem cells from mouse feeder fibroblasts. *Stem Cells Dev.* 17, 383–387. doi:10.1089/scd.2007.0138

Linge, C., Green, M. R., and Brooks, R. F. (1989). A method for removal of fibroblasts from human tissue culture systems. *Exp. Cell Res.* 185, 519–528. doi:10.1016/0014-4827(89)90320-0

Locatelli, F., Bersano, A., Ballabio, E., Lanfranconi, S., Papadimitriou, D., Strazzer, S., et al. (2009). Stem cell therapy in stroke. *Cell. Mol. Life Sci.* 66, 757–772. doi:10.1007/s00018-008-8346-1

Lohr, M., Tzouras, G., Molcanyi, M., Ernestus, R. I., Hampl, J. A., Fischer, J. H., et al. (2008). Degeneration of cholinergic rat basal forebrain neurons after experimental subarachnoid hemorrhage. *Neurosurgery* 63, 336–344. doi:10.1227/01.NEU.0000320422.54985.6D

Marinho, P. A., Vareschini, D. T., Gomes, I. C., Paulsen Bda, S., Furtado, D. R., Castilho Ldos, R., et al. (2013). Xeno-free production of human embryonic stem cells in stirred microcarrier systems using a novel animal/human-component-free medium. *Tissue Eng. Part C Methods* 19, 146–155. doi:10.1089/ten.TEC.2012.0141

Molcanyi, M., Bosche, B., Kraitsy, K., Patz, S., Zivcak, J., Riess, P., et al. (2013). Pitfalls and fallacies interfering with correct identification of embryonic stem cells implanted into the brain after experimental traumatic injury. *J. Neurosci. Methods* 215, 60–70. doi:10.1016/j.jneumeth.2013.02.012

Molcanyi, M., Riess, P., Bentz, K., Maegele, M., Hescheler, J., Schafke, B., et al. (2007). Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain. *J. Neurotrauma* 24, 625–637. doi:10.1089/neu.2006.0180

Molcanyi, M., Riess, P., Haj-Yasein, N. N., Bentz, K., Loehr, M., Kuchta, J., et al. (2009). Developmental potential of the murine embryonic stem cells transplanted into the healthy rat brain – novel insights into tumorigenesis. *Cell. Physiol. Biochem.* 24, 87–94. doi:10.1159/000227816

Oki, K., Tatarishvili, J., Wood, J., Koch, P., Wattananit, S., Mine, Y., et al. (2012). Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells* 30, 1120–1133. doi:10.1002/stem.1104

Olanow, C. W., Goetz, C. G., Kordower, J. H., Stoessl, A. J., Sossi, V., Brin, M. F., et al. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann. Neurol.* 54, 403–414. doi:10.1002/ana.10720

Paraskeva, C., Buckle, B. G., and Thorpe, P. E. (1985). Selective killing of contaminating human fibroblasts in epithelial cultures derived from colorectal tumours using an anti Thy-1 antibody-ricin conjugate. *Br. J. Cancer* 51, 131–134.

Pereira, M. C., Secco, M., Suzuki, D. E., Janjoppi, L., Rodini, C. O., Torres, L. B., et al. (2011). Contamination of mesenchymal stem-cells with fibroblasts accelerates neurodegeneration in an experimental model of Parkinson's disease. *Stem Cell. Rev.* 7, 1006–1017. doi:10.1007/s12015-011-9256-4

Perrier, A. L., Tabar, V., Barberi, T., Rubio, M. E., Bruses, J., Topf, N., et al. (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12543–12548. doi:10.1073/pnas.0404700101

Polentes, J., Jendelova, P., Cailleret, M., Braun, H., Romanyuk, N., Tropel, P., et al. (2012). Human induced pluripotent stem cells improve stroke outcome and reduce secondary degeneration in the recipient brain. *Cell Transplant.* 21, 2587–2602. doi:10.3727/096368912X653228

Rhee, Y. H., Ko, J. Y., Chang, M. Y., Yi, S. H., Kim, D., Kim, C. H., et al. (2011). Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. *J. Clin. Invest.* 121, 2326–2335. doi:10.1172/JCI45794

Richardson, R. M., Singh, A., Sun, D., Fillmore, H. L., Dietrich, D. W. III, and Bullock, M. R. (2010). Stem cell biology in traumatic brain injury: effects of injury and strategies for repair. *J. Neurosurg.* 112, 1125–1138. doi:10.3171/2009.4.JNS081087

Riess, P., Molcanyi, M., Bentz, K., Maegele, M., Simanski, C., Carlitscheck, C., et al. (2007). Embryonic stem cell transplantation after experimental traumatic brain injury dramatically improves neurological outcome, but may cause tumors. *J. Neurotrauma* 24, 216–225. doi:10.1089/neu.2006.0141

Saporta, S., Willing, A. E., Shamekh, R., Bickford, P., Paredes, D., and Cameron, D. F. (2004). Rapid differentiation of NT2 cells in Sertoli-NT2 cell tissue constructs grown in the rotating wall bioreactor. *Brain Res. Bull.* 64, 347–356. doi:10.1016/j.brainresbull.2004.09.003

Schneider, A., Spitkovsky, D., Riess, P., Molcanyi, M., Kamisetti, N., Maegele, M., et al. (2008). "The good into the pot, the bad into the crop!" – a new technology to free stem cells from feeder cells. *PLoS ONE* 3:e3788. doi:10.1371/journal.pone.0003788

Schouten, J. W., Fulp, C. T., Royo, N. C., Saatman, K. E., Watson, D. J., Snyder, E. Y., et al. (2004). A review and rationale for the use of cellular transplantation as a therapeutic strategy for traumatic brain injury. *J. Neurotrauma* 21, 1501–1538. doi:10.1089/neu.2004.21.1501

Shintani, A., Nakao, N., Kakishita, K., and Itakura, T. (2008). Generation of dopamine neurons from embryonic stem cells in the presence of the neuralizing activity of bone marrow stromal cells derived from adult mice. *J. Neurosci. Res.* 86, 2829–2838. doi:10.1002/jnr.21748

Willmann, C. A., Hemeda, H., Pieper, L. A., Lenz, M., Qin, J., Joussen, S., et al. (2013). To clone or not to clone? Induced pluripotent stem cells can be generated in bulk culture. *PLoS ONE* 8:e65324. doi:10.1371/journal.pone.0065324

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In vivo bioluminescence imaging of vascular remodeling after stroke

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Thrombolysis remains the only beneficial therapy for ischemic stroke, but is restricted to a short therapeutic window following the infarct. Currently research is focusing on spontaneous regenerative processes during the sub-acute and chronic phase. Angiogenesis, the formation of new blood vessels from pre-existing ones, was observed in stroke patients, correlates with longer survival and positively affects the formation of new neurons. Angiogenesis takes place in the border zones of the infarct, but further insight into the temporal profile is needed to fully apprehend its therapeutic potential and its relevance for neurogenesis and functional recovery. Angiogenesis is a multistep process, involving extracellular matrix degradation, endothelial cell proliferation, and, finally, new vessel formation. Interaction between vascular endothelial growth factor and its receptor 2 (VEGFR2) plays a central role in these angiogenic signaling cascades. In the present study we investigated non-invasively the dynamics of VEGFR2 expression following cerebral ischemia in a mouse model of middle cerebral artery occlusion (MCAO). We used a transgenic mouse expressing firefly luciferase under the control of the VEGFR2 promotor to non-invasively elucidate the temporal profile of VEGFR2 expression after stroke as a biomarker for VEGF/VEGFR2 signaling. We measured each animal repetitively up to 2 weeks after stroke and found increased VEGFR2 expression starting 3 days after the insult with peak values at 7 days. These were paralleled by increased VEGFR2 protein levels and increased vascular volume in peri-infarct areas at 14 days after the infarct, indicating that signaling via VEGFR2 leads to successful vascular remodeling. This study describes VEGFR2-related signaling is active at least up to 2 weeks after the infarct and results in increased vascular volume. Further, this study presents a novel strategy for the non-invasive evaluation of angiogenesis-based therapies.

Keywords: VEGFR2, flk-1, cerebral ischemia, angiogenesis, vessel density

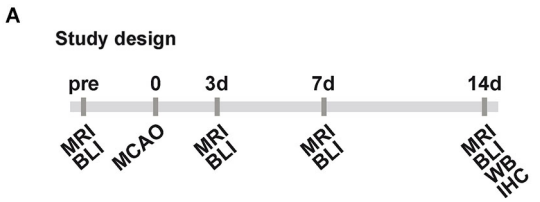
INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is recognized as a potential new therapeutic target in ischemic stroke (Slevin et al., 2006; Navaratna et al., 2009; Shibuya, 2009). Increased vascularization in areas surrounding the infarct has been observed in human (Krupinski et al., 1994; Szpak et al., 1999) as well as in animal brain tissue (Beck et al., 2000; Marti et al., 2000; Hayashi et al., 2003; Thored et al., 2007; Li et al., 2011) and is associated with improved functionality (Krupinski et al., 1994; Wang et al., 2006; Lee et al., 2007b; Reitmeir et al., 2012). Furthermore, angiogenesis is closely linked to neurogenesis and has shown positive effects on neuronal cell formation, migration and maturation (Taguchi et al., 2004). These results support the hypothesis, that angiogenesis after stroke is therapeutically advantageous.

The absence of adequate blood supply caused by the blockage of a cerebral artery leads to tissue hypoxia, which triggers the angiogenic response (Marti et al., 2000; Beck and Plate, 2009). Hypoxia inducible factor (HIF) 1α is stabilized under hypoxic conditions and dimerizes with HIF1β to form a transcription factor, which binds to the hypoxia responsible element in the promotor region of several hypoxia inducible cytokines and growth factors (Ferrara et al., 2003; Hayashi et al., 2006). The most potent angiogenic factor is the vascular endothelial growth factor (VEGF), which exerts its effect through its main receptor, i.e., vascular endothelial growth factor receptor 2 (VEGFR2; Ferrara et al., 2003; Koch et al., 2011). Activation of the VEGFR2 results in endothelial cell proliferation, migration and differentiation (Ferrara et al., 2003; Hayashi et al., 2006). Therefore, VEGFR2 plays a key role in adult angiogenesis and represents a molecular biomarker for angiogenic signaling in tissue. In a mouse

model of middle cerebral artery occlusion (MCAO), VEGFR2 was upregulated as early as 1 h poststroke, continued to increase for up to 1 week and decreased thereafter (Marti et al., 2000; Hayashi et al., 2003; Cai et al., 2009). Poststroke VEGFR2 expression was observed in neuronal and endothelial cells—neuronal VEGFR2 expression was early and transient, while its expression on endothelial cells persisted for prolonged time after the ischemic insult (Hayashi et al., 2003). Although poststroke VEGFR2 induction is not restricted to endothelial cells, its sub-acute and chronic expression is pre-dominently on endothelial cells. Furthermore, poststroke angiogenesis is VEGF/VEGFR2 signaling dependent, as inhibition of the VEGFR2 receptor resulted in decreased vascular regeneration and reduced regional cerebral blood flow (Li et al., 2011). The time profile of VEGFR2 expression after stroke is mainly based upon invasive methods like immunohistochemistry (IHC), Western blot (WB) and mRNA analysis. Up to date, only one study approached the non-invasive tracking of VEGFR2 expression as a correlate for angiogenesis after stroke (Cai et al., 2009). Using a PET-tracer for VEGF receptors Cai et al. (2009) found increased VEGFR2 expression in the ischemic hemisphere of rats until 16 days after stroke, and it subsequently decreased to almost normal levels at 23 days post-stroke. However, the tracer detected both, VEGFR2 and VEGFR1.

In the following study we utilized the VEGFR2 as a biomarker for the molecular regulation of angiogenic remodeling after stroke. We used the non-invasive imaging technique of bioluminescence to monitor the dynamic changes in VEGFR2 expression in a VEGFR2-luc reporter mouse with VEGFR2-specific photon emission (PE). We observed the temporal dynamics of VEGFR2 expression up to 2 weeks after stroke and validated the elevated VEGFR2 levels in the ischemic hemisphere by Western blotting of the receptor. Subsequently we evaluated the effect of increased VEGFR2 expression on vessel density by quantifying changes in vascular volume on immunohistological sections.



B
Group sizes

	BLI	WB	IHC
Healthy	6	5	3
Sham 14d	9	5	4
MCAO 7d	4	2	2
MCAO 14d	12	6	6

FIGURE 1 | Study design and group sizes. (A) Sham and MCAO animals were measured before (pre), and 3, 7, 14 days (3d, 7d, 14d) after 30 min MCAO with MRI and BLI. At 7d and 14d post MCAO tissue was collected for WB and IHC. **(B)** The displayed final group sizes as used per method and group. Excluded animals are not listed. (MCAO: middle cerebral artery occlusion, MRI: magnetic resonance imaging, BLI: bioluminescence imaging, WB: western blot, IHC: immunohistochemistry).

MATERIALS AND METHODS

ANIMAL MODEL

All animal experiments were conducted according to the guidelines laid out in the German Animal Welfare Act, in accordance with the European Council Directive 2010/63/EU, and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz North Rhine-Westphalia, reference number 84-02.04.2011.A123, as well as by the bioethics committee from Leiden University Medical Center, Leiden, The Netherlands, reference number 10215. A transgenic knock-in mouse model (Lyons et al., 2005) which expresses firefly luciferase under the control of the VEGFR2 promotor was used for all experiments. The animals were kept under *ad libitum* supply of food and water in a 12 h/12 h day and night cycle. All measurements and surgical interventions were performed under isoflurane anesthesia.

EXPERIMENTAL GROUPS

In total 39 male VEGFR2-luc knock-in mice (7–13 weeks old) were randomly assigned into groups of different survival times and different post mortem tissue processing. Six healthy control animals were measured with bioluminescence at three different days for the assessment of inter- and intra-animal stability of bioluminescence kinetics from the brain. Twelve animals received a sham surgery of which three had to be excluded due to death ($n = 1$) and spontaneous hyperintense brain areas on T2-weighted MR images ($n = 2$). Eighteen mice received a 30 min occlusion of the right middle cerebral artery of which two were excluded due to lack of stroke ($n = 1$) and strong weight loss ($n = 1$). Middle cerebral artery occlusion and sham animals were imaged 3 to 7 days before surgery (baseline acquisition) and 3, 7 and 14 days post surgery. Each bioluminescence imaging (BLI) session was directly followed by a magnetic resonance imaging (MRI) acquisition of T2 maps. Between day 3 and day 7 post surgery, sham and stroke animals received injections of 5-bromo-2′-deoxyuridine (BrdU, Sigma Aldrich, Taufkirchen, Germany) twice daily (50 mg/kg). At the end time point brain tissue was collected for either Western blots or IHC. An overview of the study design and final group sizes is presented in **Figure 1**.

MIDDLE CEREBRAL ARTERY OCCLUSION

The ischemic lesion was induced by transient occlusion of the right middle cerebral artery (MCAO), using the intraluminal filament model adapted from rat. The specific surgical method used in this study equals previously described MCAO in mice (Bahmani et al., 2011). Mice were anesthetized with 1–2% isoflurane in a 30/70 oxygen/air mixture and received a subcutaneous injection of 4 mg/kg buprenorphin (Temgesic, Merck, Darmstadt, Germany) for analgesia. A neck incision exposed the common carotid artery (CCA) and a silicon rubber-coated filament with a tip diameter of 170 μm (7017PK5Re, Doccol Corporation, Sharon, MA USA) was introduced into its lumen. The filament was advanced through the internal carotid artery (ICA) until it blocked the blood flow to the middle cerebral artery. Animals were allowed to recover under a red light lamp during the

occlusion period. After 30 min of occlusion, animals were re-anesthetized and reperfusion was initiated by filament removal. The CCA was permanently ligated. Sham surgery involved the partial introduction of a filament into the CCA without blocking the blood flow to the MCA. Animals were also recovered for 30 min and re-anesthetized for filament removal. The CCA was also ligated permanently. Following MCAO surgery, all animals received s.c. injections of 1 ml NaCl twice daily until the body weight stabilized.

BIOLUMINESCENCE IMAGING

One day prior to the first BLI session, mice were anesthetized in 2% isoflurane and the fur on the head was shaved to allow better photon penetration. It was not necessary to shave the animals a second time during the study. Photon emission was captured using the IVIS 100 (Perkin-Elmer, Waltham, MA, USA) equipped with a mirror system (prototype, Perkin-Elmer) consisting of two mirrors at a 45° angle to the basis (**Figure 2A**). Mice were individually anesthetized in 2% isoflurane and subsequently injected i.p. with 150 mg/kg click beetle luciferin (Promega, Madison, WI, USA) (stock solution 20 mg/ml). The acquisition of PE was directly started after luciferin injection with Living Image software (binning = 4, aperture = 1; Perkin-Elmer). Fifteen consecutive measurements of 1 min duration were performed in order to capture the inflow kinetics. Photon emission was analyzed for different regions of interest (ROIs; **Figure 2A**). Regions of interest were kept constant in size and were positioned on the photographic image using anatomical landmarks for guidance (ears, eyes; compare **Figure 2A**). Inflow kinetics and PE of the 15th min (PE15) after injection were extracted for each ROI. PE15 values from the "ischemic hemisphere" ROI and the "mirror ischemic" ROI were subsequently divided by the "intact hemisphere" and "mirror intact" ROI, respectively, in order to receive a quantitative ratio of change relative to the intact hemisphere. All values are displayed as mean ± standard deviation.

MAGNETIC RESONANCE IMAGING

Experiments were performed on a 7 Tesla Bruker Pharmascan 70/16 (Bruker Biospin, Ettlingen, Germany) with a 16 cm horizontal bore magnet and a 9 cm (inner diameter) shielded gradient, a maximum gradient strength of 300 mT/m, and a 23-mm birdcage transmit-receive RF coil (Bruker Biospin, Ettlingen, Germany). A multi-slice multi-echo (MSME) sequence (TR/TE = 4000 ms /11 ms, 16 echoes, 8 slices, slice thickness 1 mm, FOV 1.5 × 1.5 cm, matrix 128 × 128, resolution 117 × 117 μm) was measured for T2 evaluation and visualization of lesion location. T2 maps were calculated with IDL software (Exelis Visual Information Soution, Boulder, CO, USA).

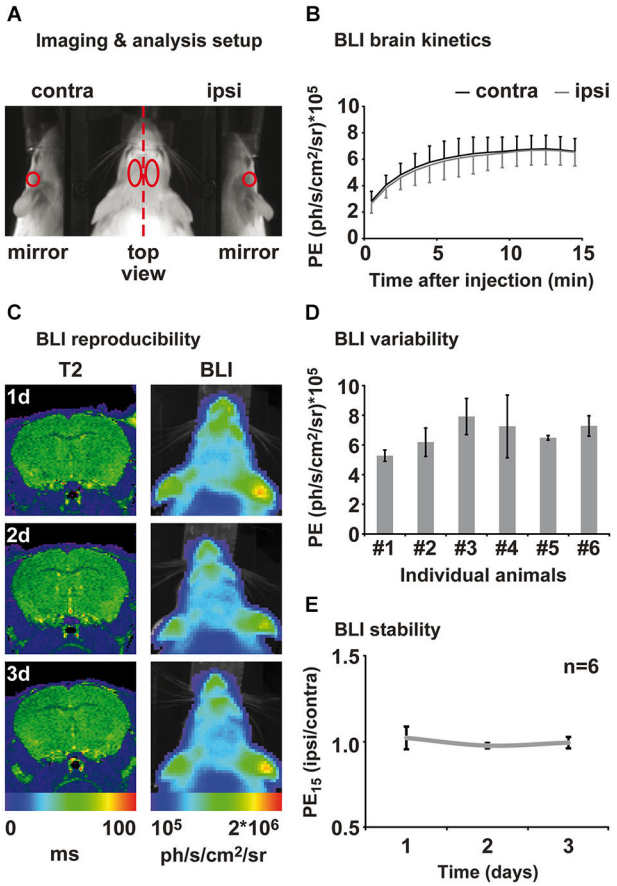


FIGURE 2 | Assessment of brain kinetics and stability of the BLI signal from healthy animals. (A) Animals were placed in a prone position on an elevated bar between two 45° mirrors. BLI was evaluated in four different regions of interest: ipsi- and contralateral top view, ipsi- and contra-lateral mirror (side) view. **(B)** Measuring PE directly after luciferin injection reveals brain specific inflow kinetics. An inflow phase between 1–10 min can be distinguished from a plateau phase of maximal PE between 12 and 15 min after injection. Emission from ipsi- and contralateral hemisphere was consistently of same magnitude. **(C)** Healthy control animals were measured repetitively for the assessment of the inter- and intra-animal variability of the BLI signal. MRI and BLI images of an exemplary healthy animal show an intact brain and stable intensity of PE over time. MRI is shown as coronal brain section, BLI is shown as horizontal planar projection of the PE onto the mouse photograph. **(D)** Three-fold measurement of each healthy animal revealed intra-individual variability of 3–30%. Inter-animal variability was around 20% to 45%. **(E)** Normalization of PE from ipsi- to contralateral hemisphere eliminates intra- and inter-animal variability and results in stable bioluminescence over time. Data is presented as mean ± standard deviation. (PE: photon emission, PE15: photon emission of the 15th minute after luciferin injection).

WESTERN BLOTTING

Animals were deeply anesthetized and killed by cervical dislocation. Brains were removed quickly and placed in ice cold phosphate buffered saline (PBS). Left and right cortex, as well as left and right striatum were dissected and directly frozen on dried ice. Tissue was stored at −80°C until further processing. Tissue was lysated in cell lysis buffer (#9803, Cell signaling Technology, Beverly, MA, USA) and treated with protease inhibitor complete Mini (CatNo 04693159001, Roche Applied Science, Indianapolis, Indiana, USA) supplemented with phenylmethylsulphonyl fluoride (PMSF, P6726, Sigma-Aldrich, Taufkirchen, Germany). Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). For each sample equal amounts of protein were electrophoresed through 8–16% SDS–PAGE gel (Invitrogen, Life Technologies, Darmstadt, Germany) and subsequently electrotransferred to nitrocellulose membranes

(ProTran, Whatman, Kent, UK). Membranes were probed with the primary antibodies for VEGFR2 (1:500; #2479, Cell Signaling Technology, Beverly, MA, USA), and β-actin (1:5000; MP Biomedical, Solon, Ohio, USA) overnight at 4°C. For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:3000 for β-actin, 1:800 for VEGFR2) followed by enhanced chemiluminescence development with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Results were analyzed using ImageJ software[1] (NIH). Regions of interest with constant size were positioned over each protein band and the integrated density was quantified followed by background subtraction and normalization to the β-actin signal. Data from the ischemic/sham hemisphere was then normalized to the intact hemisphere and displayed as mean ± standard deviation.

IMMUNOHISTOCHEMISTRY

Animals were deeply anesthetized and transcardially perfused with ice cold PBS followed by 20 ml 4% paraformaldehyde. Subsequently, brains were removed and shock frozen in −40°C methylbutane (Sigma-Aldrich, Taufkirchen, Germany). Brain tissue was stored at −80°C until further processing. Brain sections of 10 μm thickness were cut on the cryostat (Leica Microsystems, Wetzlar, Germany) and stored at −20°C. Sections for BrdU staining were pretreated with 2N hydrochloric acid for 2 h at room temperature. Prior to immunostaining, sections were preincubated at room temperature in 5% normal serum and 0.25% Triton X-100, in KPBS for 45 min. Primary antibodies were incubated overnight at 4°C. The following primary antibodies were used for double staining: anti-laminin (1:100, ab11575, Abcam, Cambridge, UK), anti-GFAP (1:200, G3898, Abcam Cambridge, UK), biotinylated *Solanum tuberosum* (potato) lectin (1:100, B-1165, Vector Laboratories, Burlingame, CA, USA), anti-BrdU (1:100, ab6326, Abcam, Cambridge, UK). Secondary antibodies were applied for 2 h at room temperature. A biotin-conjugated secondary antibody (1:200, Vector Laboratories, Burlingame, USA) was used with Alexa 488-conjugated streptavidin (1:200, Molecular Probes, Invitrogen, Life Technologies, Darmstadt, Germany). Cy2 and Cy3 (1:200, Jackson Immuno Research, West Grove, PA, USA) were used as complementary secondary antibodies for double staining, and Hoechst 33342 (1:1000 Invitrogen, Carlsbad, USA) was added during final incubation with secondary antibodies for nuclear staining. Slides were coverslipped with mounting medium (Entellan, Merck, Darmstadt, Germany). Z-stacks of BrdU/lectin positive cells were acquired with a confocal microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany). Laminin/GFAP double staining was used for vascular volume estimation. Microscopic images of whole brain sections were acquired at 4× magnification with a fluorescent microscope (BZ-9000 Keyence, Osaka, Japan). Areas of interest were defined inside the glial scar ("cortex core", "striatum core") and outside the glial scar ("cortex peri", "striatum peri"). From each area of interest three images were taken at 20× magnification from the ischemic side and from corresponding homotopic areas within the intact hemisphere, while holding exposure time constant. Using the Keyence microscope processing software, the area of staining was quantified in these ROIs by fixed thresholding and constant cut-off of clusters of small size (<100 pixels), which were regarded as unspecific dirt and were eliminated from the selection. Subsequently, the area covered by the staining was calculated for each image and a ratio was made to the corresponding image of the intact hemisphere. This procedure was chosen to conservatively control for differences in staining intensities between animal sections, although the staining was highly reproducible and in particular even across sections. A mean was calculated for each region for each animal. Subsequently, a group mean was calculated for each region. Data is presented as group mean ± standard deviation.

STATISTICS

Statistical analysis was performed with SPSS version 15 (IBM SPSS statistics, Ehningen, Germany). *In vivo* BLI data was tested for significant changes using a repeated measures analysis of variance (RM-ANOVA) with Bonferroni corrected *post hoc* comparisons. Western blot data was tested for significant changes by one way analysis of variance (one way ANOVA) with least significant difference *post hoc* comparisons between groups. Using an independent one-tailed Student's *t*-test we tested vascular volume for significant changes between the stroke and the sham group. We performed bivariate correlation analysis between bioluminescence and Western blot data as well as between bioluminescence and vascular volume data with Spearman's ρ correlation coefficient. A p-value ≤ 0.05 was considered to be statistically significant.

RESULTS

INTER- AND INTRA-ANIMAL STABILITY AND KINETICS OF THE BIOLUMINESCENCE SIGNAL

We characterized the PE kinetics from the brain of six healthy transgenic mice expressing firefly luciferase under the control of the VEGFR2 promotor using the ROIs depicted in **Figure 2A**. All animals showed increasing PE from the brain between 1 to 10 min after luciferin injection. Photon emission reached a maximum between 10 and 13 min and remained on this level in a plateau phase until the end of the measurement at 15 min post injection (**Figure 2B**). Photon emission kinetics and intensity from the right (ipsilateral) hemisphere were equal to the left (contralateral) hemisphere for each individual animal (**Figure 2B**). We therefore continued to evaluate the PE of the 15th minute after luciferin injection (PE_{15}), which represents maximum PE. Healthy animals were measured at three consecutive days for the assessment of inter- and intra-animal stability. Repetitive MRI on the healthy subjects reveals stable T2 values between time points and equality of both hemispheres. Corresponding BLI of this exemplary animal, presented as color-coded PE_{15}, confirms reproducibly equal VEGFR2 baseline expression in both hemispheres, as well as a stable PE_{15} over time (**Figure 2C**). Nevertheless, repeated measurement of the same animal resulted in PE_{15} variations from 3 to 30% of the mean and absolute signal intensity variation between animals was between 20 to 45% of the mean (**Figure 2D**). In order to investigate BLI changes over time, we corrected for inter- and intra-individual variation by normalizing the ischemic

[1]http://rsbweb.nih.gov/ij/

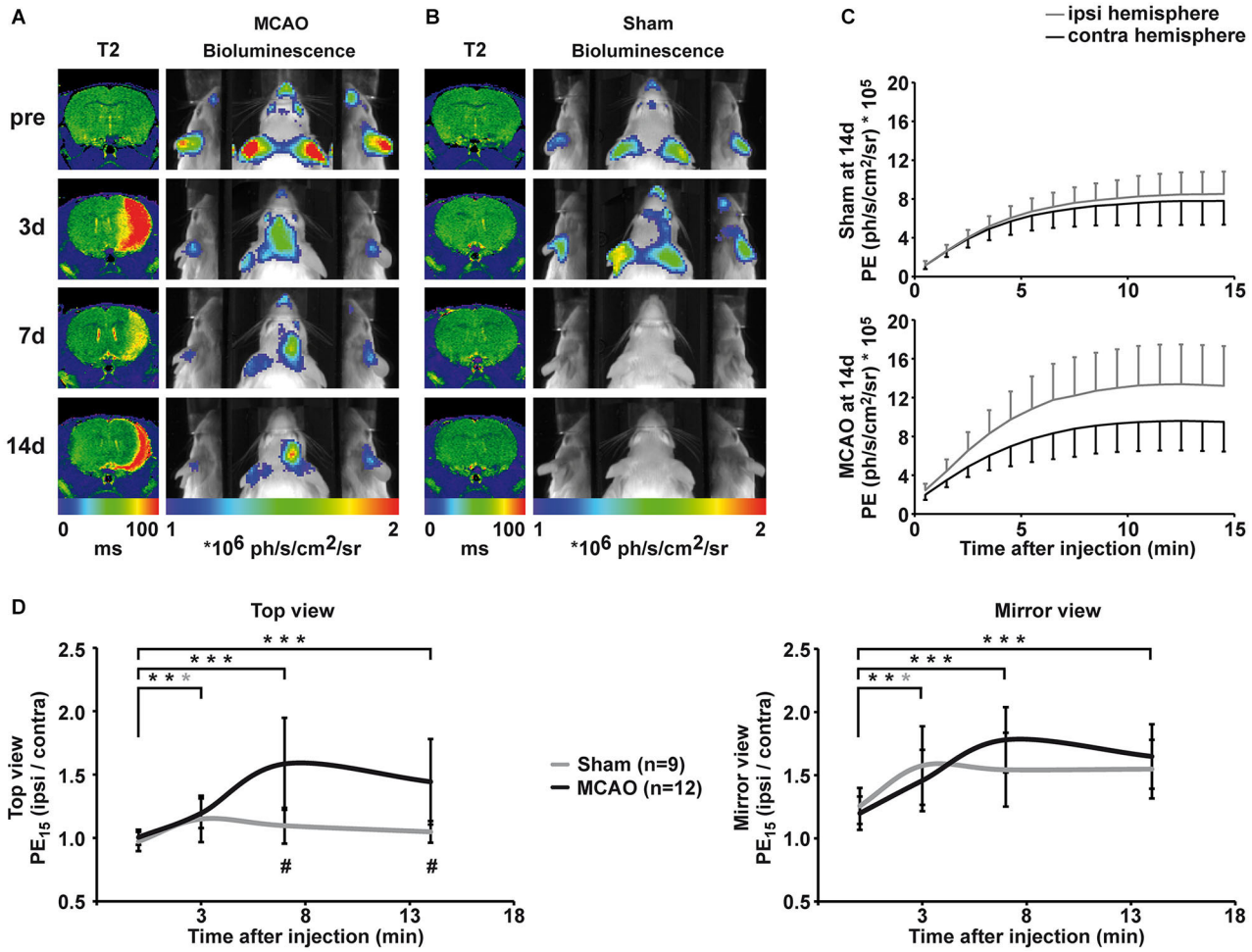


FIGURE 3 | Qualitative and quantitative evaluation of BLI changes after sham and MCAO surgery. (A) Representative longitudinal MRI and BLI data set from one animal that received MCAO surgery. MRI is shown as coronal brain section, BLI is shown as horizontal planar projection of the PE onto the mouse photograph. After MCAO, a clear lesion is visible in the right hemisphere on T2 maps. BLI signal intensity starts to increase 3d post-stroke and PE is clearly increased over the ischemic hemisphere compared to the intact hemisphere at 7d and 14d post-stroke. Increased PE from the ischemic hemisphere is distinctively visualized in the mirrors. **(B)** Representative longitudinal MRI and BLI data set from one animal that received sham surgery. Sham surgery did not result in lesion formation confirmed by unchanged T2 maps. However, sham surgery lead to a transient increase in PE from the ipsilateral side, which was best visible in the mirror system. **(C)** BLI kinetics of the ischemic and the intact hemisphere at 14d post sham surgery (upper graph) and MCAO surgery (lower graph). **(D)** Quantification of BLI changes was achieved by normalization of PE from ipsi- to contralateral hemisphere for the top view (left graph) and the mirror view (right graph). Pre-stroke PE was consistently equal in all investigated groups with a stronger variation in the mirror view. Increased PE was significantly different to pre-stroke values on day three for stroke, but also for sham animals in both views. At 7d and 14d, elevated PE in stroke animals was statistically significant compared to pre-stroke values (both $p < 0.001$) and to the sham group ($p = 0.001$, $p = 0.004$). (black = stroke, gray = sham, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # comparison between groups $p < 0.01$).

(ipsilateral) to the intact (contralateral) hemisphere. This analysis strategy shows stable equal VEGFR2 expression in healthy subjects over time (**Figure 2E**), thus allowing for reproducible and stable assessment of the hemispheric differences in VEGFR2 expression.

CEREBRAL ISCHEMIA

Cerebral ischemia was induced by 30 min occlusion of the right MCA with a silicone rubber-coated filament. Lesion size, location and development were assessed by MRI using quantitative T2 maps. Lesions appear as areas of increased T2 values on T2 maps, and a representative lesion is displayed in **Figure 3A**. Lesions were of similar size throughout all groups, including damage in the striatum and the parietal cortex, except for one animal of the 7d IHC group that showed only a small striatal infarct. At 14 days after MCAO, the ischemic hemisphere has shrunken in size, giving space to cerebrospinal fluid, which is visible as a rim of increased T2 value along the ischemic cortex.

BIOLUMINESCENCE OF VEGFR2 EXPRESSION AFTER STROKE

Before MCAO, T2 maps displayed no signs of lesion and PE was comparable for the right and left hemisphere (**Figure 3A**,

first row). Three days after MCAO, vasogenic edema resulted in increased T2 values, and a clear lesion was visible in the right hemisphere on T2 maps (**Figure 3A**, second row). Middle cerebral artery occlusion induced a significant increase in PE from the ischemic hemisphere (top view: $F_{(1.8,33.7)} = 14.8$, $p < 0.001$; mirror view: $F_{(3,45)} = 12.224$, $p < 0.001$). Photon emission from the ischemic hemisphere was already significantly increased at 3 days post MCAO compared to pre stroke values (top view: $p = 0.002$, mirror view: $p = 0.006$) indicating an early upregulation of VEGFR2 expression. Photon emission continued to increase from the ischemic hemisphere and was clearly visible on BL images on 7d and 14d after MCAO. The maximal PE increase of 58 ± 36% ($p = < 0.001$) in the ischemic hemisphere was observed 7d post MCAO in the top view (**Figure 3D**, left graph). The PE increase was even more pronounced (78 ± 26%; $p < 0.001$) when observed through the mirror system (**Figure 3D**, right graph). At the last observation time point of 14d post MCAO, the PE was still strongly increased in the ischemic hemisphere in both, the top view (44 ± 34%; $p = 0.001$) and the mirror view (65 ± 26%; $p = 0.001$). The pathological condition of ischemia does not alter the BLI kinetics described for healthy control mice. Photon emission from the ischemic hemisphere reaches the plateau phase also between 10–13 min but with higher PE than from the intact hemisphere (**Figure 3C**).

Sham surgery (introduction of the filament into the ICA but without advancing it to occlude the MCA) did not result in lesion formation (cf. T2 maps in **Figure 3B**), but, nevertheless, resulted in a transient change in PE on the ispilateral hemisphere (**Figures 3B,D**) at 3 days after surgery (top view: 15 ± 18% $p = 0.019$; mirror view: 58 ± 31% $p = 0.026$). The increase in emission was of similar magnitude as observed in the MCAO group. Although emission from the sham hemisphere stayed elevated at 7d and 14d, the change to the pre-surgery values was no longer significant (**Figure 3D**). The upregulation of VEGFR2 expression was significantly higher in MCAO animals when compared to sham animals ($F_{(1.77,33.67)} = 10.19$ $p = 0.001$) at 7d ($p = 0.001$) and at 14d ($p = 0.004$).

WESTERN BLOT ANALYSIS

Western blot analysis of healthy control animals shows little change in VEGFR2 expression between left and right hemisphere. Middle cerebral artery occlusion results in changes in VEGFR2 protein content of the ischemic hemisphere (**Figures 4A,B**). Significantly elevated levels of VEGFR2 protein were found in the ischemic cortex (40 ± 21%, $p = 0.005$) and in the ischemic striatum (32 ± 43%, $p = 0.048$) compared to healthy control animals (**Figure 4C**). Sham surgery resulted in non-significant elevation of VEGFR2 protein in the cortex (20 ± 15%), but an apparent decrease in the striatum (−7 ± 20%; **Figure 4C**).

IMMUNOHISTOCHEMISTRY

Histology was performed on the brains to determine changes in vascular volume after MCAO. 10-μm-thick brain sections were therefore stained with laminin. The stained area was interpreted as vascular volume. Glial fibrillary acidic protein staining was used for characterization of astrocyte activation in association with the ischemic lesion. Overview images were acquired for identification of the four areas of interest: the core lesion in the cortex (cortex core), the peri-infarct area of the cortical lesion (cortex peri), the striatal core lesion (striatum core), and the peri-infarct area of the striatal lesion (striatum peri). Three close-ups of 20× magnification were taken from each area of interest and from corresponding sites of the intact hemisphere with equal exposure times. **Figure 5A** shows representative close-ups of each region. As a measure for vascular volume changes, the area positive for laminin was compared between the ischemic hemisphere and the corresponding regions on the contralateral hemisphere (% area, normalized to contralateral side). Vascular volume was strongly reduced in the cortical core region in MCAO animals at 14 days after stroke (34 ± 17%, $p = 0.08$), but not in the striatal core. Peri-infarct regions of both, cortex and striatum, showed increased vascular volume, reaching significance in the striatum (29 ± 23%, $p = 0.03$; **Figure 5B**). Sham animals did not differ from healthy animals in all investigated brain regions. In each region that showed increased vascular volume, we observed endothelial cells with incorporated BrdU (**Figures 5C–E**), indicating that angiogenic signaling resulted in endothelial cell proliferation during day 3 and 7 after MCAO.

CORRELATION OF BIOLUMINESCENCE INTENSITY WITH PROTEIN EXPRESSION AND VASCULAR VOLUME

Middle cerebral artery occlusion resulted in an increase in bioluminescene intensity from the ischemic hemisphere, which was paralleled by the observation of increased VEGFR2 protein content and increased vascular volume. For the completeness of this study we performed correlation analysis of the bioluminescence signal intensity with VEGFR2 protein content on the one side, or with vascular volume on the other side (**Figure 6**). Bioluminescence intensity correlates well with VEGFR2 protein content in the cortex ($\rho = 0.474$, $p = 0.44$). No correlation was found between bioluminescence and protein content in the striatum, which is due to the stronger absorption of photons from this deeper structure. Therefore signal from the striatum will be overlayed by signal from the cortex. Also a significant correlation was observed to vascular volume in peri-infarct areas ($\rho = 0.817$, $p < 001$).

DISCUSSION

We non-invasively observed the temporal profile of VEGFR2 expression after cerebral ischemia as a molecular reporter for post-stroke pro-angiogenic signaling, while we followed lesion development with the complementary method of MRI. VEGFR2 plays a key role in post-ischemic vascular remodeling and we found increased expression lasting up to 14d post MCAO, which was paralleled by an increased vascular volume in distinct peri-infarct areas.

METHODOLOGICAL CONSIDERATIONS

Bioluminescence imaging is a very sensitive method and allows for the detection of even small changes in cell numbers (Keyaerts et al., 2008). However, it has a poor spatial resolution. For the first time we report here the additional observation of PE through two 45° angled mirrors for a better discrimination of left and right hemisphere through lateral views. Signal changes

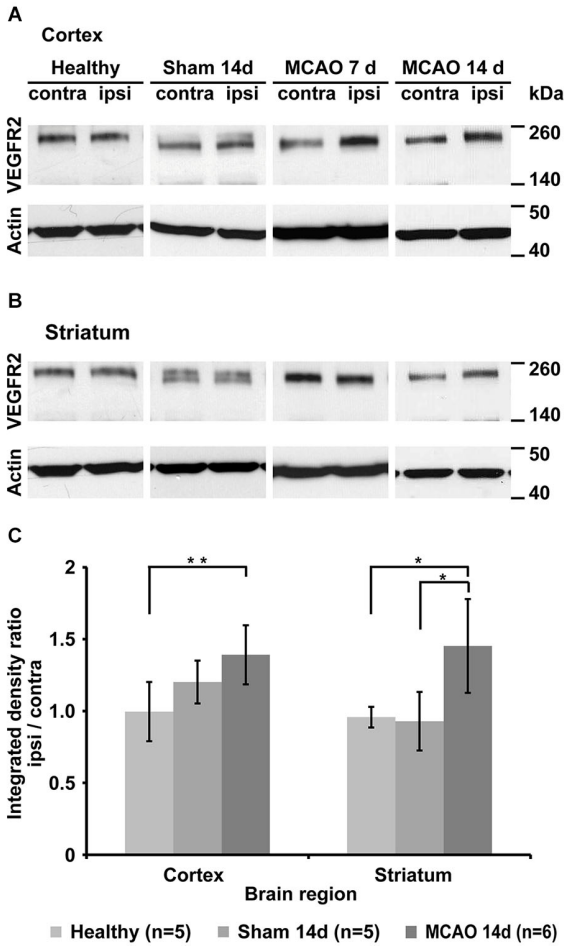


FIGURE 4 | Qualitative and quantitative evaluation of tissue VEGFR2 protein content. (A) Representative Western blots from cortical tissue samples for each group showing increased VEGFR2 (210 and 230 kDa) content in the ischemic cortex of animals that underwent MCAO. Note the strong increase in the 7d group. Sham animals and healthy control display similar levels between left and right hemisphere. **(B)** Representative Western blots from striatal tissue samples. Strongest elevation is visible in the 14d MCAO group. **(C)** Semi-quantification (including normalization to actin) and subsequent normalization to the intact hemisphere reveals significant increased VEGFR2 expression in the ischemic cortex ($p = 0.005$) and ischemic striatum ($p = 0.048$) (* $p < 0.05$, ** $p < 0.01$).

were consistently greater with intensity readouts from the mirror views, but variation of the signal was also increased, suggesting the introduction of additional noise. *In vitro*, a linear correlation exists between PE and number of luciferase expressing cells. *In vivo* this relation is affected by luciferin distribution and photon absorption and scattering by tissue (Virostko et al., 2004; Inoue et al., 2009; Keyaerts et al., 2011). Bioluminence imaging of the brain has to deal with limited substrate diffusibility through the blood brain-barrier (BBB; Berger et al., 2008). Brain pathologies, which result in a breakdown of the BBB, impose even further methodological obstacles towards the interpretation of the PE. Theoretically, an open BBB may facilitate luciferin inflow on the ischemic hemisphere, thus resulting in higher PE due to higher substrate availability, possibly mistaken for higher luciferase content. However, we can exclude the open BBB as dominating confounding factor in this animal model and study, since sham animals with intact BBB have PE increase equal to MCAO animals with open BBB at 3d post surgery. If carotid artery occlusion (sham surgery) disturbs the BBB integrity, it will be of much less intensity than by stroke. Hence, if the increase at that time point was only due to increased luciferin content rather than increased expression of VEGFR2 (and therefore luciferase), then we should have had observed stronger signal in MCAO compared to sham. Furthermore, increased wash-in of luciferin due to an open BBB would result in faster slopes of the signal kinetics. This, however, was not seen at all. We can therefore safely conclude that BBB breakdown in this model does not affect the bioluminescence reaction and that the increase in PE can be solely attributed to increased VEGFR2 expression.

VEGFR2 UPREGULATION DURING POST-STROKE ANGIOGENESIS

In order to non-invasively monitor VEGFR2 expression following stroke, we made use of a transgenic mouse expressing luciferase

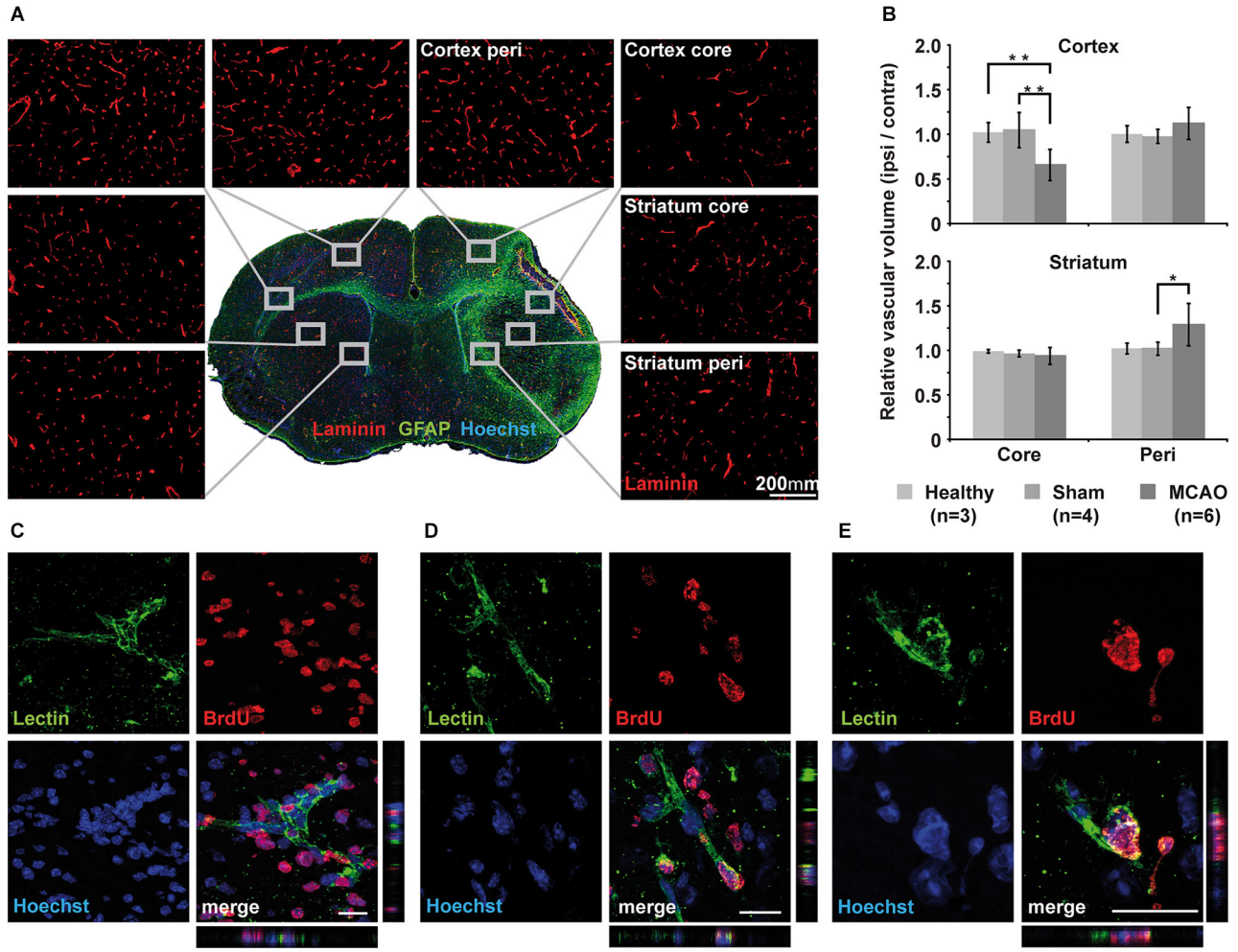


FIGURE 5 | Immunohistochemical analysis of vascular changes. (A) Immunohistochemistry of laminin for vascular volume estimation. Representative close-ups of 20x magnification for each region of interest visualize changes in vessel density. **(B)** Quantification and subsequent normalization to the intact hemisphere confirms decreased vascular volume in the core region of the cortex but not in the core region of the striatum. Vascular volume increases significantly in the striatal peri-infarct zone (p = 0.03) but does not reach significance in peri-infarct cortex (p = 0.08). Sham animals showed little changes in corresponding areas of the brain. **(C–E)** Three examples of Z-stacks of representative BrdU+/lectin+newly formed endothelial cells from the peri-infarct striatum **(C)** and the cortex **(D,E)**. Scale bar 20 μm.

under the control of the VEGFR2 promotor (Lyons et al., 2005). We observed increasing bioluminescence intensity after cerebral ischemia in the ischemic hemisphere, which peaked at 7d post-stroke. In agreement with previous reports (Marti et al., 2000; Hayashi et al., 2003; Cai et al., 2009), our results show a continuous increase in VEGFR2 expression from 3d to 7d post-stroke. We observe still significantly elevated expression at 14d post-stroke, which was confirmed by increased VEGFR2 protein content in the ischemic hemisphere compared to the intact hemisphere. Semi-quantitative analysis of regional VEGFR2 content in the brain by Western blotting indicates strong expression in the ischemic striatum and cortex, since the amount of VEGFR2 was increased by as much as 40%. Also sham surgery resulted in a transient increase in BLI signal from the sham hemisphere, indicating also upregulated VEGFR2 expression. Western blot results show a slightly increased VEGFR2 content at 14d post surgery in the right cortex, but not in the striatum of sham animals. Sham surgery involved the introduction of a filament only into the CCA. Upon removal of the filament, the CCA was ligated permanently to control for blood flow changes induced by the permanent ligation of the CCA in the stroke group. Common carotid artery occlusion is used as a model for mild hypoxia and chronic cerebral hypoperfusion (Hecht et al., 2012; Pimentel-Coelho et al., 2012). Following the occlusion of the ICA, Hecht et al. (2012) observed reduction by 80% in overall cerebral blood flow, while cortical perfusion was not notably changed. Unilateral CCA occlusion did not result in neuronal cell death within the territory of the MCAO (Pimentel-Coelho et al., 2012), but at 21d after ICA occlusion, slightly increased vessel density was noted in the ipsilateral cortex (Hecht et al., 2012). Although our study did not indicate a strong increase

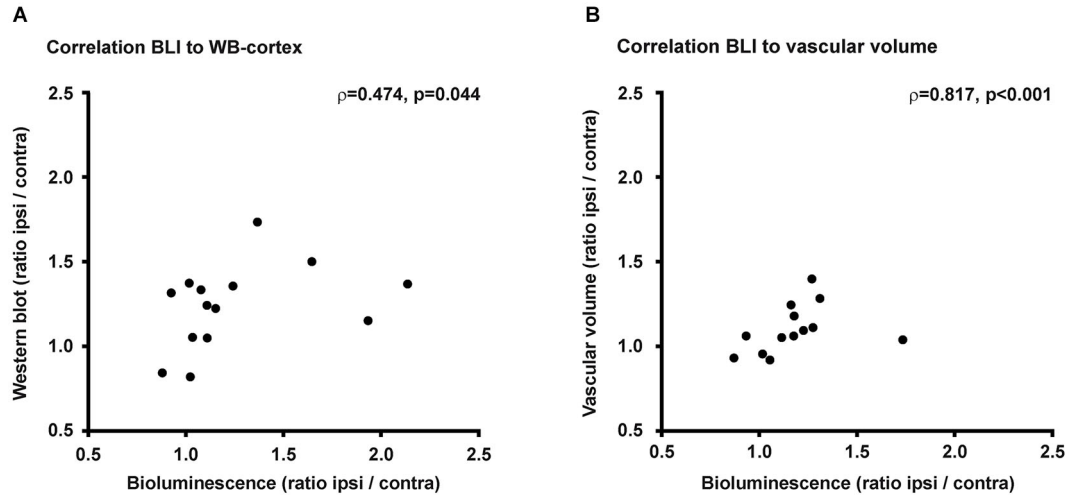


FIGURE 6 | Correlations. (A) Changes of PE within the ischemic hemisphere correlates to VEGFR2 protein changes in the ischemic cortex (ρ = 0.474, p = 0.044). **(B)** Photon emission increase also correlates to vascular volume changes within the ischemic hemisphere (ρ = 0.817, $p < 0.001$).

in cortical or striatal vascular volume, BLI was sensitive enough to detect the minor changes in VEGFR2 expression induced by mild hypoxia after CCA occlusion.

VASCULAR VOLUME INCREASE IN PERI-INFARCT AREAS

We investigated the vascular volume in core regions and in peri-infarct regions of both, the cortex and the striatum. Our MCAO model resulted in strong reduction in vascular volume within the cortical core region. Similar observations were made by Bosomtwi et al. (2011) in a distal MCAO rat model where only the cortex is affected. Yet, in the region defined as striatum core, we detected no decrease in vessel density. High upregulation of VEGFR2 in the striatum, as indicated by our WB results, may account for early enhanced endothelial survival due to protective properties of VEGF2 (Ferrara, 2004; Shibuya, 2006; Lee et al., 2007a; Hermann and Zechariah, 2009), and may even have been followed by stabilization of existing or recruitment of new vessels. In accordance with previous studies (Marti et al., 2000; Hayashi et al., 2003; Thored et al., 2007; Li et al., 2011; Reitmeir et al., 2012), we observed increased vascular volume in the peri-infarct striatum (29 $\pm$ 23%) and peri-infarct cortex (12% $\pm$ 17%). Vascular volume increase in the striatal peri-infarct was most pronounced in the most dorsal part of the striatum close to the subventricular zone (SVZ), which has been described as highly angiogenic (Ohab et al., 2006; Thored et al., 2007). Strong endothelial cell proliferation had been observed in this area next to the SVZ between 1 and 2 weeks, and consequently increased vessel density persisted up to 16 weeks (Thored et al., 2007). Other studies reported endothelial cell proliferation starting as early as 24 h after MCAO in mice (Hayashi et al., 2003), and increased vessel density was detectable at 2–3d post-stroke (Marti et al., 2000; Hayashi et al., 2003). The number of microvessels remained increased up to 21d (Hayashi et al., 2003). In both regions with increased vascular volume, striatal and cortical peri-infarct, we detected proliferating endothelial cells, which were newly generated in the first week after stroke, due to BrdU incorporation. As new vessels can also form without endothelial cell proliferation by splitting existing vessels through the insertion of tissue pillars, intussusception (Adams and Alitalo, 2007) and peripheral bone marrow-derived endothelial progenitor cells can contribute to vessel formation or stabilization (Zhang et al., 2002) we restrained from quantification of BrdU positive endothelial cells as this would underestimate the intensity of increased vascular volume.

STROKE-INDUCED ANGIOGENIC SIGNALING CAN BE NON-INVASIVELY QUANTIFIED WITH THE VEGFR2-luc MOUSE MODEL

The bioluminescence signal change reflected increased VEGFR2 expression within the ischemic hemisphere. Western blot analysis revealed increased VEGFR2 protein concentration in both, ischemic striatum and cortex. In both regions, increased VEGFR2 expression translated into increased vascular volume. A positive correlation, not very strong but significant, between bioluminescence intensity and VEGFR2 protein content within the cortex was observed and renders this mouse model an optimal model for non-invasive tracking of VEGFR2 regulation in the brain. No correlation was observed to VEGFR2 content of the striatum. As BLI signal recorded by the CCD camera is a two-dimensional image from the three-dimensional structure of the brain, signals from deeper structures like the striatum are subjected to stronger absorption by overlaying tissue than signals from structures closer to the brain surface like the cortex. Hence, the detected bioluminescence signal in this model is strongly dominated by its contribution from cortex. A significant correlation was also detected between PE intensity and vascular volume. Such a correlation was not expected because of the time delay between VEGFR2 expression and the occurrence of laminated microvessels, and due to additional expression of VEGFR2 by cells other than proliferating endothelial cells (Hayashi et al., 2003). VEGFR2 is mainly expressed on

endothelial cells and functions as transducer of survival, proliferation, migration and differentiation cues (Ferrara et al., 2003). Following stroke, the number of VEGFR2 positive endothelial cells was strongly increased in the penumbra (Li et al., 2011), but also post-ischemic neurons started to express this receptor (Hayashi et al., 2003; Beck and Plate, 2009). However, neuronal VEGFR2 expression was an early and transient effect, declining already 3 days after ischemia, while its expression remained strong in endothelial cells (Hayashi et al., 2003). Vascular endothelial growth factor receptor 2 expression was also reported on astrocytes (Issa et al., 1999) and microglia/macrophages (Lennmyr et al., 1998) following stroke, however, other groups could not reproduce these results (Krum et al., 2002). Further, a distinct pattern, where astrocytes express VEGFR1 and endothelial cells express VEGFR2 was suggested (Krum et al., 2008). Therefore, we believe it is justified to neglect the effect of neuronal and glial poststroke VEGFR2 in our study. The changes in VEGFR2 levels we observe in the chronic phase after stroke represent predominantly changes of VEGFR2 on endothelial cells. New vessel formation is not restricted to on-site endothelial cell proliferation but can occur also by intussusception (Adams and Alitalo, 2007) and by the contribution of peripheral bone marrow-derived endothelial progenitor cells (Zhang et al., 2002). Also in these processes signaling via the VEGFR2 plays a pivotal role. Poststroke angiogenesis is VEGF/VEGFR2 signaling dependent, as selectively blocking the VEGFR2 leads to decreased endothelial cell proliferation and reduced cerebral blood flow (Li et al., 2011). We can summarize that pro-angiogenic signaling via the VEGF—VEGFR2 pathway is a general necessity for new vessel formation, and non-invasive observation of the VEGFR2 expression provides longitudinal information about poststroke neurovascular dynamics.

CONCLUSION

The present study established the value of the VEGFR2-luc mouse model for non-invasive tracking of VEGFR2 expression as a correlate for neurovascular remodeling in stroke pathology. We have deciphered non-invasively and quantitatively a temporal profile of VEGFR2 expression for the first 2 weeks after stroke. Also, we have observed, that chronic VEGFR2 expression correlates well with vascular volume increase in cortical peri-infarct regions. Future studies will benefit from this newly validated non-invasive tool for investigations of angiogenesis promoting therapies.

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REFERENCES

Adams, R. H., and Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* 8, 464–478. doi: 10.1038/nrm2183

Bahmani, P., Schellenberger, E., Klohs, J., Steinbrink, J., Cordell, R., Zille, M., et al. (2011). Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide and TUNEL staining. *J. Cereb. Blood Flow Metab.* 31, 1311–1320. doi: 10.1038/jcbfm.2010.233

Beck, H., Acker, T., Wiessner, C., Allegrini, P. R., and Plate, K. H. (2000). Expression of angiopoietin-1, angiopoietin-2 and tie receptors after middle cerebral artery occlusion in the rat. *Am. J. Pathol.* 157, 1473–1483. doi: 10.1016/s0002-9440(10)64786-4

Beck, H., and Plate, K. H. (2009). Angiogenesis after cerebral ischemia. *Acta Neuropathol.* 117, 481–496. doi: 10.1007/s00401-009-0483-6

Berger, F., Paulmurugan, R., Bhaumik, S., and Gambhir, S. S. (2008). Uptake kinetics and biodistribution of 14C-D-luciferin—a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: impact on bioluminescence based reporter gene imaging. *Eur. J. Nucl. Med. Mol. Imaging* 35, 2275–2285. doi: 10.1007/s00259-008-0870-6

Bosomtwi, A., Chopp, M., Zhang, L., Zhang, Z. G., Lu, M., and Jiang, Q. (2011). Mean microvessel segment length and radius after embolic stroke: comparison of magnetic resonance imaging (MRI) and laser scanning confocal microscopy (LSCM). *Brain Res.* 1381, 217–227. doi: 10.1016/j.brainres.2011.01.009

Cai, W., Guzman, R., Hsu, A. R., Wang, H., Chen, K., Sun, G., et al. (2009). Positron emission tomography imaging of poststroke angiogenesis. *Stroke* 40, 270–277. doi: 10.1161/STROKEAHA.108.517474

Ferrara, N. (2004). Vascular endothelial growth factor: basic science and clinical progress. *Endocr. Rev.* 25, 581–611. doi: 10.1210/er.2003-0027

Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat. Med.* 9, 669–676. doi: 10.1038/nm0603-669

Hayashi, T., Deguchi, K., Nagotani, S., Zhang, H., Sehara, Y., Tsuchiya, A., et al. (2006). Cerebral ischemia and angiogenesis. *Curr. Neurovasc. Res.* 3, 119–129. doi: 10.2174/156720206776875902

Hayashi, T., Noshita, N., Sugawara, T., and Chan, P. H. (2003). Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. *J. Cereb. Blood Flow Metab.* 23, 166–180. doi: 10.1097/00004647-200302000-00004

Hecht, N., He, J., Kremenetskaia, I., Nieminen, M., Vajkoczy, P., and Woitzik, J. (2012). Cerebral hemodynamic reserve and vascular remodeling in C57/BL6 mice are influenced by age. *Stroke* 43, 3052–3062. doi: 10.1161/STROKEAHA.112.653204

Hermann, D. M., and Zechariah, A. (2009). Implications of vascular endothelial growth factor for postischemic neurovascular remodeling. *J. Cereb. Blood Flow Metab.* 29, 1620–1643. doi: 10.1038/jcbfm.2009.100

Inoue, Y., Kiryu, S., Izawa, K., Watanabe, M., Tojo, A., and Ohtomo, K. (2009). Comparison of subcutaneous and intraperitoneal injection of D-luciferin for in vivo bioluminescence imaging. *Eur. J. Nucl. Med. Mol. Imaging* 36, 771–779. doi: 10.1007/s00259-008-1022-8

Issa, R., Krupinski, J., Bujny, T., Kumar, S., Kaluza, J., and Kumar, P. (1999). Vascular endothelial growth factor and its receptor, KDR, in human brain tissue after ischemic stroke. *Lab. Invest.* 79, 417–425.

Keyaerts, M., Heneweer, C., Gainkam, L. O., Caveliers, V., Beattie, B. J., Martens, G. A., et al. (2011). Plasma protein binding of luciferase substrates influences sensitivity and accuracy of bioluminescence imaging. *Mol. Imaging Biol.* 13, 59–66. doi: 10.1007/s11307-010-0325-x

Keyaerts, M., Verschueren, J., Bos, T. J., Tchouate-Gainkam, L. O., Peleman, C., Breckpot, K., et al. (2008). Dynamic bioluminescence imaging for quantitative tumour burden assessment using IV or IP administration of D: -luciferin: effect on intensity, time kinetics and repeatability of photon emission. *Eur. J. Nucl. Med. Mol. Imaging* 35, 999–1007. doi: 10.1007/s00259-007-0664-2

Koch, S., Tugues, S., Li, X., Gualandi, L., and Claesson-Welsh, L. (2011). Signal transduction by vascular endothelial growth factor receptors. *Biochem. J.* 437, 169–183. doi: 10.1042/BJ20110301

Krum, J. M., Mani, N., and Rosenstein, J. M. (2002). Angiogenic and astroglial responses to vascular endothelial growth factor administration in adult rat brain. *Neuroscience* 110, 589–604. doi: 10.1016/s0306-4522(01)00615-7

Krum, J. M., Mani, N., and Rosenstein, J. M. (2008). Roles of the endogenous VEGF receptors flt-1 and flk-1 in astroglial and vascular remodeling after brain injury. *Exp. Neurol.* 212, 108–117. doi: 10.1016/j.expneurol.2008.03.019

Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., and Wang, J. M. (1994). Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke* 25, 1794–1798. doi: 10.1161/01.str.25.9.1794

Lee, S., Chen, T. T., Barber, C. L., Jordan, M. C., Murdock, J., Desai, S., et al. (2007a). Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 130, 691–703. doi: 10.1016/j.cell.2007.06.054

Lee, H. J., Kim, K. S., Park, I. H., and Kim, S. U. (2007b). Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model. *PLoS One* 2:e156. doi: 10.1371/journal.pone.0000156

Lennmyr, F., Ata, K. A., Funa, K., Olsson, Y., and Terent, A. (1998). Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. *J. Neuropathol. Exp. Neurol.* 57, 874–882. doi: 10.1097/00005072-199809000-00009

Li, W. L., Fraser, J. L., Yu, S. P., Zhu, J., Jiang, Y. J., and Wei, L. (2011). The role of VEGF/VEGFR2 signaling in peripheral stimulation-induced cerebral neurovascular regeneration after ischemic stroke in mice. *Exp. Brain Res.* 214, 503–513. doi: 10.1007/s00221-011-2849-y

Lyons, S. K., Clermont, A. O., Neben, T. Y., Campbell, K., Coffee, R., Hunter, J., et al. (2005). Non-invasive in vivo bioluminescent imaging of tumor angiogenesis in mice. *AACR Meeting Abstracts* 2005, 903-b.

Marti, H. J., Bernaudin, M., Bellail, A., Schoch, H., Euler, M., Petit, E., et al. (2000). Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am. J. Pathol.* 156, 965–976. doi: 10.1016/s0002-9440(10)64964-4

Navaratna, D., Guo, S., Arai, K., and Lo, E. H. (2009). Mechanisms and targets for angiogenic therapy after stroke. *Cell Adh. Migr.* 3, 216–223. doi: 10.4161/cam.3.2.8396

Ohab, J. J., Fleming, S., Blesch, A., and Carmichael, S. T. (2006). A neurovascular niche for neurogenesis after stroke. *J. Neurosci.* 26, 13007–13016. doi: 10.1523/jneurosci.4323-06.2006

Pimentel-Coelho, P. M., Michaud, J. P., and Rivest, S. (2012). Effects of mild chronic cerebral hypoperfusion and early amyloid pathology on spatial learning and the cellular innate immune response in mice. *Neurobiol. Aging* 34, 679–693. doi: 10.1016/j.neurobiolaging.2012.06.025

Reitmeir, R., Kilic, E., Reinboth, B. S., Guo, Z., Elali, A., Zechariah, A., et al. (2012). Vascular endothelial growth factor induces contralesional corticobulbar plasticity and functional neurological recovery in the ischemic brain. *Acta Neuropathol.* 123, 273–284. doi: 10.1007/s00401-011-0914-z

Shibuya, M. (2006). Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J. Biochem. Mol. Biol.* 39, 469–478. doi: 10.5483/bmbrep.2006.39.5.469

Shibuya, M. (2009). Brain angiogenesis in developmental and pathological processes: therapeutic aspects of vascular endothelial growth factor. *FEBS J.* 276, 4636–4643. doi: 10.1111/j.1742-4658.2009.07175.x

Slevin, M., Kumar, P., Gaffney, J., Kumar, S., and Krupinski, J. (2006). Can angiogenesis be exploited to improve stroke outcome? Mechanisms and therapeutic potential. *Clin. Sci. (Lond).* 111, 171–183. doi: 10.1042/cs20060049

Szpak, G. M., Lechowicz, W., Lewandowska, E., Bertrand, E., Wierzba-Bobrowicz, T., and Dymecki, J. (1999). Border zone neovascularization in cerebral ischemic infarct. *Folia Neuropathol.* 37, 264–268.

Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., et al. (2004). Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J. Clin. Invest.* 114, 330–338. doi: 10.1172/jci200420622

Thored, P., Wood, J., Arvidsson, A., Cammenga, J., Kokaia, Z., and Lindvall, O. (2007). Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke* 38, 3032–3039. doi: 10.1161/strokeaha.107.488445

Virostko, J., Chen, Z., Fowler, M., Poffenberger, G., Powers, A. C., and Jansen, E. D. (2004). Factors influencing quantification of in vivo bioluminescence imaging: application to assessment of pancreatic islet transplants. *Mol. Imaging* 3, 333–342. doi: 10.1162/1535350042973508

Wang, Y., Galvan, V., Gorostiza, O., Ataie, M., Jin, K., and Greenberg, D. A. (2006). Vascular endothelial growth factor improves recovery of sensorimotor and cognitive deficits after focal cerebral ischemia in the rat. *Brain Res.* 1115, 186–193. doi: 10.1016/j.brainres.2006.07.060

Zhang, Z. G., Zhang, L., Jiang, Q., and Chopp, M. (2002). Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circ. Res.* 90, 284–288. doi: 10.1161/hh0302.104460

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A review of novel optical imaging strategies of the stroke pathology and stem cell therapy in stroke

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Transplanted stem cells can induce and enhance functional recovery in experimental stroke. Invasive analysis has been extensively used to provide detailed cellular and molecular characterization of the stroke pathology and engrafted stem cells. But post mortem analysis is not appropriate to reveal the time scale of the dynamic interplay between the cell graft, the ischemic lesion and the endogenous repair mechanisms. This review describes non-invasive imaging techniques which have been developed to provide complementary *in vivo* information. Recent advances were made in analyzing simultaneously different aspects of the cell graft (e.g., number of cells, viability state, and cell fate), the ischemic lesion (e.g., blood–brain-barrier consistency, hypoxic, and necrotic areas) and the neuronal and vascular network. We focus on optical methods, which permit simple animal preparation, repetitive experimental conditions, relatively medium-cost instrumentation and are performed under mild anesthesia, thus nearly under physiological conditions. A selection of recent examples of optical intrinsic imaging, fluorescence imaging and bioluminescence imaging to characterize the stroke pathology and engrafted stem cells are discussed. Special attention is paid to novel optimal reporter genes/probes for genetic labeling and tracking of stem cells and appropriate transgenic animal models. Requirements, advantages and limitations of these imaging platforms are critically discussed and placed into the context of other non-invasive techniques, e.g., magnetic resonance imaging and positron emission tomography, which can be joined with optical imaging in multimodal approaches.

Keywords: optical neuroimaging, non-invasive, stem cell therapy, stroke, bioluminescence imaging, fluorescence imaging

INTRODUCTION

The stroke pathology and regeneration processes induced by endogenous mechanisms or engrafted stem cells have been studied extensively. Invasive studies – including immunohistochemistry, autoradiography, electrophysiology, and molecular biology – revealed the ischemic cascade of pathological and protective signaling events (Zhang and Chopp, 2009; Iadecola and Anrather, 2011). New neurons are found in the rat striatum after experimental stroke (Arvidsson et al., 2002), but neurogenesis and functional neuronal integration seem alone not to be able to restore brain function. In this line, exogenous stem cells, e.g., neural stem cells (NSCs) derived from embryonic or induced-pluripotent stem cells, have been implanted in experimental rodent models of stroke and found to increase functional recovery in many studies (Bliss et al., 2007; Oki et al., 2012). However, the interplay of stem cells with the injured host tissue and the mode of action of engrafted cells in the longitudinal time profile of stroke regeneration have to be deciphered before clinical translation.

We review here optical imaging as one promising approach to shed new light on structural and functional components of stem cell therapy in stroke. We introduced fluorescence and bioluminescence imaging (FLI and BLI) which have been extensively developed in the last decade to meet the criteria of a highly sensitive and minimally invasive set-up (**Figure 1**). We provide a selection of recent publications related to stroke and/or stem cell transplantation in rodents, which we find appropriate to introduce current possibilities and constraints of optical neuroimaging. In addition, selected references – in line with most preclinical optical imaging studies – refer to mice or rats and exclude, e.g., stroke studies in non-human primates (Bihel et al., 2010) or the zebrafish (Walcott and Peterson, 2014).

OPTICAL NEUROIMAGING

Molecular imaging aims to visualize cellular and molecular events – related to physiological or pathophysiological processes – in the living subject, e.g., by genetically linked imaging reporters (Massoud et al., 2008a). Based on the first non-invasive experiments with superficial sources, optical neuroimaging has been so far most effectively implemented for brain tumor studies (Massoud et al., 2008b) and less for neurological disease models or endogenous/exogenous NSCs in which sensitivity is essential. Compared to superficial sources, the brain appears to be a very difficult organ to be penetrated and explored by light. The natural multilayer barrier of blood, meninges, bone and skin covers all neural cells. Despite the extensive technical developments in optical imaging, major challenges of light absorption and scattering, autofluorescence, low spectral resolution and quantification still need to be considered (Shah and Weissleder, 2005; Hillman, 2007; Sutton et al., 2008). Among

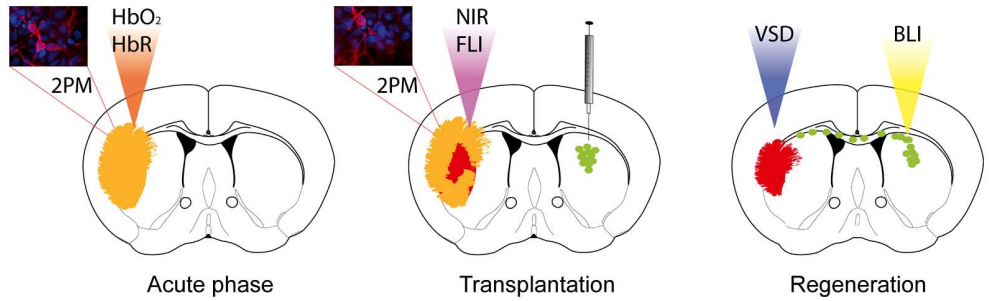

FIGURE 1 | Optical neuroimaging strategies to target the stroke pathology and regeneration processes upon stem cell grafting. Two-photon microscopy (2PM) can be used to monitor blood flow changes during the acute phase, as well as changes in the cortical neuronal network over time. Optical intrinsic imaging utilizes the different absorption behavior of oxygenated (HbO_2) and deoxygenated hemoglobin (HbR) to resolve changes in blood flow. Near-infrared probes have been designed to target, e.g., apoptotic cells or immune cells specifically by fluorescence imaging (FLI). Functional reorganization can be monitored by application of voltage-sensitive dye (VSD) imaging. Transplanted stem cells expressing bioluminescence and fluorescence imaging reporters allow longitudinal monitoring by bioluminescence imaging (BLI) and histological validation.

these physical limitations, light absorption and scattering are the main cause that affects *in vivo* optical approaches. Absorption is mainly driven by pigments/chromophores (hemoglobin and bilirubin in the blood, myoglobin in the muscles, pheo- and eumelanin in the skin) and also by water and lipids (**Figure 2**). Brain tissue requires continuous blood supply, which implies strong light attenuation by absorption. Efficient light propagation through the brain is provided in a naturally existing window of low absorption in the near-infrared (NIR, ∼700–900 nm; Frangioni, 2003). Despite absorption effects, light is scattered at inter- and intracellular membrane boundaries due to differences in the refractive index n (ratio of the speed of light in vacuum and speed of light in the material), e.g., extracellular fluids ($n = 1.335$) and triglycerides ($n = 1.491$; Ross, 1967). The brain parenchyma is composed of many of these specific boundaries, most prominently white and gray matter, leading to light scatter. Improving the imaging set-up, e.g., by advanced technical devices or imaging reporters with higher sensitivity will certainly facilitate imaging in small animals. But it should be noted that the physical factors leading to light attenuation and scattering and the insertion of an imaging transgene definitely limit the application on small animals and preclude optical neuroimaging in humans. As this review is focused on recent optical imaging applications but not on the physical principles, we refer the interested reader to a technical review of Schulz and Semmler (2008) and the comprehensive book Molecular imaging: Principles and Practice edited by Weissleder and Gambhir (2010). The following two chapters introduce FLI and BLI techniques with a focus on optical neuroimaging, describe useful imaging reporters and recent studies. Finally, we discuss the importance of cell-specific imaging and the benefit of combining different imaging techniques.

FLUORESCENCE IMAGING

In vivo FLI uses a set-up similar to fluorescence microscopy consisting of a light source, fluorescence filters and a sensitive charge-coupled device (CCD) camera. But in addition, the set-up is housed in a light-tight chamber to collect fluorescence emission exclusively from the anesthetized animal at the macroscopic level (Rao et al., 2007). To penetrate the mouse skull efficiently, excitation with NIR laser light either through the mouse head [transillumination fluorescence imaging (TFI)] or from top [fluorescence reflectance imaging (FRI)] is applied (Frangioni, 2003; Klohs et al., 2006). New systems can perform transillumination while the mouse is rotated through 360° to allow photon acquisition from multiple projections [fluorescence tomography (FMT); Deliolanis and Ntziachristos, 2013]. Nevertheless, some applications still require exposure of the skull and removal of the skin. In general, only NIR fluorescence is efficient enough for *in vivo* neuroimaging due to the strong attenuation of shorter wavelengths (<700 nm). FLI has been adapted to the mouse brain application in the micro- and macroscale to visualize stem cells or stroke-related functional changes based on fluorescent proteins (FPs), fluorescent dyes, and endogenous chromophores.

FLUORESCENCE PROTEINS

The discovery of the green FP (GFP) from the jellyfish *Aequorea victoria* paved the way for a universal marker for cell structures and cellular processes detectable by fluorescence microscopy (Chalfie et al., 1994). The diversity of FPs has increased since then tremendously by mutating the original GFP sequence and cloning FP from distant species like crustaceans. Such FPs can be expressed in mammalian cells, including stem cells and transgenic mice without signs of toxicity (Shaner et al., 2005). Smart multi-label approaches like the brainbow toolbox have been developed to mark neurons with many different FPs (Cai et al., 2013). However, *in vivo* FLI is challenged by a variety of factors: the excitation/emission wavelength, the brightness being determined by the quantum yield (QY, the ratio of photons emitted to photons absorbed during excitation), the extinction coefficient (EC, determining how strongly light is absorbed), the FP maturation rate, photostability, pH stability, and aggregation potential (Chudakov et al., 2010). FPs for *in vivo* neuroimaging will profit from novel – much more efficient – NIR probes (Shcherbakova and Verkhusha, 2013).

FLUORESCENT DYES

Several chemical probes have proven long term labeling of stem cells, including chloromethylfluorescein diacetate (CMFDA or

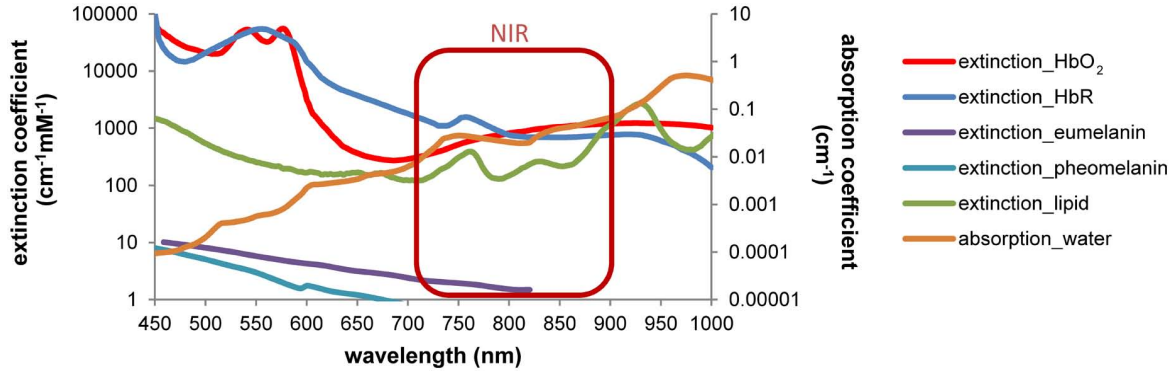

FIGURE 2 | Wavelength-dependent absorption in tissue restricts the optical imaging spectrum. Light absorption by hemoglobin, melanin, lipids and water is wavelength-dependent. Differences can be used to discriminate, e.g., oxy- and deoxyhemoglobin by spectral analysis. A window of low absorption exists in the near-infrared range (~700–900 nm). Absorption and extinction coefficients from Palmer and Williams (1974), Cheong et al. (1990), Pope and Fry (1997), Prahl (1999) and Sarna and Swartz (2006), displayed in log scale.

CellTracker) and long chain carbocyanine dyes (like DiI, DiO, DiD, and CM-DiI; Sutton et al., 2008; Mäkinen et al., 2013; Boehm-Sturm et al., 2014). CMFDA and DiD labels were found to be stable for up to 4 weeks in human ES-cell derived neural cells *in vitro* (Mäkinen et al., 2013). Stem cell labeling prior to implantation is efficient but cell tracking is restricted to *ex vivo* fluorescence microscopy (Jablonska et al., 2010; Boehm-Sturm et al., 2014). *In vivo* data has been acquired with NIR cyanine dyes (Cy) – especially Cy5.5 (excitation 675 nm, emission 694 nm). Targeted probes have been designed to visualize key components of the stroke pathology. Inflammatory processes are monitored by an fluorescence-labeled antibody against the inflammatory receptor CD40 expressed on immune cells (Klohs et al., 2008) and dead cells can be targeted by the cell death marker Annexin A5 (Bahmani et al., 2011). In addition, Zhang et al. (2012) investigated an optical method to detect fibrin deposition, which leads to thrombosis – responsible for 80% of human stroke. In a mouse model of thromboembolic stroke, a NIR probe was injected, which is recognized by the activated coagulation factor XIII (FXIIIa), an important mediator of thrombosis or fibrinolytic resistance (**Figure 3**). Numerous probes have the potential to visualize stroke-related pathologies and host reactions, e.g., blood–brain-barrier (BBB) breakdown, infiltrating immune cells and (de-) myelination (Wang et al., 2011; Eaton et al., 2013) but have not been tested in animal models of stroke yet. NIR probes have been successfully used to simultaneously image cell death and BBB-disruption in traumatic brain injury (Smith et al., 2012). Smart probes have been designed to report activity of matrix metalloproteinases, which are highly upregulated after stroke (Klohs et al., 2009). In order to overcome the low sensitivity of FPs to label stem cells before transplantation, the new class of 2–8 nm small fluorescent quantum dots (QDs) holds great promise to overcome the low sensitivity of FPs to label cells. QDs provide surpassing absorbance, high QY, narrow emission bands and high resistance to photobleaching (Frangioni, 2003; Michalet et al., 2005). Sugiyama et al. (2011) could show that bone marrow stromal cells, labeled with QDs for the NIR, can be detected non-invasively up to 8 weeks after transplantation in the rat brain.

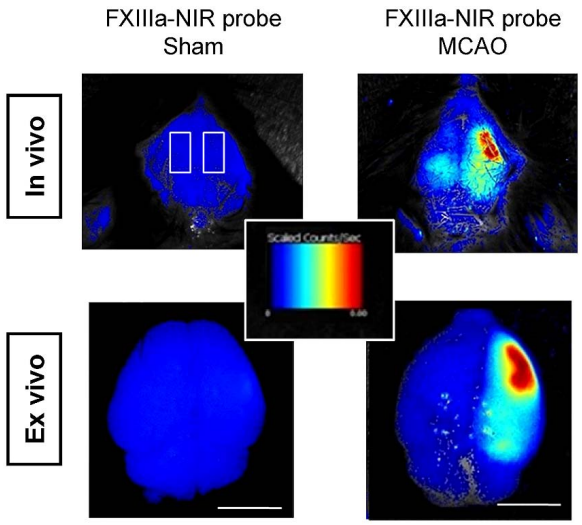

FIGURE 3 | *In vivo* FLI of thromboembolic-stroke. Fibrin deposition is visualized with a near-infrared probe against the activated coagulation factor XIII (FXIIIa; A15) in a thromboembolic model of stroke. Scale bar 5 mm. Images adapted with permission from Zhang et al. (2012).

FLUORESCENCE NEUROIMAGING AT THE MICRO- AND MACROSCALE

Two-photon microscopy (2PM) is the method of choice to obtain detailed structural information of neural tissue *in vivo* [also referred to 2P laser scanning microscopy (2PLSM)]. A pulsed infrared laser is used to excite fluorophores by the combined power of two long-wavelength photons (Sigler and Murphy, 2010), which promotes better sample penetration, higher resolution, less light scatter, and less photo-damage compared to *in vivo* confocal microscopy (Belluscio, 2005). 2PM has also been applied to freely moving animals equipped with a fiber-based endoscopic system (Helmchen et al., 2001). Using 2PM implies the limitation to the first cortical layers within the mouse brain, imaging subcortical structures requires a cranial window (Dombeck et al., 2010; Shih et al., 2012). Fluorophores are essential for 2PM,

either by injectable tracers, like intravenous bolus injection of fluorescein-conjugated dextran to target blood vessels and blood flow (Shih et al., 2012), or FPs expressed by specific cell types (Belluscio, 2005). Label-free 2PM of the living mouse brain has been reported by Witte et al. (2011) by using intrinsic non-linear light interactions, referred to second- and third-harmonic generation, which can be applied to visualize, e.g., myelin (Farrar et al., 2011). Zhang and Murphy (2007) and Sigler and Murphy (2010) could identify with 2PM to which extent reduced blood flow in stroke leads to changes in synaptic circuitry. Recently, three-photon microscopy (3PM) extended the depth resolution to 1 mm by using longer wavelengths (1,700 nm, equivalent to a one-photon excitation of ∼560 nm), which are less attenuated by tissue and appropriate to excite a variety of existing fluorophores (Horton et al., 2013).

A common way to measure neuronal activity indirectly *in vivo* is functional magnetic resonance imaging (MRI) on the basis of the hemodynamic response, thus the locally dynamic changes in oxy- and deoxyhemoglobin (HbO_2 and HbR). Differences in absorption of HbO_2 and HbR can be used by intrinsic imaging to record cortical activity on a sub-second time scale based on the changes in blood oxygenation (Ts'o et al., 1990). The exposed cortex is illuminated sequentially by light of different wavelengths (multi-spectral reflectance imaging). Images are recorded which are particularly sensitive to changes in HbO_2 and HbR concentration. At wavelengths where HbO_2 and HbR absorption is the same (isosbestic points), changes in total hemoglobin concentration can be measured (Hillman, 2007). Abookasis et al. (2009) used NIR illumination in ischemic rat barrel cortex to generate maps of light absorption, scattering properties and tissue hemoglobin concentration. Dynamic changes in cerebral blood flow are acquired by laser speckle-flow imaging, which is using a similar set-up to intrinsic imaging but with a laser diode as light source. The laser speckle pattern is caused by the coherent laser light scattering within the brain, which is dependent on the movement of red blood cells over time (Hillman, 2007). A cranial imaging window is necessary for repeated light illumination of the exposed cortex (Armitage et al., 2010). Both systems can be combined for simultaneous blood oxygenation and flow imaging during stroke in rats (Steimers et al., 2011). However, blood flow dynamics and neuronal structures are still best resolved with 2PLSM (Shih et al., 2012).

Grinvald and colleagues paved the way for a direct method to optically record neuronal activity: voltage sensitive-dyes (VSDs; Orbach et al., 1985) and voltage sensitive-proteins (VSFPs; Sakai et al., 2001). Both, VSDs and VSFPs respond to changes in transmembrane voltage in the millisecond time scale, by changing their fluorescence properties [but resolving single action potentials is still limited (Akemann et al., 2009)]. Several studies have recently applied VSDs on experimental models of stroke to image functional reorganization of forelimb cortex areas (Brown et al., 2009; **Figure 4A**) and long-lasting impairments on the processing of sensory stimuli by the forelimb somatosensory cortex (Sweetnam and Brown, 2013). Weerakkody et al. (2013) applied VSD imaging to study the cortical activity after implantation of NSCs into the ventricle of naïve mice. They found that high-density engraftment of non-integrating NSCs leads to functional defects in cortical layers (Weerakkody et al., 2013; **Figure 4B**).

BIOLUMINESCENCE IMAGING

In vivo BLI uses, similar to fluorescence imaging (FLI), a CCD camera housed in a light-tight chamber to collect photon emission from the anesthetized animal. But instead of an excitation source, photons are emitted when the intracellular enzyme luciferase oxidizes its substrate luciferin. As luciferase is only expressed in transgenic mammalian cells, there is only negligible BLI background signal and emitted photons can be detected with surpassing sensitivity through the intact skull (Keyaerts et al., 2012a) – even from freely moving awake animals (Keyaerts et al., 2012b). BLI is a high throughput technique, scalable from *in vitro* to *in vivo*, highly non-invasive and ease of use. Similar to FLI, the spatial resolution is limited to several mm (Massoud and Gambhir, 2003). Although the number of photons emitted is proportional to the number of luciferase molecules (De Wet et al., 1987), quantification of *in vivo* BLI is also challenged by biological factors (e.g., substrate bio-distribution, luciferase expression, stability, and inhibition) and physical factors (e.g., emission wavelength, light attenuation by overlying tissue; Keyaerts et al., 2012a). The percentage of transmitted light is linearly decreasing with depth of the bioluminescent source in the rodent brain (Pesnel et al., 2011). However, this could be used to predict the extent of optical attenuation for correct quantification (Virostko and Jansen, 2009). BLI quantification *in vitro* relies on excess conditions of ATP and oxygen. But *in vivo* only 5% of the systemic administered luciferin reaches the brain (Berger et al., 2008), as it has to pass several biological barriers to reach the cell of interest and distribution is dependent on the hemodynamic rate (Keyaerts et al., 2012a). Luciferin can pass freely through the BBB, but efflux transporters like ABCG2 actively pump it back to the lumen (Bakhsheshian et al., 2013) and BBB disruption during stroke may affect biodistribution. Recently, luciferin derivates have been developed to boost sensitivity especially for the mouse brain even at lower doses (Evans et al., 2014). In addition, we proposed an optimized neuroimaging protocol, which minimizes inhibitory effects on the luciferase-luciferin reaction while maximizing the photon detection by a factor of two over conventional experimental protocols and provides detection of 3,000 NSCs *in vivo* (Aswendt et al., 2013; **Figure 5A**).

LUCIFERASES FOR *IN VIVO* IMAGING

Luciferase enzymes occur naturally in numerous luminous species, such as the North American firefly [firefly luciferase (Fluc)], click beetles [click beetle luciferase (CBR)], the sea pansy *Renilla reniformis* [Renilla luciferase (Rluc)], and the copepod *Gaussia princeps* [Gaussia luciferase (Gluc); Mezzanotte et al., 2013]. There are species-specific changes in the substrate, co-factors and emitted wavelength. Gluc and Rluc emit in the blue spectrum and therefore are not optimal for *in vivo* use, as it gets strongly absorbed when traveling through tissue (see **Figure 1**). Fluc and CBR, on the other hand, have a strong component above 600 nm (**Figure 6**) resulting in less absorption (Zhao et al., 2005). Changes in one single amino acid of the luciferase can already result in wavelength shifts. Extensive mutagenesis was

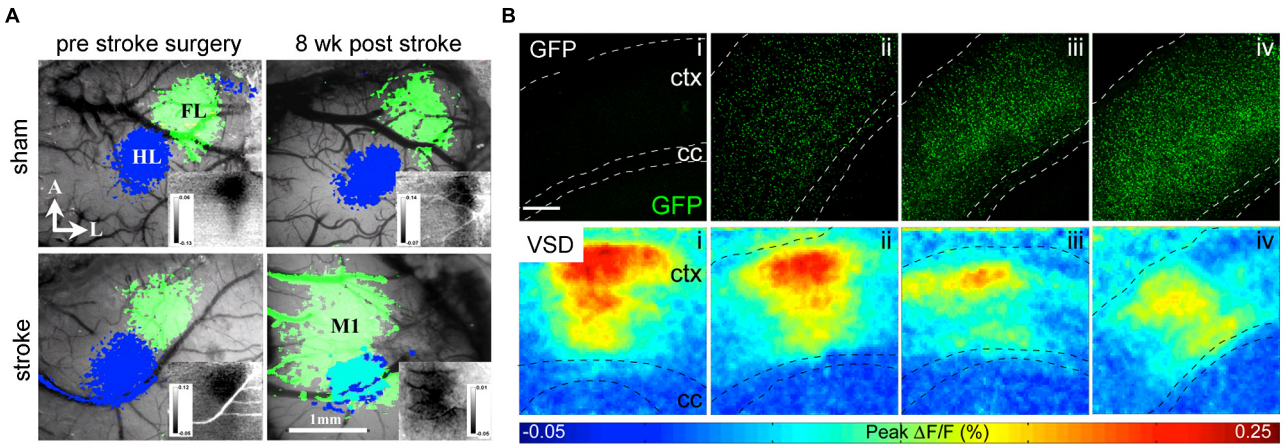

FIGURE 4 | VSD imaging detects macroscopic changes in functional networks after stroke and implantation of NSCs. (A) Changes of the functional representation of the forelimb (FL) cortex area toward the peri-infarct primary motor cortex M1 and hindlimb (HL) area appear 8 weeks upon photothrombotic stroke but not sham surgery. Reprinted by permission from MacMillan Publisher Ltd: Molecular Therapy (Brown et al., 2009). **(B)** With increasing number of cortical grafts (confocal images upper row), the amplitude of cortical activation is reduced as displayed in color-coded maps (lower row) of cortical activation depicting the maximum of mean fluorescence intensity change (F/F_0) within 1024 ms recording interval. Images adapted with permission from Weerakkody et al. (2013).

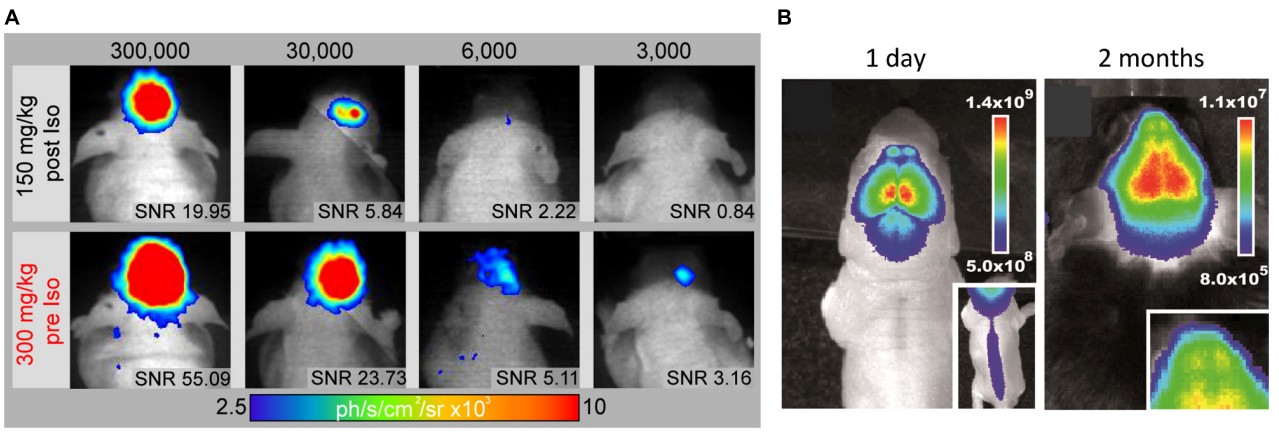

FIGURE 5 | *In vivo* monitoring of endogenous and implanted stem cells. (A) The improved BLI protocol (300 mg/kg Luciferin pre Isofluran anaesthesia) promotes substantial higher photon emission compared to standard protocol (150 mg/kg Luciferin post anesthesia) and the lowers the detection limit to 3,000 engrafted NSCs *in vivo*. Signal-to-noise ratio (SNR) above 3 was defined as a limit for reliable cell graft detection. **(B)** Bioluminescence images of a 1 day and 2 month old doublecortin-luciferase mouse for *in vivo* imaging of endogenous neurogenesis (Insert shows neurogenesis in the spinal cord and the olfactory bulbs, respectively). Images modified with permission from Aswendt et al. (2013) and Couillard-Despres et al. (2008).

performed to create red and green shifts and improve pH-tolerance and thermostability (Miloud et al., 2011; Jathoul et al., 2012). To answer the question, which luciferase fits best the brain application, we recently evaluated the codon-optimized Luc2, the red codon-optimized mutant PpyRE9 (Liang et al., 2012), the codon-optimized hRluc and the green CBG99. BLI signal from transduced cells *in vivo* after intracerebral transplantation showed photon emission decrease in the order of Luc2, CBG99, PpyRE9 to hRluc (Mezzanotte et al., 2013). Most importantly, as the green part of emission spectra gets strongly absorbed, luciferases with a high QY and red-shifted emission are preferable. Multicolor BLI, e.g., to distinguish two cell types becomes possible by combining Luc2/hRluc [through the not cross-reacting substrates luciferin and coelenterazine (Bhaumik and Gambhir, 2002)] or CBG99/PpyRE9 [through spectral unmixing of the green and red emission light (Mezzanotte et al., 2013)].

BIOLUMINE SCENE IMAGING OF ENDOGENOUS AND EXOGENOUS STEM CELLS FOR STROKE REPAIR

Stem cell therapy has already been proven beneficial for stroke recovery (Bliss et al., 2007; Oki et al., 2012), however, the mechanisms of action needs further investigation. BLI provides unique information about the dynamics of NSCs, such as location, migration and proliferation. Exogenous stem cells have to be transgenically modified to express a luciferase protein in order to allow longitudinal *in vivo* optical imaging after implantation.

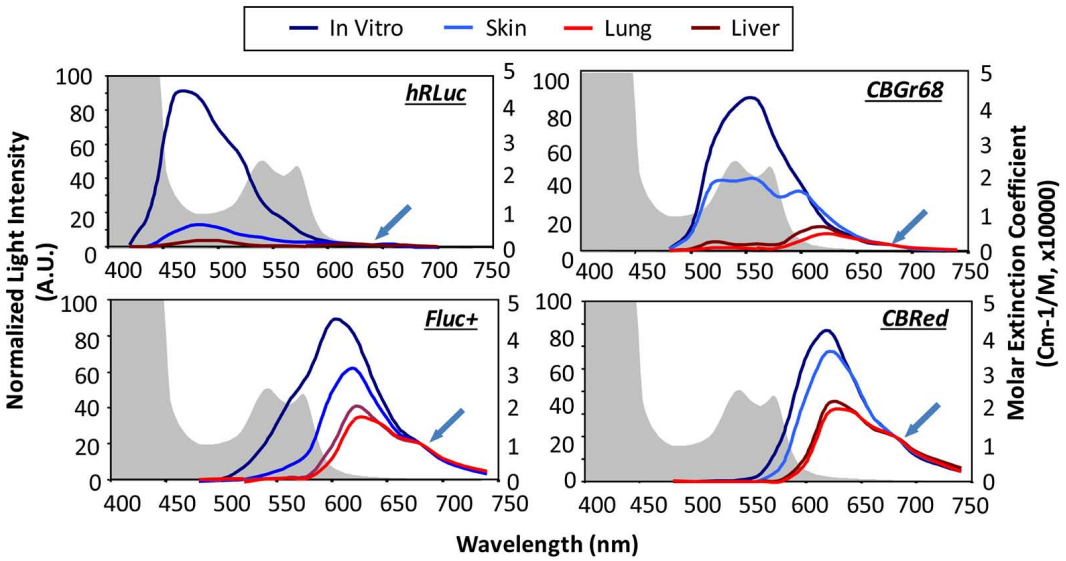

FIGURE 6 | *In vivo* emission spectra of bioluminescent reporters. Emission spectra from luciferase expression in skin, lung, and liver are compared with those from labeled cells in culture showing tissue-induced light attenuation in the blue spectrum. Photon fluxes are normalized to the values at 680 nm for beetle luciferases and 640 nm for Renilla luciferase (indicated at green arrow), where absorption is minimal. Hemoglobin absorption curves are plotted as background (shaded in gray). hRLuc, Renilla reniformis; CBGr68, click beetle green; CBRed, click beetle red; Fluc+, Firefly luciferase. Reproduced with permission from Zhao et al. (2005).

By using bicistronic vectors for expression of two transgenes, e.g., a bioluminescent reporter for *in vivo* BLI together with a fluorescent reporter (e.g., EGFP) provides the opportunity to also detect engrafted cells by invasive methods such as histology, microscopy, RT-PCR and Western blot. Notably, some luciferases, as Fluc, are dependent on ATP, thus serving as a non-invasive viability marker of engrafted stem cells to assess survival *in vivo*. Using this biochemical relationship Boehm-Sturm et al. (2014) investigated whether the peri-infarct region is a location permissive for stem cell survival. Their multimodal approach of 19F-MRI and BLI revealed that human NSCs after intracerebral implantation into the peri-infarct region show same survival behavior as when implanted into healthy brain tissue. *In vivo* BLI has also been used to assess the recruitment of NSCs in stroke mice after contralateral parenchymal, intra-ventricular (Kim et al., 2004) or intra-arterial injection (Rosenblum et al., 2012). Murin neural precursor transfected to express Fluc showed strong migrational activity toward the lesioned hemisphere when transplanted contralaterally. Despite the rather poor spectral resolution of BLI compared to other imaging modalities like MRI, Kim et al. (2004) could detect the midline crossing as early as 7 days after grafting (Quattromani et al., 2014). Also intraventricular injection of this cell line into stroke mice resulted in strong recruitment to the lesioned area (Quattromani et al., 2014). Intra-arterially injected murine neural progenitors transduced to express Gluc were found to reach the brain, with largest recruitment to the brain when injected 3 days after hypoxia-ischemia (Cordeau et al., 2008).

The second source of stem cells for stroke repair is the pool of endogenous stem cells, e.g., within the subventricular zone. These cells can be either targeted by *in vivo* transduction with a viral vector to express Fluc (Reumers et al., 2008), or by designing a neural progenitor-specific reporter mouse, in which Fluc expression is controlled by the doublecortin (DCX) promotor (Couillard-Despres et al., 2008). Both approaches allow quantitative observation of adult neurogenesis (**Figure 5B**) and represent useful non-invasive tools to investigate the therapeutic potential of endogenous stem cells for stroke recovery. Using conditional viral vectors, Vandeputte et al. (2014) labeled Nestin-positive cells of the subventricular zone prior to induction of photothrombotic stroke and followed the recruitment to the lesion for 90 days. Photothrombotic stroke is a variation of ischemic stroke models, which produces localized cortical strokes based on microvascular thrombosis after localized photosensitive dye activation. Already 2 days after stroke, the number of endogenous neural progenitor cells increased and translocated to the lesion site, peaking at 14 days and declining thereafter (Vandeputte et al., 2014). The time profile of the endogenous neural progenitor cell proliferation in response to ischemic stroke of the middle cerebral artery territory was investigated using the DCX reporter mouse model (Adamczak, 2013). A similar early rise in proliferation was observed within the first week. Other aspects of neural replacement have been investigated by non-invasive BLI. For example neural responses to stroke by Gravel et al. (2011) using a multimodal transgenic mouse targeting the growth associated protein GAP-43. BLI could show that the nervous tissue-specific GAP-43 is silent in adult neurons, but up-regulated after neural injury and contributes to neurite outgrowth as part of the regeneration process after stroke. Post-stroke neurogenesis is effected by the inflammatory response (Kokaia et al., 2012), which can be monitored with a transgenic mouse model expressing Fluc under the control of the toll-like receptor (TLR) two promotor (Lalancette-Hébert et al.,

2009). TLRs are expressed by cells of the innate immune system to identify damage-associated patterns released during cell damage. Quattromani et al. (2014) successfully used this mouse model to visualize that the inflammatory response in stroke mice is decreased when the animals had access to enriched environment in their cages. The inflammation post stroke was also found to be sex dependent using a transgenic mouse model with Fluc restricted to GFAP positive cells, predominantly astrocytes. While male mice showed a correlation of astrogliosis and infarct volume, such a correlation was missing in female mice (Cordeau et al., 2008).

FUTURE DIRECTIONS

CELL FATE IMAGING

Besides the possibility to track the viability of transplanted NSCs by BLI, luciferases have been linked to neural cell specific promoters to monitor differentiation. Such system was successfully used to trace the *in vivo* activation of neuronal differentiation by coupling Fluc to the NeuroD promoter which is active in neuronal precursor cells (Oh et al., 2013). Weak cell specific promoters can be enhanced by coupling them to a two-step transcriptional amplification (TSTA; Hwang do et al., 2008). An efficient *in vitro* method to increase reliability of BLI quantification was described as dual-reporter systems, which uses a constitutive promoter to image the localization, viability and quantity of transplanted cells and a cell specific promoter to monitor the differentiation degree in real-time based on two different luciferases (Kern et al., 2013; Xu et al., 2014). The dual-reporter system can be used to answer important questions, such as (i) the optimal type and number of NSCs to be administered, (ii) the route of administration, and (iii) the best time to administer cells after injury with higher accuracy compared to single reporter assays.

COMBINATION OF NON-INVASIVE IMAGING TECHNIQUES

Optical imaging provides best sensitivity of 10^{-15}–10^{-17} mol/l for visualization of stem cell therapy in stroke (Massoud and Gambhir, 2003). Among many recent technical developments, optoacoustic (photoacoustic) imaging holds great potential to visualize both, the stem cell graft location (Jokerst et al., 2012) and progression/reorganization of the stroke lesion (Kneipp et al., 2014) with one non-invasive imaging device. However, also the combination of optical imaging with other non-invasive imaging techniques like positron emission tomography (PET) and MRI is of interest, as complementary functional and structural information can be derived. A novel multimodal approach is the combination of PET and optical intrinsic imaging to characterize cortical spreading depression in ischemic rats (Gramer et al., 2014). PET imaging may also be useful to quantify the metabolic rate (via the glucose consumption) and oxygen distribution, two important factors in stroke pathology, and confounding factors for BLI. Another multimodal approach is the combination of MRI with BLI to characterize transplanted NSCs in the stroke pathology. Firefly bioluminescence was used as a viability marker while transplant location was visualized with iron oxide labeling by MRI for 8 weeks (Daadi et al., 2009). Sequential MRI/BLI was successfully employed to investigate the amount and distribution of intra-arterially (i.a.) versus intra-venously (i.v.) injected stem cells in an ischemia model, showing brain accumulation after i.a. but not after i.v. injection (Pendharkar et al., 2010). We have combined the advanced 19F MRI technique for unambiguous graft location on high-resolution structural MR images with BLI for monitoring cell viability (Boehm-Sturm et al., 2014). There, the murine NSCs were transplanted next to the stroke lesion in nude mice. But the graft location had no effect on the decline in cell survival over 14 days observation period. Further technical developments are needed to improve sensitivity, spatial resolution and integration of imaging techniques to facilitate co-registration of quantitative data (e.g., on database references as the Allen brain atlas, http://www.brain-map.org/). The combination of optical imaging with MRI data could be used to create 3D reconstructions to relocate cell function back to the anatomical structure, which is highly resolved by MRI (100 μm spatial resolution).

We believe that the application of the non-invasive imaging tools presented here will be essential to fully understand stroke regeneration and the potential of engrafted stem cells in pre-clinical trials – the prerequisite of an effective clinical therapy.

AUTHOR CONTRIBUTIONS

Markus Aswendt, Joanna Adamczak and Annette Tennstaedt wrote the manuscript.

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REFERENCES

Abookasis, D., Lay, C. C., Mathews, M. S., Linskey, M. E., Frostig, R. D., and Tromberg, B. J (2009). Imaging cortical absorption, scattering, and hemodynamic response during ischemic stroke using spatially modulated near-infrared illumination. *J. Biomed. Opt.* 14, 024033. doi: 10.1117/1.3116709

Adamczak, J. (2013). *Watching the Healing Brain: Multimodal and Non-invasive Imaging of Regenerative Processes after Experimental Cerebral Ischemia.* Ph.D. thesis, Faculty of Mathematics and Natural Sciences, University of Cologne, Cologne.

Akemann, W., Middleton, S. J., and Knöpfel, T. (2009). Optical imaging as a link between cellular neurophysiology and circuit modeling. *Front. Cell. Neurosci.* 3:5. doi: 10.3389/neuro.03.005.2009

Armitage, G. A., Todd, K. G., Shuaib, A., and Winship, I. R. (2010). Laser speckle contrast imaging of collateral blood flow during acute ischemic stroke. *J. Cereb. Blood Flow Metab.* 30, 1432–1436. doi: 10.1038/jcbfm.2010.73

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Aswendt, M., Adamczak, J., Couillard-Despres, S., and Hoehn, M. (2013). Boosting bioluminescence neuroimaging: an optimized protocol for brain studies. *PLoS ONE* 8:e55662. doi: 10.1371/journal.pone.0055662

Bahmani, P., Schellenberger, E., Klohs, J., Steinbrink, J., Cordell, R., Zille, M., et al. (2011). Visualization of cell death in mice with focal cerebral ischemia using fluorescent annexin A5, propidium iodide, and TUNEL staining. *J. Cereb. Blood Flow Metab.* 31, 1311–1320. doi: 10.1038/jcbfm.2010.233

Bakhsheshian, J., Wei, B. R., Chang, K. E., Shukla, S., Ambudkar, S. V., Simpson, R. M., et al. (2013). Bioluminescent imaging of drug efflux at the blood–brain barrier mediated by the transporter ABCG2. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20801–20806. doi: 10.1073/pnas.1312159110

Belluscio, L. (2005). Two-photon imaging in live rodents. *Curr. Protoc. Neurosci.* Chap. 2, Unit 2.9. doi: 10.1002/0471142301.ns0209s32

Berger, F., Paulmurugan, R., Bhaumik, S., and Gambhir, S. S. (2008). Uptake kinetics and biodistribution of 14C-D-luciferin–a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: impact on bioluminescence based reporter gene imaging. *Eur. J. Nucl. Med. Mol. Imaging* 35, 2275–2285. doi: 10.1007/s00259-008-0870-6

Bhaumik, S., and Gambhir, S. S. (2002). Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc. Natl. Acad. Sci. U.S.A.* 99, 377–382. doi: 10.1073/pnas.012611099

Bihel, E., Pro-Sistiaga, P., Letourneur, A., Toutain, J., Saulnier, R., Insausti, R., et al. (2010). Permanent or transient chronic ischemic stroke in the non-human primate: behavioral, neuroimaging, histological, and immunohistochemical investigations. *J. Cereb. Blood Flow Metab.* 30, 273–285. doi: 10.1038/jcbfm.2009.209

Bliss, T., Guzman, R., Daadi, M., and Steinberg, G. K., (2007). Cell transplantation therapy for stroke. *Stroke* 38, 817–826. doi: 10.1161/01.STR.0000247888.25985.62

Boehm-Sturm, P., Aswendt, M., Minassian, A., Michalk, S., Mengler, L., Adamczak, J., et al. (2014). A multi-modality platform to image stem cell graft survival in the naïve and stroke-damaged mouse brain. *Biomaterials* 35, 2218–2226. doi: 10.1016/j.biomaterials.2013.11.085

Brown, C. E., Aminoltejari, K., Erb, H., Winship, I. R., and Murphy, T. H. (2009). *In vivo* voltage-sensitive dye imaging in adult mice reveals that somatosensory maps lost to stroke are replaced over weeks by new structural and functional circuits with prolonged modes of activation within both the peri-infarct zone and distant sites. *J. Neurosci.* 29, 1719–1734. doi: 10.1523/JNEUROSCI.4249-08.2009

Cai, D., Cohen, K. B., Luo, T., Lichtman, J. W., and Sanes, J. R. (2013). Improved tools for the Brainbow toolbox. *Nat. Methods* 10, 540–547. doi: 10.1038/nmeth.2450

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805. doi: 10.1126/science.8303295

Cheong, W. F. F., Prahl, S. A., and Welch, A. J. (1990). A review of the optical properties of biological tissues. *IEEE J. Quantum Electron.* 26, 2166–2185. doi: 10.1109/3.64354

Chudakov, D. M., Matz, M. V., Lukyanov, S., and Lukyanov, K. A. (2010). Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* 90, 1103–1163. doi: 10.1152/physrev.00038.2009

Cordeau, P. Jr., Lalancette-Hébert, M., Weng, Y. C., and Kriz, J. (2008). Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury. *Stroke* 39, 935–942. doi: 10.1161/STROKEAHA.107.501460

Couillard-Despres, S., Finkl, R., Winner, B., Ploetz, S., Wiedermann, D., Aigner, R., et al. (2008). *In vivo* optical imaging of neurogenesis: watching new neurons in the intact brain. *Mol. Imaging* 7, 28–34.

Daadi, M. M., Li, Z., Arac, A., Grueter, B. A., Sofilos, M., Malenka, R. C., et al. (2009). Molecular and magnetic resonance imaging of human embryonic stem cell-derived neural stem cell grafts in ischemic rat brain. *Mol. Ther.* 17, 1282–1291. doi: 10.1038/mt.2009.104

De Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7, 725–737.

Deliolanis, N. C., and Ntziachristos, V. (2013). Fluorescence molecular tomography of brain tumors in mice. *Cold Spring Harb. Protoc.* 2013, 438–443. doi: 10.1101/pdb.prot074245

Dombeck, D. A., Harvey, C. D., Tian, L., Looger, L. L., and Tank, D. W. (2010). Functional imaging of hippocampal place cells at cellular resolution during virtual navigation. *Nat. Neurosci.* 13, 1433–1440. doi: 10.1038/nn.2648

Eaton, V. L., Vasquez, K. O., Goings, G. E., Hunter, Z. N., Peterson, J. D., and Miller, S. D. (2013). Optical tomographic imaging of near infrared imaging agents quantifies disease severity and immunomodulation of experimental autoimmune encephalomyelitis *in vivo*. *J. Neuroinflammation* 10, 138. doi: 10.1186/1742-2094-10-138

Evans, M. S., Chaurette, J. P., Adams, S. T. Jr., Reddy, G. R., Paley, M. A., Aronin, N., et al. (2014). A synthetic luciferin improves bioluminescence imaging in live mice. *Nat. Methods* 11, 393–395. doi: 10.1038/nmeth.2839

Farrar, M. J., Wise, F. W., Fetcho, J. R., and Schaffer, C. B. (2011). *In vivo* imaging of myelin in the vertebrate central nervous system using third harmonic generation microscopy. *Biophys. J.* 100, 1362–1371. doi: 10.1016/j.bpj.2011.01.031

Frangioni, J. V. (2003). *In vivo* near-infrared fluorescence imaging. *Curr. Opin. Chem. Biol.* 7, 626–634. doi: 10.1016/j.cbpa.2003.08.007

Gramer, M., Feuerstein, D., Steimers, A., Takagaki, M., Kumagai, T., Sué, M., et al. (2014). Device for simultaneous positron emission tomography, laser speckle imaging and RGB reflectometry: validation and application to cortical spreading depression and brain ischemia in rats. *Neuroimage* 94C, 250–262. doi: 10.1016/j.neuroimage.2014.03.027

Gravel, M., Weng, Y. C., and Kriz, J. (2011). Model system for live imaging of neuronal responses to injury and repair. *Mol. Imaging* 10, 434–445.

Helmchen, F., Fee, M. S., Tank, D. W., and Denk, W. (2001). A miniature head-mounted two-photon microscope. high-resolution brain imaging in freely moving animals. *Neuron* 31, 903–912. doi: 10.1016/S0896-6273(01)00421-4

Hillman, E. M. C. (2007). Optical brain imaging *in vivo*: techniques and applications from animal to man. *J. Biomed. Opt.* 12, 051402. doi: 10.1117/1.2789693

Horton, N. G., Wang, K., Kobat, D., Clark, C. G., Wise, F. W., Schaffer, C. B., et al. (2013). *In vivo* three-photon microscopy of subcortical structures within an intact mouse brain. *Nat. Photonics* 7, 205–209. doi: 10.1038/nphoton.2012.336

Hwang do, W., Kang, J. H., Jeong, J. M., Chung, J. K., Lee, M. C., Kim, S., et al. (2008). Noninvasive *in vivo* monitoring of neuronal differentiation using reporter driven by a neuronal promoter. *Eur. J. Nucl. Med. Mol. Imaging* 35, 135–145. doi: 10.1007/s00259-007-0561-8

Iadecola, C., and Anrather, J. (2011). Stroke research at a crossroad: asking the brain for directions. *Nat. Neurosci.* 14, 1363–1368. doi: 10.1038/nn.2953

Jablonska, A., Kozlowska, H., Markiewicz, I., Domanska-Janik, K., and Lukomska, B. (2010). Transplantation of neural stem cells derived from human cord blood to the brain of adult and neonatal rats. *Acta Neurobiol. Exp.* (*Wars*). 70, 337–350.

Jathoul, A., Law, E., Gandelman, O., Pule, M., Tisi, L., and Murray, J. (2012). "Development of a pH-tolerant thermostable photinus pyralis luciferase for brighter *in vivo* imaging," in *Bioluminescence - Recent Advances in Oceanic Measurements and Laboratory Applications*, ed. D. Lapota (Rijeka: InTech).

Jokerst, J. V., Thangaraj, M., Kempen, P. J., Sinclair, R., and Gambhir, S. S. (2012). Photoacoustic imaging of mesenchymal stem cells in living mice via silica-coated gold nanorods. *ACS Nano* 6, 5920–5930. doi: 10.1021/nn302042y

Kern, I., Xu, R., Julien, S., Suter, D. M., Preynat-Seauve, O., Baquié, M., et al. (2013). Embryonic stem cell-based screen for small molecules: cluster analysis reveals four response patterns in developing neural cells. *Curr. Med. Chem.* 20, 710–723. doi: 10.2174/092986713804999330

Keyaerts, M., Caveliers, V., and Lahoutte, T. (2012a). Bioluminescence imaging: looking beyond the light. *Trends Mol. Med.* 18, 164–172. doi: 10.1016/j.molmed.2012.01.005

Keyaerts, M., Remory, I., Caveliers, V., Breckpot, K., Bos, T. J., Poelaert, J., et al. (2012b). Inhibition of firefly luciferase by general anesthetics: effect on *in vitro* and *in vivo* bioluminescence imaging. *PLoS ONE* 7:e30061. doi: 10.1371/journal.pone.0030061

Kim, D. E., Schellingerhout, D., Ishii, K., Shah, K., and Weissleder, R. (2004). Imaging of stem cell recruitment to ischemic infarcts in a murine model. *Stroke* 35, 952–957. doi: 10.1161/01.STR.0000120308.21946.5D

Klohs, J., Baeva, N., Steinbrink, J., Bourayou, R., Boettcher, C., Royl, G., et al. (2009). *In vivo* near-infrared fluorescence imaging of matrix metalloproteinase activity after cerebral ischemia. *J. Cereb. Blood Flow Metab.* 29, 1284–1292. doi: 10.1038/jcbfm.2009.51

Klohs, J., Gräfe, M., Graf, K., Steinbrink, J., Dietrich, T., Stibenz, D., et al. (2008). *In vivo* imaging of the inflammatory receptor CD40 after cerebral ischemia using a fluorescent antibody. *Stroke* 39, 2845–2852. doi: 10.1161/STROKEAHA.107.509844

Klohs, J., Steinbrink, J., Nierhaus, T., Bourayou, R., Lindauer, U., Bahmani, P., et al. (2006). Noninvasive near-infrared imaging of fluorochromes within the brain of live mice: an *in vivo* phantom study. *Mol. Imaging* 5, 180–187.

Kneipp, M., Turner, J., Hambauer, S., Krieg, S. M., Lehmberg, J., Lindauer, U., et al. (2014). Functional real-time optoacoustic imaging of middle cerebral artery occlusion in mice. *PLoS ONE* 9:e96118. doi: 10.1371/journal.pone.0096118

Kokaia, Z., Martino, G., Schwartz, M., and Lindvall, O. (2012). Cross-talk between neural stem cells and immune cells: the key to better brain repair? *Nat. Neurosci.* 15, 1078–1087. doi: 10.1038/nn.3163

Lalancette-Hébert, M., Phaneuf, D., Soucy, G., Weng, Y. C., and Kriz, J. (2009). Live imaging of Toll-like receptor 2 response in cerebral ischaemia reveals a role of olfactory bulb microglia as modulators of inflammation. *Brain* 132, 940–954. doi: 10.1093/brain/awn345

Liang, Y., Walczak, P., and Bulte, J. W. (2012). Comparison of red-shifted firefly luciferase Ppy RE9 and conventional Luc2 as bioluminescence imaging reporter genes for *in vivo* imaging of stem cells. *J. Biomed. Opt.* 17, 016004. doi: 10.1117/1.JBO.17.1.016004

Mäkinen, M., Joki, T., Ylä-Outinen, L., Skottman, H., Narkilahti, S., and Aänismaa, R. (2013). Fluorescent probes as a tool for cell population tracking in spontaneously active neural networks derived from human pluripotent stem cells. *J. Neurosci. Methods* 215, 88–96. doi: 10.1016/j.jneumeth.2013.02.019

Massoud, T. F., and Gambhir, S. S. (2003). Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* 17, 545–580. doi: 10.1101/gad.1047403

Massoud, T. F., Singh, A., and Gambhir, S. S. (2008a). Noninvasive molecular neuroimaging using reporter genes: part I, principles revisited. *AJNR Am. J. Neuroradiol.* 29, 229–234. doi: 10.3174/ajnr.A0864

Massoud, T. F., Singh, A., and Gambhir, S. S. (2008b). Noninvasive molecular neuroimaging using reporter genes: part II, experimental, current, and future applications. *AJNR Am. J. Neuroradiol.* 29, 409–418. doi: 10.3174/ajnr.A0863

Mezzanotte, L., Aswendt, M., Tennstaedt, A., Hoeben, R., Hoehn, M., and Löwik, C. (2013). Evaluating reporter genes of different luciferases for optimized *in vivo* bioluminescence imaging of transplanted neural stem cells in the brain. *Contrast Media Mol. Imaging* 8, 505–513. doi: 10.1002/cmmi.1549

Michalet, X., Pinaud, F. F., Bentolila, L. A., Tsay, J. M., Doose, S., Li, J. J., et al. (2005). Quantum dots for live cells, *in vivo* imaging, and diagnostics. *Science* 307, 538–544. doi: 10.1126/science.1104274

Miloud, T., Henrich, C., and Hämmerling, G. J. (2011). Quantitative comparison of click beetle and firefly luciferases for *in vivo* bioluminescence imaging. *J. Biomed. Opt.* 12, 054018. doi: 10.1117/1.2800386

Oh, H. J., Hwang do, W., Youn, H., and Lee, D. S. (2013). *In vivo* bioluminescence reporter gene imaging for the activation of neuronal differentiation induced by the neuronal activator neurogenin 1 (Ngn1) in neuronal precursor cells. *Eur. J. Nucl. Med. Mol. Imaging* 40, 1607–1617. doi: 10.1007/s00259-013-2457-0

Oki, K., Tatarishvili, J., Wood, J., Koch, P., Wattananit, S., Mine, Y., et al. (2012). Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells* 30, 1120–1133. doi: 10.1002/stem.1104

Orbach, H. S., Cohen, L. B., and Grinvald, A. (1985). Optical mapping of electrical activity in rat somatosensory and visual cortex. *J. Neurosci.* 5, 1886–1895.

Palmer, K. F., and Williams, D. (1974). Optical properties of water in the near infrared. *J. Opt. Soc. Am.* 64, 1107. doi: 10.1364/JOSA.64.001107

Pendharkar, A. V., Chua, J. Y., Andres, R. H., Wang, N., Gaeta, X., Wang, H., et al. (2010). Biodistribution of neural stem cells after intravascular therapy for hypoxic-ischemia. *Stroke* 41, 2064–2070. doi: 10.1161/STROKEAHA.109.575993

Pesnel, S., Pillon, A., Créancier, L., Guilbaud, N., Bailly, C., Kruczynski, A., et al. (2011). Quantitation in bioluminescence imaging by correction of tissue absorption for experimental oncology. *Mol. Imag. Biol.* 13, 646–652. doi: 10.1007/s11307-010-0387-9

Pope, R. M., and Fry, E. S. (1997). Absorption spectrum (380–700 nm) of pure water. II. Integrating cavity measurements. *Appl. Opt.* 36, 8710–8723. doi: 10.1364/AO.36.008710

Prahl, S. (1999). *Optical Absorption of Hemoglobin.* Available at: http://omlc.org/spectra/hemoglobin/ [accessed December 15, 1999].

Quattromani, M. J., Cordeau, P., Ruscher, K., Kriz, J., and Wieloch, T. (2014). Enriched housing down-regulates the Toll-like receptor 2 response in the mouse brain after experimental stroke. *Neurobiol. Dis.* 66C, 66–73. doi: 10.1016/j.nbd.2014.02.010

Rao, J., Dragulescu-Andrasi, A., and Yao, H. (2007). Fluorescence imaging *in vivo*: recent advances. *Curr. Opin. Biotechnol.* 18, 17–25. doi: 10.1016/j.copbio.2007.01.003

Reumers, V., Deroose, C. M., Krylyshkina, O., Nuyts, J., Geraerts, M., Mortelmans, L., et al. (2008). Noninvasive and quantitative monitoring of adult neuronal stem cell migration in mouse brain using bioluminescence imaging. *Stem Cells* 26, 2382–2390. doi: 10.1634/stemcells.2007-1062

Rosenblum, S., Wang, N., Smith, T. N., Pendharkar, A. V., Chua, J. Y., Birk, H., et al. (2012). Timing of intra-arterial neural stem cell transplantation after hypoxia-ischemia influences cell engraftment, survival, and differentiation. *Stroke* 43, 1624–1631. doi: 10.1161/STROKEAHA.111.637884

Ross, K. F. A. (1967). *Phase Contrast and Interference Microscopy for Cell Biologists.* London: Edward Arnold Publishers.

Sakai, R., Repunte-Canonigo, V., Raj, C. D., and Knöpfel, T. (2001). Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. *Eur. J. Neurosci.* 13, 2314–2318. doi: 10.1046/j.0953-816x.2001.01617.x

Sarna, T., and Swartz, H. A. (2006). "The physical properties of melanins," in *The Pigmentary System Physiology and Pathophysiology,* eds J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King, W. S. Oetting, and J.-P. Ortonne (Oxford: Blackwell Publishing), 311–341.

Schulz, R. B., and Semmler, W. (2008). Fundamentals of optical imaging. *Handb. Exp. Pharmacol.* 85, 3–22. doi: 10.1007/978-3-540-72718-7_1

Shah, K., and Weissleder, R. (2005). Molecular optical imaging: applications leading to the development of present day therapeutics. *NeuroRx* 2, 215–225. doi: 10.1602/neurorx.2.2.215

Shaner, N. C., Steinbach, P. A., and Tsien, R. Y. (2005). A guide to choosing fluorescent proteins. *Nat. Methods* 2, 905–909. doi: 10.1038/nmeth819

Shcherbakova, D. M., and Verkhusha, V. V. (2013). Near-infrared fluorescent proteins for multicolor *in vivo* imaging. *Nat. Methods* 10, 751–754. doi: 10.1038/nmeth.2521

Shih, A. Y., Driscoll, J. D., Drew, P. J., Nishimura, N., Schaffer, C. B., and Kleinfeld, D. (2012). Two-photon microscopy as a tool to study blood flow and neurovascular coupling in the rodent brain. *J. Cereb. Blood Flow Metab.* 32, 1277–1309. doi: 10.1038/jcbfm.2011.196

Sigler, A., and Murphy, T. H. (2010). *In vivo* 2-photon imaging of fine structure in the rodent brain: before, during, and after stroke. *Stroke* 41, S117–S123. doi: 10.1161/STROKEAHA.110.594648

Smith, B. A., Xie, B. W., van Beek, E. R., Que, I., Blankevoort, V., Xiao, S., et al. (2012). Multicolor fluorescence imaging of traumatic brain injury in a cryolesion mouse model. *ACS Chem. Neurosci.* 3, 530–537. doi: 10.1021/cn3000197

Steimers, A., Gramer, M., Takagaki, M., Graf, R., and Kohl-Bareis, M. (2011). Simultaneous imaging of haemoglobin oxygenation and blood flow with RGB reflectometry and LASCA during stroke in rats. *Diffuse Optical Imaging III* 8088, 808808. doi: 10.1117/12.889438

Sugiyama, T., Kuroda, S., Osanai, T., Shichinohe, H., Kuge, Y., Ito, M., et al. (2011). Near-infrared fluorescence labeling allows noninvasive tracking of bone marrow stromal cells transplanted into rat infarct brain. *Neurosurgery* 68, 1036–1047; discussion 1047. doi: 10.1227/NEU.0b013e318208f891

Sutton, E. J., Henning, T. D., Pichler, B. J., Bremer, C., and Daldrup-Link, H. E. (2008). Cell tracking with optical imaging. *Eur. Radiol.* 18, 2021–2032. doi: 10.1007/s00330-008-0984-z

Sweetnam, D. A., and Brown, C. E. (2013). Stroke induces long-lasting deficits in the temporal fidelity of sensory processing in the somatosensory cortex. *J. Cereb. Blood Flow Metab.* 33, 91–96. doi: 10.1038/jcbfm.2012.135

Ts'o, D. Y., Frostig, R. D., Lieke, E. E., and Grinvald, A. (1990). Functional organization of primate visual cortex revealed by high resolution optical imaging. *Science* 249, 417–420. doi: 10.1126/science.2165630

Vandeputte, C., Reumers, V., Aelvoet, S. A., Thiry, I., De Swaef, S., Van den Haute, C., et al. (2014). Bioluminescence imaging of stroke-induced endogenous neural stem cell response. *Neurobiol. Dis.* 69, 144–155. doi: 10.1016/j.nbd.2014.05.014

Virostko, J., and Jansen, E. D. (2009). Validation of bioluminescent imaging techniques. *Methods Mol. Biol.* 574, 15–23. doi: 10.1007/978-1-60327-321-3_2

Walcott, B. P., and Peterson, R. T. (2014). Zebrafish models of cerebrovascular disease. *J. Cereb. Blood Flow Metab.* 34, 571–577. doi: 10.1038/jcbfm.2014.27

Wang, C., Wu, C., Popescu, D. C., Zhu, J., Macklin, W. B., Miller, R. H., et al. (2011). Longitudinal near-infrared imaging of myelination. *J. Neurosci.* 31, 2382–2390. doi: 10.1523/JNEUROSCI.2698-10.2011

Weerakkody, T. N., Patel, T. P., Yue, C., Takano, H., Anderson, H. C., Meaney, D. F., et al. (2013). Engraftment of nonintegrating neural stem cells differentially

perturbs cortical activity in a dose-dependent manner. *Mol. Ther.* 21, 2258–2267. doi: 10.1038/mt.2013.163

Weissleder, R., and Gambhir, S. S. (2010). *Molecular Imaging: Principles and Practice.* Shelton: Peoples Medical Publishing House.

Witte, S., Negrean, A., Lodder, J. C., de Kock, C. P., Testa Silva, G., Mansvelder, H. D., et al. (2011). Label-free live brain imaging and targeted patching with third-harmonic generation microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5970–5975. doi: 10.1073/pnas.1018743108

Xu, R., Feyeux, M., Julien, S., Nemes, C., Albrechtsen, M., Dinnyés, A., et al. (2014). Screening of bioactive peptides using an embryonic stem cell-based neurodifferentiation assay. *AAPS J.* 16, 400–412. doi: 10.1208/s12248-014-9578-7

Zhang, S., and Murphy, T. H. (2007). Imaging the impact of cortical microcirculation on synaptic structure and sensory-evoked hemodynamic responses *in vivo*. *PLoS Biol.* 5:e119. doi: 10.1371/journal.pbio.0050119

Zhang, Y., Fan, S., Yao, Y., Ding, J., Wang, Y., Zhao, Z., et al. (2012). *In vivo* near-infrared imaging of fibrin deposition in thromboembolic stroke in mice. *PLoS ONE* 7:e30262. doi: 10.1371/journal.pone.0030262

Zhang, Z. G., and Chopp, M. (2009). Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurol.* 8, 491–500. doi: 10.1016/S1474-4422(09)70061-4

Zhao, H., Doyle, T. C., Coquoz, O., Kalish, F., Rice, B. W., and Contag, C. H. (2005). Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection *in vivo*. *J. Biomed. Opt.* 10, 41210. doi: 10.1117/1.2032388

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Involvement of calpains in adult neurogenesis: implications for stroke

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Calpains are ubiquitous proteases involved in cell proliferation, adhesion and motility. In the brain, calpains have been associated with neuronal damage in both acute and neurodegenerative disorders, but their physiological function in the nervous system remains elusive. During brain ischemia, there is a large increase in the levels of intracellular calcium, leading to the activation of calpains. Inhibition of these proteases has been shown to reduce neuronal death in a variety of stroke models. On the other hand, after stroke, neural stem cells (NSC) increase their proliferation and newly formed neuroblasts migrate towards the site of injury. However, the process of forming new neurons after injury is not efficient and finding ways to improve it may help with recovery after lesion. Understanding the role of calpains in the process of neurogenesis may therefore open a new window for the treatment of stroke. We investigated the involvement of calpains in NSC proliferation and neuroblast migration in two highly neurogenic regions in the mouse brain, the dentate gyrus (DG) and the subventricular zone (SVZ). We used mice that lack calpastatin, the endogenous calpain inhibitor, and calpains were also modulated directly, using calpeptin, a pharmacological calpain inhibitor. Calpastatin deletion impaired both NSC proliferation and neuroblast migration. Calpain inhibition increased NSC proliferation, migration speed and migration distance in cells from the SVZ. Overall, our work suggests that calpains are important for neurogenesis and encourages further research on their neurogenic role. Prospective therapies targeting calpain activity may improve the formation of new neurons following stroke, in addition to affording neuroprotection.

Keywords: calpains, calpastatin, hippocampus, migration, neurogenesis, proliferation, stroke, subventricular zone

INTRODUCTION

Stroke is currently one of the main causes of brain damage and long-term disability. For this reason, therapeutic approaches aiming at repairing the lesion would highly beneficiate patients with this condition, in addition to providing neuroprotection against further damage. It is now well established that after the brain is formed new neurons are still produced throughout the adult mammalian life in discrete areas of the central nervous system, an event designated as neurogenesis (Gage, 2000). Neural stem cells (NSC), which can be found in the subventricular zone (SVZ) of the lateral ventricles and in the dentate gyrus (DG) of the hippocampus, constitute a pool of cells that exit these proliferative niches, migrate as neuroblasts towards regions where they are required and differentiate into mature neurons that integrate the neuronal circuitry (Aimone et al., 2014). Cells from the SVZ migrate through the rostral migratory stream (RMS) onto the olfactory bulbs, and are thought to be responsible for maintaining and reorganizing the interneuron system in the olfactory bulbs. In contrast, cells from the DG migrate shorter distances, from the subgranular zone (SGZ) into the granular zone (GZ) of the hippocampus, provide a substrate for additional brain plasticity and are crucial for spatial learning and memory (Imayoshi et al., 2008). Adult neurogenesis in rodents and other mammals is well established and has been widely characterized (for review, see Christian et al., 2014; Jessberger and Gage, 2014; Lim and Alvarez-Buylla, 2014). In humans, adult neurogenesis was characterized more recently, particularly in the hippocampus (Spalding et al., 2013) and in the striatum (Ernst et al., 2014).

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; $Cast^{+/+}$, wild type; $Cast^{-/-}$, calpastatin knock-out; DCX, doublecortin; DG, dentate gyrus; EdU, 5-ethynyl-2'-deoxyuridine; GZ, granular zone; NeuN, neuronal nuclei; NSC, neural stem cells; PCNA, proliferating cell nuclear antigen; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone.

Interestingly, stroke is followed by increased proliferation of NSC, and new neuroblasts migrate towards the site of injury (Arvidsson et al., 2002). In humans, there is also evidence of increased neurogenesis after stroke, even in older patients (Jin et al., 2006; Macas et al., 2006; Martí-Fàbregas et al., 2010; Popa-Wagner et al., 2014). Increased neurogenesis likely represents an endogenous attempt to regenerate cells lost in lesioned regions (Romanko et al., 2004). However, this process is limited and ineffective, since few neuroblasts reach the damaged region. In fact, the majority of the neuroblasts die before differentiating into functional mature neurons, thus failing the integration into the neuronal circuitry (Kaneko and Sawamoto, 2009; Ma et al., 2009). Only about 0.2% of dead neurons are replaced by newly generated neurons, which are not sufficient to compensate for neurological deficits (Arvidsson et al., 2002). The study of new strategies to promote post-injury repair may therefore focus in ways of enhancing neurogenesis in the adult brain.

After brain damage, the levels of intracellular calcium dramatically rise due to excitotoxicity, which leads to the activation of several proteases, including calpains (Neumar et al., 2001). Calpains are a family of calcium-dependent proteases that are involved in numerous processes, including cell adhesion and motility (cytoskeletal/membrane attachments), signal transduction pathways, cell cycle (proliferation), regulation of gene expression, apoptosis, and even long-term potentiation (for review, see Goll et al., 2003). The observed effects of calpain inhibition may be very different, depending on the cell type examined. In cell migration, for example, studies have shown that calpain inhibition impairs the migration ability of pancreatic beta-cells (Parnaud et al., 2005), vascular smooth muscle cells (Paulhe et al., 2001), T-cells (Rock et al., 2000), lung endothelial cells (Qiu et al., 2006), among others; calpain inhibition also increases the spreading ability of neutrophils (Lokuta et al., 2003). Moreover, there are also studies that show either a decrease (Croce et al., 1999) or an increase (Kuchay et al., 2012) in platelet spreading with the inhibition of calpains. Therefore, how calpains function in different cells and which are the signals that trigger their activity remain unclear.

In the brain, most of the studies regarding calpains focus on their involvement in neuronal damage. In animal models of brain ischemia, several calpain inhibitors have shown to be neuroprotective, being able to reduce neuronal damage caused by this pathology (Bartus et al., 1994; Hong et al., 1994; Li et al., 1998; Markgraf et al., 1998; Frederick et al., 2008; Koumura et al., 2008; Peng et al., 2011). However, there is little information on the physiological roles of calpains in cells from the central nervous system. Since calpains can influence cell proliferation and migration in other systems and NSC also share these functions, we investigated whether modulation of calpains can affect neurogenesis. We did this by analyzing the changes in NSC proliferation and neuroblast migration in the major neurogenic niches in the brain, the DG and the SVZ. Understanding the involvement of calpains in the modulation of NSC proliferation and neuroblast migration may help in the development of new strategies to improve post-injury brain repair.

RESULTS AND DISCUSSION

To study the effect of calpains on neurogenesis, we modulated calpain activity using inhibitors. One of the best approaches available to identify calpain functions *in vivo* is by altering the expression of calpastatin (Takano et al., 2005), the only known endogenous calpain inhibitor (Murachi, 1984). We did this by using mice lacking calpastatin ($Cast^{-/-}$). We also used a pharmacological calpain inhibitor, calpeptin. Migration and proliferation of NSC in the hippocampus were traced with different thymidine analogs, as illustrated in **Figure 1** and described in the methods section.

The effect of calpastatin deletion on the proliferation of NSC in the SGZ of the hippocampus was investigated by administering a thymidine analog, 5-ethynyl-2'-deoxyuridine (EdU), to both wild type ($Cast^{+/+}$) and $Cast^{-/-}$ mice on the day before their sacrifice. Incorporation of EdU in SGZ cells decreased in $Cast^{-/-}$ mice (5.7 $\pm$ 0.2 cells/section, $p = 0.0278$) (**Figures 2B,C**), when compared to $Cast^{+/+}$ mice (7.7 $\pm$ 0.9 cells/section) (**Figures 2A,C**). This shows that proliferation is affected when calpastatin is absent. In physiological conditions, NSC in the DG are formed in the SGZ, where they proliferate, and then migrate as neuroblasts into the GZ (Ming and Song, 2011). Since the number of EdU-positive cells in the GZ was not altered (0.4 $\pm$ 0.1 cells/section in $Cast^{+/+}$ and 0.3 $\pm$ 0.1 cells/section in $Cast^{-/-}$, $p = 0.6738$) (**Figure 2D**), the decreased number of EdU-positive cells observed in the SGZ was probably not due to enhanced migration into the GZ, supporting the idea of a decrease in the proliferation caused by loss of calpastatin. Since Sox2 controls NSC maintenance in the hippocampus (Favaro et al., 2009; Ehm et al., 2010), co-localization of this transcription factor with either EdU (**Figure 2E**) or proliferating cell nuclear antigen (PCNA; **Figure 2F**) was performed to show that dividing cells are NSC.

As previously reported, $Cast^{-/-}$ mice do not present increased intrinsic calpain activity (Takano et al., 2005), but the absence of calpastatin is expected to deregulate calpain activity, by loss of inhibition, when calpain activation is needed during cell proliferation. Suppression of calpain activity has been reported to reduce the proliferation of different types of cells, such as vascular smooth muscle cells (Ariyoshi et al., 1998), Chinese hamster ovary cell colonies (Xu and Mellgren, 2002), osteoblasts (Shimada et al., 2008), chondrocytes (Kashiwagi et al., 2010) and lung endothelial cells (Qiu et al., 2006). More recently, the inhibition of calpains was also shown to decrease the proliferation of mouse NSC lines (Santos et al., 2012). Different cells may act differently under different stimuli, so the exact mechanisms through which calpains

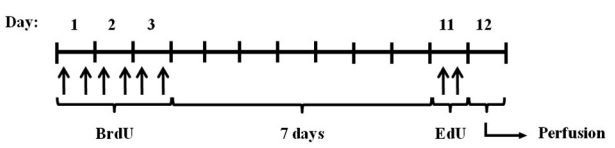

FIGURE 1 | Experimental procedure. $Cast^{+/+}$ and $Cast^{-/-}$ mice were treated with BrdU for 3 days (every 12 h), and after 7 days with EdU (twice, 2 h apart). The animals were then sacrificed by transcardial perfusion, on day 12.

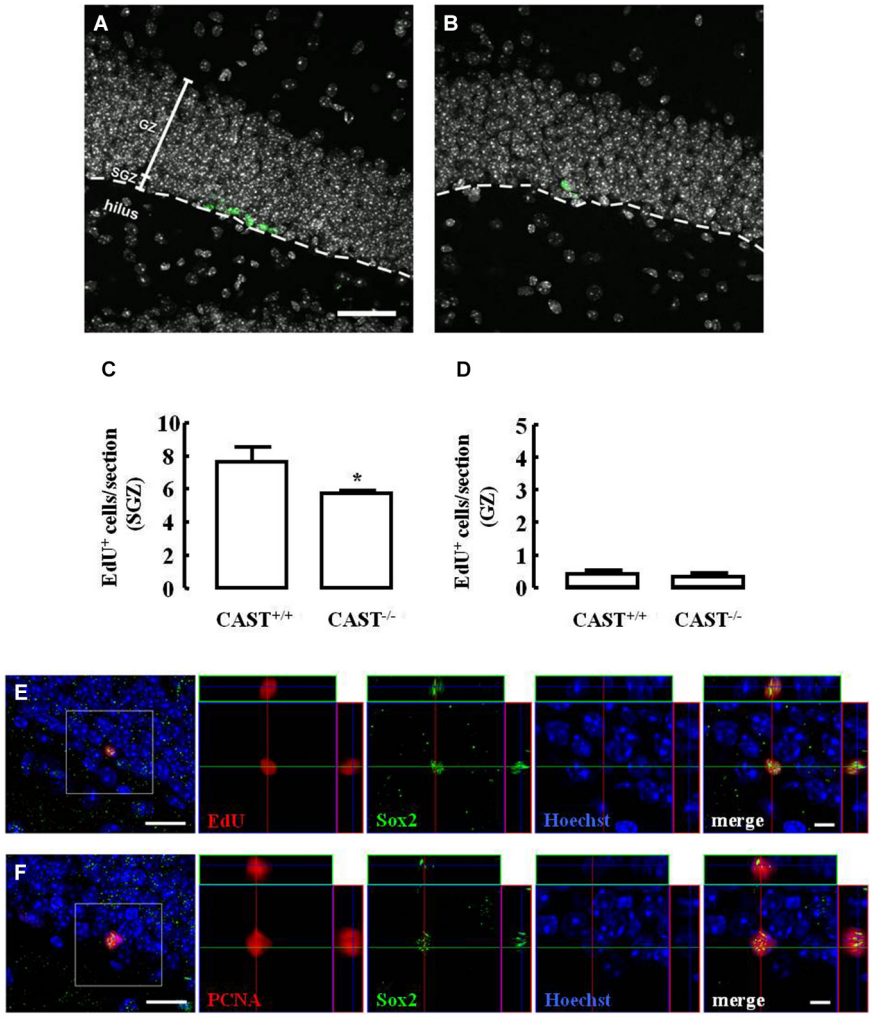

FIGURE 2 | Calpastatin deficiency impairs NSC proliferation in the SGZ. Representative images from hippocampal brain sections of *Cast*$^{+/+}$ **(A)** and *Cast*$^{-/-}$ **(B)** mice, showing EdU-positive cells in green and nuclei, labeled with Hoechst 33342, in gray. Number of EdU-positive cells in the SGZ **(C)** and the GZ **(D)** of the hippocampus. Co-localization of EdU **(E)** or proliferating cell nuclear antigen (PCNA) **(F)**, in red, with the transcription factor Sox2, in green; nuclei are labeled with Hoechst 33342, in blue. Scale bars: 40 μm for **(A)** and **(B)**; 10 μm for the left panels of **(E)** and **(F)**; 5 μm for the right panels of **(E)** and **(F)**. Means ± SEM of at least 3 independent experiments. Two-tailed *t*-test, $^{*}p < 0.05$ (significantly different from *Cast*$^{+/+}$).

affect cell proliferation must be studied in further detail, to clarify these differences.

To analyze whether calpastatin deletion affected the migration of newly formed NSC in the DG, another thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), was administered to the mice on days 9–11 prior to their sacrifice. The analysis of migration in the DG was performed by three different methods: distribution of BrdU-positive cells in the SGZ and the GZ, doublecortin (DCX) immunoreactivity, and distance of BrdU-positive/DCX-positive cells from the SGZ into the GZ. The number of BrdU-positive cells in *Cast*$^{+/+}$ mice (**Figure 3A**) was 15.7 ± 4.8 cells/section in the SGZ (**Figure 3C**) and 3.3 ± 1.3 cells/section in the GZ (**Figure 3D**). In *Cast*$^{-/-}$ mice (**Figure 3B**), we observed a similar distribution of BrdU-positive cells in the DG, with 13.1 ± 1.3 cells/section in the SGZ ($p = 0.6184$, **Figure 3C**) and 3.4 ± 0.5 cells/section in the GZ ($p = 0.9329$, **Figure 3D**). While the analysis of BrdU distribution did not suggest a decreased number of newborn cells migrating into the GZ of *Cast*$^{-/-}$ mice, the overall neuroblast migration in the DG was reduced, as determined by measuring the percentage of DCX-positive area (1.5 ± 0.1% in *Cast*$^{-/-}$ and 2.7 ± 0.3% in *Cast*$^{+/+}$, $p = 0.0049$) (**Figures 4A,B,C**). Moreover, BrdU-positive/DCX-positive cells, which are indicative of migratory newborn cells, presented shorter migration distances in *Cast*$^{-/-}$ mice (13.5 ± 0.6 μm, $p = 0.0003$) (**Figures 4D,F**), comparing to *Cast*$^{+/+}$ mice (20.8 ± 1.1 μm) (**Figures 4D,E**). These results suggest an impairment of neuroblast migration in the DG of calpastatin-deficient mice, in addition to decreased NSC proliferation. Moreover, this effect on neuroblast migration was not limited to the DG, we also observed an impairment of cell migration in the RMS of *Cast*$^{-/-}$ mice (1899.9 ± 575.3 μm^2, $p = 0.0039$) (**Figures 5B,C**), when compared to *Cast*$^{+/+}$ mice (4259.2 ± 764.8 μm^2) (**Figures 5A,C**).

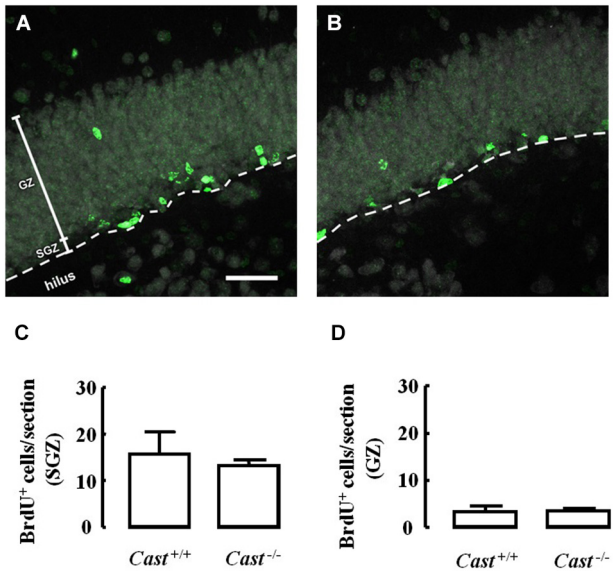

FIGURE 3 | Calpastatin deficiency does not alter the distribution of BrdU-positive cells in the DG. Representative images from hippocampal brain sections of *Cast*$^{+/+}$ **(A)** and *Cast*$^{-/-}$ **(B)** mice, showing BrdU-positive cells in green and nuclei, labeled with NeuN, in gray. Number of BrdU-positive cells in the SGZ **(C)** and the GZ **(D)** of the hippocampus. Scale bar: 40 μm. Means ± SEM of 5 independent experiments. Two-tailed *t*-test, $p > 0.05$.

Calpains have been shown to be involved in the migration of a variety of cell types, particularly by regulating cell spreading, speed and adherence, focal contact formation, filopodial and lamellipodial protusion formation and chemokinesis, with the involvement of signaling proteins such as Rac1, RhoA, Cdc42 and phospholipase C (Potter et al., 1998; Croce et al., 1999; Paulhe et al., 2001; Lokuta et al., 2003; Parnaud et al., 2005; Kuchay et al., 2012). Even though calpain inhibition results in decreased migration for most cell types already studied, there are reports showing that calpain inhibition increases the spreading ability of neutrophils (Lokuta et al., 2003) and, more recently, platelets (Kuchay et al., 2012). Although further research is needed to better understand the physiological roles of calpains, the results here presented suggest that promoting calpain activation may impair the formation of newborn neuronal cells, by interfering with the first stages of neurogenesis.

Excitotoxicity after stroke activates calpains, and several studies have already demonstrated that calpain inhibition is effective in reducing brain damage in animal models of this pathology. If calpain inhibition could enhance post-injury neurogenesis in addition to providing neuroprotection, it could be a potential therapeutic option to reduce brain damage after stroke. In this context, we investigated the effect of calpain inhibition on NSC proliferation and neuroblast migration in culture, using the calpain inhibitor calpeptin. Cell proliferation, as analyzed by EdU incorporation (**Figure 6A**), was lower in *Cast*$^{-/-}$ cells (24.2 ± 0.9%) than in *Cast*$^{+/+}$ cells (28.4 ± 1.0%), which is consistent with our *in vivo* observations. Calpeptin reversed this effect (29.7 ± 0.8%, $p < 0.01$), indicating that calpains may in fact be mediating cell proliferation. Moreover, calpeptin also increased cell proliferation in *Cast*$^{+/+}$ cells (32.0 ± 0.7%, $p < 0.05$). With the goal of enhancing post-injury neurogenesis, this slight rather

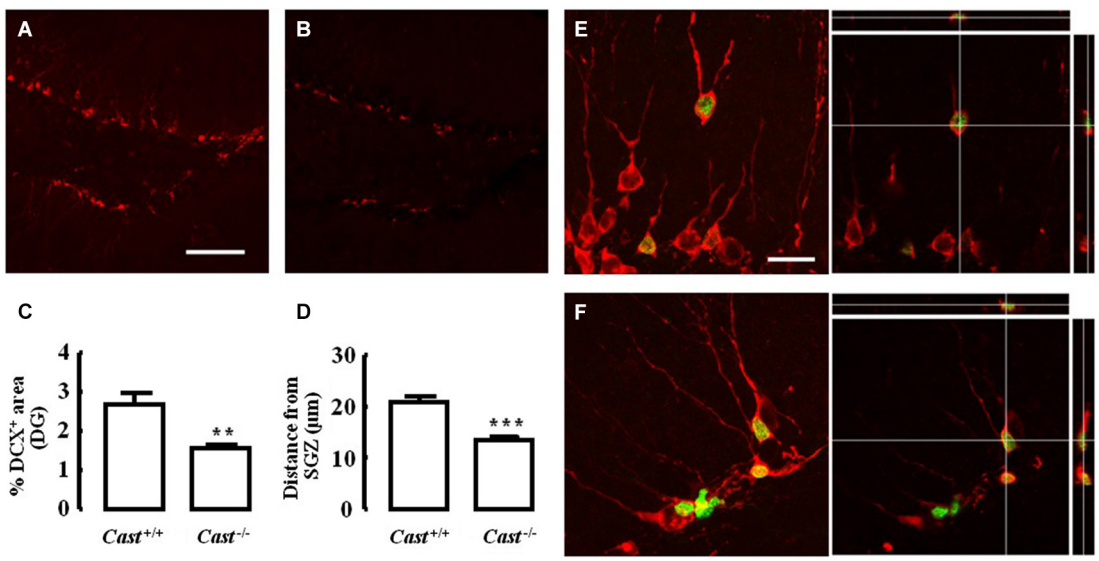

FIGURE 4 | Calpastatin deficiency decreases DCX immunoreactivity and migration distance of newborn cells in the DG. Representative images from hippocampal brain sections of *Cast*$^{+/+}$ **(A)** and **(E)** and *Cast*$^{-/-}$ **(B)** and **(F)** mice, showing migrating neuroblasts in red, labeled for DCX, and BrdU-positive cells in green. Percentage of DCX immunoreactivity in the DG **(C)**. Distance migrated from the SGZ by the BrdU-positive/DCX-positive cells present in the GZ **(D)**. Co-localization of BrdU and DCX is shown in the right panels of **(E)** and **(F)**. Scale bars: 100 μm for **(A)** and **(B)**; 20 μm for **(E)** and **(F)**. Means ± SEM of at least 3 independent experiments. Two-tailed *t*-test, $^{**}p < 0.01$ and $^{***}p < 0.001$ (significantly different from *Cast*$^{+/+}$).

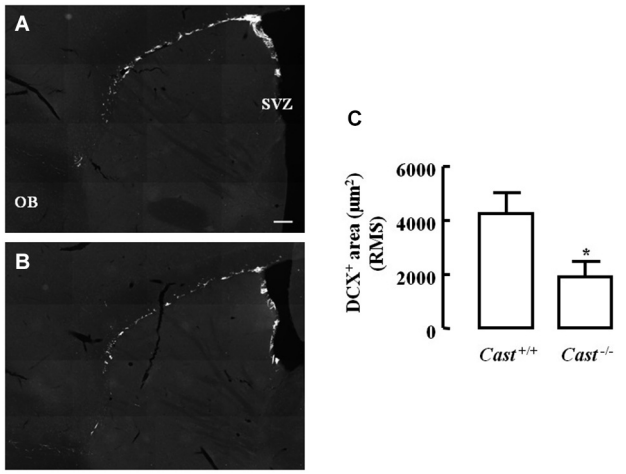

FIGURE 5 | Calpastatin deficiency decreases DCX immunoreactivity in the RMS. Representative images from sagittal striatal brain sections of *Cast*$^{+/+}$ **(A)** and *Cast*$^{-/-}$ **(B)**, showing migrating neuroblasts in gray, labeled for DCX. OB, olfactory bulb. DCX-positive area in the RMS **(C)**. Scale bar: 200 μm. Means ± SEM of 5 independent experiments. Two-tailed *t*-test, $*p < 0.05$ (significantly different from *Cast*$^{+/+}$).

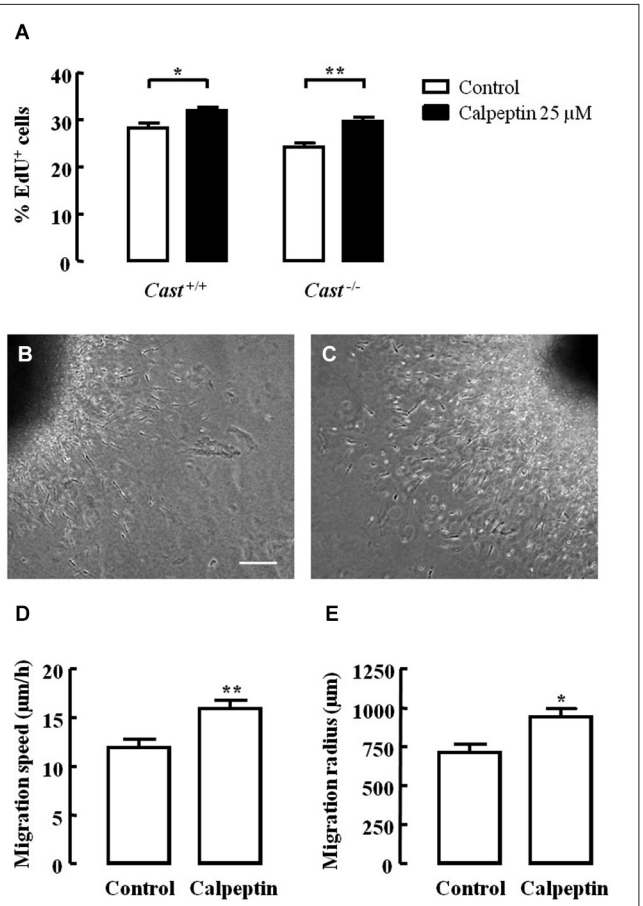

FIGURE 6 | Calpain inhibition increases NSC proliferation and cell migration in *Cast*$^{+/+}$ cells and rescues cell proliferation in *Cast*$^{-/-}$ cells. Percentage of living cells positive for EdU **(A)**. Representative images of SVZ explants untreated **(B)** or with calpeptin 25 μM **(C)**. Migration speed **(D)** and radius **(E)** of cells migrating out of the SVZ explants. Means ± SEM of at least 3 independent experiments. Two-way ANOVA (Bonferroni's post-test) **(A)** or two-tailed *t*-test **(D)** and **(E)**, $*p < 0.05$ and $**p < 0.01$ (significantly different from control). Scale bar: 100 μm.

than high increase in cell proliferation is actually preferable, since it lowers the risk of undifferentiated mass growth, while still somewhat increasing the number of newborn cells that may later on replace lost neurons. Nevertheless, increasing neuroblast migration and consequent neuronal differentiation, integration and survival is important for successfully improving brain repair after stroke.

Finally, we also assessed cell migration in SVZ explants isolated from wild type mice. Calpeptin (**Figure 6C**) increased both the migration speed (16.0 ± 0.8 μm/h, $p = 0.0063$) (**Figure 6D**) and the migration distance (943.0 ± 51.9 μm, $p = 0.0120$) (**Figure 6E**) of cells leaving the explants, when compared to untreated cells (migration speed 12.0 ± 0.8 μm/h; migration distance 715.0 ± 50.2 μm) (**Figures 6B,D,E**). This translates into another advantage for the potential use of calpain inhibition to enhance post-injury neurogenesis, i.e., improving migration. However, studies on how calpain inhibition may affect neuronal differentiation, integration and survival are still needed in order to corroborate the idea of using calpain inhibitors to treat brain damage after stroke.

Calpains are key players in the neuronal damage that occurs after stroke (Bano and Nicotera, 2007; Bevers and Neumar, 2008). For this reason, some of the strategies that have been developed in order to limit neuronal death after ischemic lesion have focused on interfering with calpain function. Several calpain inhibitors were already shown to be neuroprotective in animal models of brain ischemia (Bartus et al., 1994; Hong et al., 1994; Li et al., 1998; Markgraf et al., 1998; Frederick et al., 2008; Koumura et al., 2008; Peng et al., 2011). Furthermore, overexpression of calpastatin (Cao et al., 2007) and calpain silencing (Bevers et al., 2010) have also been shown to be effective in reducing neuronal death caused by stroke. The results presented here show that the lack of calpastatin hinders proliferation of NSC in the SGZ of the hippocampus, as well as migration of newborn cells into the GZ and in the RMS of adult mice. Moreover, we show that calpain inhibition increases SVZ-derived NSC proliferation and cell migration in wild type cells. We propose that, in addition to their neuroprotective effect, the use of calpain inhibitors results in an enhancement of post-injury brain repair. This constitutes an added benefit for potential clinical application for the treatment of stroke. Neuroprotection afforded by inhibition of calpains was also observed in other pathologies in which calpains contribute to the neuronal damage. These include traumatic brain injury (Saatman et al., 1996; Schoch et al., 2012), Alzheimer's disease (Rao et al., 2008), Parkinson's disease (Crocker et al., 2003), spinal cord injury (Ray et al., 2000), diabetic retinopathy (Shanab et al., 2012), acute optic neuritis (Das et al., 2013) and optic nerve crush (Araujo Couto et al., 2004).

Stem cells offer a promising approach for brain repair after stroke. In addition to the enhancement of endogenous neurogenesis, other approaches recently reported include

transplantation after stroke of NSC obtained from human fetal brain (Mine et al., 2013), induced pluripotent stem cells (Tornero et al., 2013) or stem cells from other origins (for review, see Lindvall and Kokaia, 2011; Hermann et al., 2014; Ikegame et al., 2014). Directly inducing the conversion of astrocytes into neurogenic cells is also being regarded as a possibility for post-injury brain repair (Niu et al., 2013; Magnusson et al., 2014). However, these approaches are currently hampered by limited cell survival due to the unfavorable conditions present in the lesioned areas. Such unfavorable conditions seem to be particularly worsened with age, conditioning the outcome of brain repair, partly by reducing the natural increase of endogenous neurogenesis (Popa-Wagner et al., 2014). Nonetheless, stem cell therapies have also shown to at least partially improve recovery after stroke in aged rodents (Jin et al., 2010; Zhang et al., 2013; Balseanu et al., 2014; Tatarishvili et al., 2014). Thus, combined therapies pose a promising approach to enhance post-injury brain repair, both in young and aged brains.

Overall, inhibiting calpains may improve the outcome of brain repair strategies based on cell therapy by both limiting neuronal damage and enhancing neurogenesis.

MATERIAL AND METHODS

ANIMALS

Twenty-week old calpastatin knock-out mice ($Cast^{-/-}$, $n = 5$) (Takano et al., 2005), in a C57Bl6 background, and their wild type littermates ($Cast^{+/+}$, $n = 5$), were used in this study. The mice were kept in our animal facilities, in a room with controlled temperature (21 ± 1°C) and humidity (55%), with food and water *ad libitum* in a 12 h dark:light cycle. All experiments were performed in accordance with institutional and European guidelines (2010/63/EU) for the care and use of laboratory animals.

LABELING OF DIVIDING CELLS WITH THYMIDINE ANALOGS

The experimental procedure was conducted as illustrated in **Figure 1**. Briefly, the animals (5 $Cast^{-/-}$ and 5 $Cast^{+/+}$) were treated for 3 days with BrdU (Sigma Aldrich, St Louis, MO, USA), through i.p. injections of 50 mg/kg every 12 h. After 7 days, EdU was administrated (Invitrogen, Paisley, UK), through i.p. injections of 50 mg/kg, twice, 2 h apart. This was performed in order to assess cell migration and cell proliferation, respectively. On day 12, the mice were perfused transcardially with 0.9 % NaCl followed by 4 % paraformaldehyde. The brains were removed and coronal hippocampal sections and sagittal striatal sections were obtained by cryosectioning (30 μm thick, in 6-series). The sections were stored in an antifreeze solution, at 4°C.

IMMUNOHISTOCHEMISTRY AND DETECTION OF THYMIDINE ANALOGS

Free-floating coronal hippocampal sections were processed for immunohistochemistry against BrdU, DCX and neuronal nuclei (NeuN), or double-labeled against PCNA and Sox2, or EdU and Sox2. DNA denaturation for BrdU staining was performed by treating the sections with 1 M HCl for 20 min at 65°C. Antigen retrieval for Sox2 or PCNA stainings was performed by treating the sections with 10 mM citric acid, pH 6.0, for 20 min at 95°C. The sections were then blocked for 1 h with 5 % normal horse or goat serum (Vector Laboratories Inc., Burlingame, CA, USA), respectively, in 0.25 % Triton X-100 in 0.01 M PBS. Slices were incubated with the primary antibodies, mouse anti-BrdU (1:80; DAKO, Glostrup, Denmark) or rat anti-BrdU (1:50, AbD Serotec, Oxford, UK), goat anti-DCX (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NeuN (1:200; Millipore Corporation, Billerica, MA, USA), mouse anti-PCNA (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-Sox2 (1:250; Millipore Corporation, Billerica, MA, USA) for 48 h at 4°C. The sections were then incubated with the correspondent secondary antibody (1:200), in 2 % block (normal horse or goat serum, accordingly), conjugated with Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 633 (Invitrogen, Paisley, UK), for 2 h in the dark, at room temperature. Labeling for EdU was performed by click-chemistry with Alexa Fluor 488 or 594 azide, according to the manufacturer instructions (Invitrogen, Paisley, UK) and, with the exception of the BrdU staining, nuclei were stained with Hoechst 33342 (2 μg/ml; Invitrogen, Paisley, UK) for 10 min. Free-floating sagittal striatal sections were stained against DCX. The sections were mounted on gelatin-coated slides with DAKO fluorescence mounting medium (DAKO, Glostrup, Denmark).

ANALYSIS OF INCORPORATION OF BrdU AND EdU

BrdU-positive and EdU-positive cells in the SGZ, the first layer of cells adjacent to the hilus, and in the GZ of 5 mid sections of the hippocampus (spanning 180 μm of the dorsal hippocampus) were counted for each animal (Liu et al., 1998; Brunson et al., 2001; Salazar-Colocho et al., 2008; Kim et al., 2009; Carreira et al., 2010), directly under an epifluorescence microscope (Axioskop 2 Plus, Zeiss, Jena, Germany), by one blinded observer. Cell counting was carried out in both upper and lower blades of the DG.

DCX IMMUNOREACTIVITY AND MIGRATION MEASUREMENTS IN THE DG

DCX immunoreactivity in the DG and migration distances of BrdU-positive/DCX-positive cells in the GZ were determined in images acquired in a laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany), using ImageJ (version 1.43u, National Institutes of Health, Bethesda, MD, USA) with the LSM Toolbox Plugin. The quantification of the DCX-positive area was performed using a threshold analysis in 5 mid sections of the hippocampus of each animal. This consisted in defining the optimal threshold for stained *vs.* non stained cells and calculating the area stained with DCX (Komitova et al., 2005; Carreira et al., 2010). The migration measurements, in turn, were performed in a total of approximately 30 cells from 5 images acquired for each $Cast^{+/+}$ or $Cast^{-/-}$ mouse, by determining the distance between the nucleus of the cell and the boundary between the SGZ and the hilus, perpendicularly to that delimitation.

DCX IMMUNOREACTIVITY IN THE RMS

DCX immunoreactivity in the RMS was determined using ImageJ (version 1.43u, National Institutes of Health, Bethesda, MD, USA), in images acquired in an inverted microscope

(Axio Observer Z1, Zeiss, Jena, Germany), using the Mosaix module from the AxioVision software Rel. 4.8.2 (Zeiss, Jena, Germany), in order to capture the entire RMS on each section. DCX-positive area was measured for each image in 3 boxes of 250 μm × 250 μm, randomly placed along the length of the RMS, a method similar to what was previously described (Kuhn et al., 1997).

NSC PROLIFERATION IN SVZ CULTURES

NSC were isolated from the SVZ of P0-3 *Cast*$^{+/+}$ and *Cast*$^{-/-}$ mice and maintained in culture, as previously described (Morte et al., 2013). Dissociated NSC (n = 3) were plated on coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma Aldrich, St Louis, MO, USA) until 60–70% confluency was reached, and then treated with calpeptin 25 μM (Tocris Bioscience, Bristol, UK) for 6 h (untreated cells were used as controls). The cells were kept with EdU 10 μM for the last 4 h before fixation with 4% paraformaldehyde/4% sucrose. EdU incorporation was processed using a commercially available kit (Click-iT®EdU Alexa Fluor®488 HCS Assay, Invitrogen, Paisley, UK) and nuclei were stained with 1 μg/ml Hoechst 33342, for 5 min. EdU-positive cells were counted in images acquired in an Axio Imager Z2 microscope (Zeiss, Jena, Germany).

NSC MIGRATION SVZ EXPLANTS

SVZ explants were prepared from wild type C57Bl6 mice (P5–7) and cultured for 72 h in 70% Matrigel (BD Biosciences, San Jose, CA, USA) in CCM1 medium (Hyclone, Logan, UT, USA) supplemented with 1% Pen/Strep and 1% B27 (Invitrogen, Paisley, UK) (Wichterle et al., 1997; de Chevigny et al., 2006), in the presence (n = 5) or absence (n = 8) of calpeptin 25 μM. Migration distances were measured in 5 explants per culture and migrating cells were imaged for 3 h, at 3 min intervals, under an Eclipse TE200 inverted microscope (Nikon Corporation, Tokyo, Japan), using the Metamorph 6.3v1 software (Molecular Devices, Sunnyvale, CA, USA), in order to calculate the migration speed of cells leaving the explants.

STATISTICAL ANALYSIS

The data are presented as means ± SEM. Statistical significance was determined using a two-tailed t-test or a two-way ANOVA, as indicated in the figure legends, using GraphPad Prism 5 software. Differences were considered significant when $p < 0.05$.

AUTHOR CONTRIBUTIONS

Vanessa M. Machado, Inês M. Araújo: Conception and design of the work, acquisition, analysis, interpretation of data, drafting of manuscript;

Maria I. Morte, Bruno P. Carreira, Maria M. Azevedo: Acquisition, analysis, interpretation of data;

Jiro Takano, Nobuhisa Iwata, Takaomi C. Saido: Conception and design of the work, analysis, interpretation of data;

Hannelore Asmussen, Alan R. Horwitz: Conception and design of the work, acquisition, analysis, interpretation of data;

Caetana M. Carvalho: Conception and design of the work, interpretation of data.

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REFERENCES

Aimone, J. B., Li, Y., Lee, S. W., Clemenson, G. D., Deng, W., and Gage, F. H. (2014). Regulation and function of adult neurogenesis: from genes to cognition. *Physiol. Rev.* 94, 991–1026. doi: 10.1152/physrev.00004.2014

Araujo Couto, L., Sampaio Narciso, M., Hokoç, J. N., and Blanco Martinez, A. M. (2004). Calpain inhibitor 2 prevents axonal degeneration of opossum optic nerve fibers. *J. Neurosci. Res.* 77, 410–419. doi: 10.1002/jnr.20170

Ariyoshi, H., Okahara, K., Sakon, M., Kambayashi, J., Kawashima, S., Kawasaki, T., et al. (1998). Possible involvement of m-calpain in vascular smooth muscle cell proliferation. *Arterioscler. Thromb. Vasc. Biol.* 18, 493–498. doi: 10.1161/01.atv.18.3.493

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Balseanu, A. T., Buga, A. M., Catalin, B., Wagner, D. C., Boltze, J., Zagrean, A. M., et al. (2014). Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. *Front. Aging Neurosci.* 6:130. doi: 10.3389/fnagi.2014.00130

Bano, D., and Nicotera, P. (2007). Ca2+ signals and neuronal death in brain ischemia. *Stroke* 38, 674–676. doi: 10.1161/01.str.0000256294.46009.29

Bartus, R. T., Baker, K. L., Heiser, A. D., Sawyer, S. D., Dean, R. L., Elliott, P. J., et al. (1994). Postischemic administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. *J. Cereb. Blood Flow Metab.* 14, 537–544. doi: 10.1038/jcbfm.1994.67

Bevers, M. B., Ingleton, L. P., Che, D., Cole, J. T., Li, L., Da, T., et al. (2010). RNAi targeting micro-calpain increases neuron survival and preserves hippocampal function after global brain ischemia. *Exp. Neurol.* 224, 170–177. doi: 10.1016/j.expneurol.2010.03.007

Bevers, M. B., and Neumar, R. W. (2008). Mechanistic role of calpains in postischemic neurodegeneration. *J. Cereb. Blood Flow Metab.* 28, 655–673. doi: 10.1038/sj.jcbfm.9600595

Brunson, K. L., Eghbal-Ahmadi, M., Bender, R., Chen, Y., and Baram, T. Z. (2001). Long-term, progressive hippocampal cell loss and dysfunction induced by early-life administration of corticotropin-releasing hormone reproduce the effects of early-life stress. *Proc. Natl. Acad. Sci. U S A* 98, 8856–8861. doi: 10.1073/pnas.151224898

Cao, G., Xing, J., Xiao, X., Liou, A. K., Gao, Y., Yin, X. M., et al. (2007). Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J. Neurosci.* 27, 9278–9293. doi: 10.1523/jneurosci.2826-07.2007

Carreira, B. P., Morte, M. I., Inácio, A., Costa, G., Rosmaninho-Salgado, J., Agasse, F., et al. (2010). Nitric oxide stimulates the proliferation of neural stem cells bypassing the epidermal growth factor receptor. *Stem Cells* 28, 1219–1230. doi: 10.1002/stem.444

Christian, K. M., Song, H., and Ming, G. L. (2014). Functions and dysfunctions of adult hippocampal neurogenesis. *Annu. Rev. Neurosci.* 37, 243–262. doi: 10.1146/annurev-neuro-071013-014134

Croce, K., Flaumenhaft, R., Rivers, M., Furie, B., Furie, B. C., Herman, I. M., et al. (1999). Inhibition of calpain blocks platelet secretion, aggregation and spreading. *J. Biol. Chem.* 274, 36321–36327. doi: 10.1074/jbc.274.51.36321

Crocker, S. J., Smith, P. D., Jackson-Lewis, V., Lamba, W. R., Hayley, S. P., Grimm, E., et al. (2003). Inhibition of calpains prevents neuronal and behavioral

deficits in an MPTP mouse model of Parkinson's disease. *J. Neurosci.* 23, 4081–4091.

Das, A., Guyton, M. K., Smith, A., Wallace, G. T., Mcdowell, M. L., Matzelle, D. D., et al. (2013). Calpain inhibitor attenuated optic nerve damage in acute optic neuritis in rats. *J. Neurochem.* 124, 133–146. doi: 10.1111/jnc.12064

de Chevigny, A., Lemasson, M., Saghatelyan, A., Sibbe, M., Schachner, M., and Lledo, P. M. (2006). Delayed onset of odor detection in neonatal mice lacking tenascin-C. *Mol. Cell. Neurosci.* 32, 174–186. doi: 10.1016/j.mcn.2006.04.002

Ehm, O., Göritz, C., Covic, M., Schäffner, I., Schwarz, T. J., Karaca, E., et al. (2010). RBPJkappa-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus. *J. Neurosci.* 30, 13794–13807. doi: 10.1523/jneurosci.1567-10.2010

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., et al. (2014). Neurogenesis in the striatum of the adult human brain. *Cell* 156, 1072–1083. doi: 10.1016/j.cell.2014.01.044

Favaro, R., Valotta, M., Ferri, A. L., Latorre, E., Mariani, J., Giachino, C., et al. (2009). Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. *Nat. Neurosci.* 12, 1248–1256. doi: 10.1038/nn.2397

Frederick, J. R., Chen, Z., Bevers, M. B., Ingleton, L. P., Ma, M., and Neumar, R. W. (2008). Neuroprotection with delayed calpain inhibition after transient forebrain ischemia. *Crit. Care Med.* 36, S481–S485. doi: 10.1097/ccm.0b013e31818a8ec8

Gage, F. H. (2000). Mammalian neural stem cells. *Science* 287, 1433–1438. doi: 10.1126/science.287.5457.1433

Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003). The calpain system. *Physiol. Rev.* 83, 731–801. doi: 10.1152/physrev.00029.2002

Hermann, D. M., Peruzzotti-Jametti, L., Schlechter, J., Bernstock, J. D., Doeppner, T. R., and Pluchino, S. (2014). Neural precursor cells in the ischemic brain - integration, cellular crosstalk and consequences for stroke recovery. *Front. Cell. Neurosci.* 8:291. doi: 10.3389/fncel.2014.00291

Hong, S. C., Goto, Y., Lanzino, G., Soleau, S., Kassell, N. F., and Lee, K. S. (1994). Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* 25, 663–669. doi: 10.1161/01.str.25.3.663

Ikegame, Y., Yamashita, K., Nakashima, S., Nomura, Y., Yonezawa, S., Asano, Y., et al. (2014). Fate of graft cells: what should be clarified for development of mesenchymal stem cell therapy for ischemic stroke? *Front. Cell. Neurosci.* 8:322. doi: 10.3389/fncel.2014.00322

Imayoshi, I., Sakamoto, M., Ohtsuka, T., Takao, K., Miyakawa, T., Yamaguchi, M., et al. (2008). Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat. Neurosci.* 11, 1153–1161. doi: 10.1038/nn.2185

Jessberger, S., and Gage, F. H. (2014). Adult neurogenesis: bridging the gap between mice and humans. *Trends Cell Biol.* 24, 558–563. doi: 10.1016/j.tcb.2014.07.003

Jin, K., Mao, X., Xie, L., Greenberg, R. B., Peng, B., Moore, A., et al. (2010). Delayed transplantation of human neural precursor cells improves outcome from focal cerebral ischemia in aged rats. *Aging Cell* 9, 1076–1083. doi: 10.1111/j.1474-9726.2010.00638.x

Jin, K., Wang, X., Xie, L., Mao, X. O., Zhu, W., Wang, Y., et al. (2006). Evidence for stroke-induced neurogenesis in the human brain. *Proc. Natl. Acad. Sci. U S A* 103, 13198–13202. doi: 10.1073/pnas.0603512103

Kaneko, N., and Sawamoto, K. (2009). Adult neurogenesis and its alteration under pathological conditions. *Neurosci. Res.* 63, 155–164. doi: 10.1016/j.neures.2008.12.001

Kashiwagi, A., Schipani, E., Fein, M. J., Greer, P. A., and Shimada, M. (2010). Targeted deletion of Capn4 in cells of the chondrocyte lineage impairs chondrocyte proliferation and differentiation. *Mol. Cell. Biol.* 30, 2799–2810. doi: 10.1128/mcb.00157-10

Kim, H. J., Leeds, P., and Chuang, D. M. (2009). The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in the ischemic brain. *J. Neurochem.* 110, 1226–1240. doi: 10.1111/j.1471-4159.2009.06212.x

Komitova, M., Mattsson, B., Johansson, B. B., and Eriksson, P. S. (2005). Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. *Stroke* 36, 1278–1282. doi: 10.1161/01.str.0000166197.94147.59

Koumura, A., Nonaka, Y., Hyakkoku, K., Oka, T., Shimazawa, M., Hozumi, I., et al. (2008). A novel calpain inhibitor, ((1S)-1((((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl)-3-methylbutyl) carbamic acid 5-methoxy-3-oxapentyl ester, protects neuronal cells from cerebral ischemia-induced damage in mice. *Neuroscience* 157, 309–318. doi: 10.1016/j.neuroscience.2008.09.007

Kuchay, S. M., Wieschhaus, A. J., Marinkovic, M., Herman, I. M., and Chishti, A. H. (2012). Targeted gene inactivation reveals a functional role of calpain-1 in platelet spreading. *J. Thromb. Haemost.* 10, 1120–1132. doi: 10.1111/j.1538-7836.2012.04715.x

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J., and Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.* 17, 5820–5829.

Li, P. A., Howlett, W., He, Q. P., Miyashita, H., Siddiqui, M., and Shuaib, A. (1998). Postischemic treatment with calpain inhibitor MDL 28170 ameliorates brain damage in a gerbil model of global ischemia. *Neurosci. Lett.* 247, 17–20. doi: 10.1016/s0304-3940(98)00266-3

Lim, D. A., and Alvarez-Buylla, A. (2014). Adult neural stem cells stake their ground. *Trends Neurosci.* 37, 563–571. doi: 10.1016/j.tins.2014.08.006

Lindvall, O., and Kokaia, Z. (2011). Stem cell research in stroke: how far from the clinic? *Stroke* 42, 2369–2375. doi: 10.1161/strokeaha.110.599654

Liu, J., Solway, K., Messing, R. O., and Sharp, F. R. (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci.* 18, 7768–7778.

Lokuta, M. A., Nuzzi, P. A., and Huttenlocher, A. (2003). Calpain regulates neutrophil chemotaxis. *Proc. Natl. Acad. Sci. U S A* 100, 4006–4011. doi: 10.1073/pnas.0636533100

Ma, D. K., Bonaguidi, M. A., Ming, G. L., and Song, H. (2009). Adult neural stem cells in the mammalian central nervous system. *Cell Res.* 19, 672–682. doi: 10.1038/cr.2009.56

Macas, J., Nern, C., Plate, K. H., and Momma, S. (2006). Increased generation of neuronal progenitors after ischemic injury in the aged adult human forebrain. *J. Neurosci.* 26, 13114–13119. doi: 10.1523/jneurosci.4667-06.2006

Magnusson, J. P., Göritz, C., Tatarishvili, J., Dias, D. O., Smith, E. M., Lindvall, O., et al. (2014). A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science* 346, 237–241. doi: 10.1126/science.346.6206.237

Markgraf, C. G., Velayo, N. L., Johnson, M. P., Mccarty, D. R., Medhi, S., Koehl, J. R., et al. (1998). Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke* 29, 152–158. doi: 10.1161/01.str.29.1.152

Martí-Fàbregas, J., Romaguera-Ros, M., Gómez-Pinedo, U., Martínez-Ramírez, S., Jiménez-Xarrié, E., Marín, R., et al. (2010). Proliferation in the human ipsilateral subventricular zone after ischemic stroke. *Neurology* 74, 357–365. doi: 10.1212/WNL.0b013e3181cbccec

Mine, Y., Tatarishvili, J., Oki, K., Monni, E., Kokaia, Z., and Lindvall, O. (2013). Grafted human neural stem cells enhance several steps of endogenous neurogenesis and improve behavioral recovery after middle cerebral artery occlusion in rats. *Neurobiol. Dis.* 52, 191–203. doi: 10.1016/j.nbd.2012.12.006

Ming, G. L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70, 687–702. doi: 10.1016/j.neuron.2011.05.001

Morte, M. I., Carreira, B. P., Machado, V., Carmo, A., Nunes-Correia, I., Carvalho, C. M., et al. (2013). Evaluation of proliferation of neural stem cells in vitro and in vivo. *Curr. Protoc. Stem Cell Biol.* Chapter 2, Unit 2D.14. doi: 10.1002/9780470151808.sc02d14s24

Murachi, T. (1984). Calcium-dependent proteinases and specific inhibitors: calpain and calpastatin. *Biochem. Soc. Symp.* 49, 149–167.

Neumar, R. W., Meng, F. H., Mills, A. M., Xu, Y. A., Zhang, C., Welsh, F. A., et al. (2001). Calpain activity in the rat brain after transient forebrain ischemia. *Exp. Neurol.* 170, 27–35. doi: 10.1006/exnr.2001.7708

Niu, W., Zang, T., Zou, Y., Fang, S., Smith, D. K., Bachoo, R., et al. (2013). In vivo reprogramming of astrocytes to neuroblasts in the adult brain. *Nat. Cell Biol.* 15, 1164–1175. doi: 10.1038/ncb2843

Parnaud, G., Hammar, E., Rouiller, D. G., and Bosco, D. (2005). Inhibition of calpain blocks pancreatic beta-cell spreading and insulin secretion. *Am. J. Physiol. Endocrinol. Metab.* 289, E313–E321. doi: 10.1152/ajpendo.00006.2005

Paulhe, F., Bogyo, A., Chap, H., Perret, B., and Racaud-Sultan, C. (2001). Vascular smooth muscle cell spreading onto fibrinogen is regulated by calpains and phospholipase C. *Biochem. Biophys. Res. Commun.* 288, 875–881. doi: 10.1006/bbrc.2001.5859

Peng, S., Kuang, Z., Zhang, Y., Xu, H., and Cheng, Q. (2011). The protective effects and potential mechanism of Calpain inhibitor Calpeptin against focal cerebral ischemia-reperfusion injury in rats. *Mol. Biol. Rep.* 38, 905–912. doi: 10.1007/s11033-010-0183-2

Popa-Wagner, A., Buga, A. M., Doeppner, T. R., and Hermann, D. M. (2014). Stem cell therapies in preclinical models of stroke associated with aging. *Front. Cell. Neurosci.* 8:347. doi: 10.3389/fncel.2014.00347

Potter, D. A., Tirnauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco, K. A., et al. (1998). Calpain regulates actin remodeling during cell spreading. *J. Cell Biol.* 141, 647–662. doi: 10.1083/jcb.141.3.647

Qiu, K., Su, Y., and Block, E. R. (2006). Use of recombinant calpain-2 siRNA adenovirus to assess calpain-2 modulation of lung endothelial cell migration and proliferation. *Mol. Cell. Biochem.* 292, 69–78. doi: 10.1007/s11010-006-9219-2

Rao, M. V., Mohan, P. S., Peterhoff, C. M., Yang, D. S., Schmidt, S. D., Stavrides, P. H., et al. (2008). Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. *J. Neurosci.* 28, 12241–12254. doi: 10.1523/jneurosci.4119-08.2008

Ray, S. K., Matzelle, D. C., Wilford, G. G., Hogan, E. L., and Banik, N. L. (2000). E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. *Brain Res.* 867, 80–89. doi: 10.1016/s0006-8993(00)02260-5

Rock, M. T., Dix, A. R., Brooks, W. H., and Roszman, T. L. (2000). Beta1 integrin-mediated T cell adhesion and cell spreading are regulated by calpain. *Exp. Cell Res.* 261, 260–270. doi: 10.1006/excr.2000.5048

Romanko, M. J., Rola, R., Fike, J. R., Szele, F. G., Dizon, M. L., Felling, R. J., et al. (2004). Roles of the mammalian subventricular zone in cell replacement after brain injury. *Prog. Neurobiol.* 74, 77–99. doi: 10.1016/j.pneurobio.2004.07.001

Saatman, K. E., Murai, H., Bartus, R. T., Smith, D. H., Hayward, N. J., Perri, B. R., et al. (1996). Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. *Proc. Natl. Acad. Sci. U S A* 93, 3428–3433. doi: 10.1073/pnas.93.8.3428

Salazar-Colocho, P., Lanciego, J. L., Del Rio, J., and Frechilla, D. (2008). Ischemia induces cell proliferation and neurogenesis in the gerbil hippocampus in response to neuronal death. *Neurosci. Res.* 61, 27–37. doi: 10.1016/j.neures.2008.01.008

Santos, D. M., Xavier, J. M., Morgado, A. L., Sola, S., and Rodrigues, C. M. (2012). Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation. *PLoS One* 7:e33468. doi: 10.1371/journal.pone.0033468

Schoch, K. M., Evans, H. N., Brelsfoard, J. M., Madathil, S. K., Takano, J., Saido, T. C., et al. (2012). Calpastatin overexpression limits calpain-mediated proteolysis and behavioral deficits following traumatic brain injury. *Exp. Neurol.* 236, 371–382. doi: 10.1016/j.expneurol.2012.04.022

Shanab, A. Y., Nakazawa, T., Ryu, M., Tanaka, Y., Himori, N., Taguchi, K., et al. (2012). Metabolic stress response implicated in diabetic retinopathy: the role of calpain and the therapeutic impact of calpain inhibitor. *Neurobiol. Dis.* 48, 556–567. doi: 10.1016/j.nbd.2012.07.025

Shimada, M., Greer, P. A., Mcmahon, A. P., Bouxsein, M. L., and Schipani, E. (2008). In vivo targeted deletion of calpain small subunit, Capn4, in cells of the osteoblast lineage impairs cell proliferation, differentiation and bone formation. *J. Biol. Chem.* 283, 21002–21010. doi: 10.1074/jbc.m710354200

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., et al. (2013). Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153, 1219–1227. doi: 10.1016/j.cell.2013.05.002

Takano, J., Tomioka, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., et al. (2005). Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J. Biol. Chem.* 280, 16175–16184. doi: 10.1074/jbc.m414552200

Tatarishvili, J., Oki, K., Monni, E., Koch, P., Memanishvili, T., Buga, A. M., et al. (2014). Human induced pluripotent stem cells improve recovery in stroke-injured aged rats. *Restor. Neurol. Neurosci.* 32, 547–558. doi: 10.3233/RNN-140404

Tornero, D., Wattananit, S., Grønning Madsen, M., Koch, P., Wood, J., Tatarishvili, J., et al. (2013). Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain* 136, 3561–3577. doi: 10.1093/brain/awt278

Wichterle, H., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Direct evidence for homotypic, glia-independent neuronal migration. *Neuron* 18, 779–791. doi: 10.1016/s0896-6273(00)80317-7

Xu, Y., and Mellgren, R. L. (2002). Calpain inhibition decreases the growth rate of mammalian cell colonies. *J. Biol. Chem.* 277, 21474–21479. doi: 10.1074/jbc.m111689200

Zhang, L., Yi, L., Chopp, M., Kramer, B. C., Romanko, M., Gosiewska, A., et al. (2013). Intravenous administration of human umbilical tissue-derived cells improves neurological function in aged rats after embolic stroke. *Cell Transplant.* 22, 1569–1576. doi: 10.3727/096368912x658674

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Influence of the extracellular matrix on endogenous and transplanted stem cells after brain damage

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The limited regeneration capacity of the adult central nervous system (CNS) requires strategies to improve recovery of patients. In this context, the interaction of endogenous as well as transplanted stem cells with their environment is crucial. An understanding of the molecular mechanisms could help to improve regeneration by targeted manipulation. In the course of reactive gliosis, astrocytes upregulate Glial fibrillary acidic protein (GFAP) and start, in many cases, to proliferate. Beside GFAP, subpopulations of these astroglial cells coexpress neural progenitor markers like Nestin. Although cells express these markers, the proportion of cells that eventually give rise to neurons is limited in many cases *in vivo* compared to the situation *in vitro*. In the first section, we present the characteristics of endogenous progenitor-like cells and discuss the differences in their neurogenic potential *in vitro* and *in vivo*. As the environment plays an important role for survival, proliferation, migration, and other processes, the second section of the review describes changes in the extracellular matrix (ECM), a complex network that contains numerous signaling molecules. It appears that signals in the damaged CNS lead to an activation and de-differentiation of astrocytes, but do not effectively promote neuronal differentiation of these cells. Factors that influence stem cells during development are upregulated in the damaged brain as part of an environment resembling a stem cell niche. We give a general description of the ECM composition, with focus on stem cell-associated factors like the glycoprotein Tenascin-C (TN-C). Stem cell transplantation is considered as potential treatment strategy. Interaction of transplanted stem cells with the host environment is critical for the outcome of stem cell-based therapies. Possible mechanisms involving the ECM by which transplanted stem cells might improve recovery are discussed in the last section.

Keywords: brain damage, stem cells, stem cell niche, reactive gliosis, extracellular matrix

INTRODUCTION

Regeneration of the adult central nervous system (CNS) after damage is limited in mammals. This causes severe problems for patients who suffer from CNS lesions or stroke. Regeneration of the peripheral nervous system is more effective, differences in the cellular response are attributed to this discrepancy (Brosius Lutz and Barres, 2014). Several approaches have been tested in animal models to improve functional recovery of patients, for instance neutralization of inhibitory factors by injection of blocking antibodies or by enzymatic degradation (Zhao et al., 2013). Transplantation of stem cells are another promising strategy and effort is made to examine their effect on regeneration in clinical trials (Savitz et al., 2014). For an efficient treatment, an understanding of the cellular and molecular mechanisms underlying the observed limitations is of interest.

In the first section we describe the effect of CNS damage on astrocytes that become reactive and in many cases start to re-express neural progenitor markers. The second section is focused on the extracellular matrix (ECM) and factors in this matrix expressed by these astrocytes and other cell types. The third section is dedicated to the question how transplanted stem cells can interact with the host and by which mechanisms they might improve regeneration.

LESIONS OF THE CENTRAL NERVOUS SYSTEM ACTIVATE ENDOGENOUS PROGENITOR-LIKE CELLS

After stroke or lesion, astrocytes, microglia, and other cell types of the immune system are activated in a process called reactive gliosis (reviewed by Donnelly and Popovich, 2008; Burda and Sofroniew, 2014). Astrocytes change their morphology and subpopulations of them upregulate markers typical of immature neural progenitors during development (**Figure 1**). In severe cases, a glial scar is formed by astrocytes that intermingle with fibroblasts before they segregate (Bundesen et al., 2003). In the following first section of the review, we want to give a short overview of the markers these astroglial subtypes express, of their spatial distribution and origin, and of the neurogenic potential these cells might have *in vivo*.

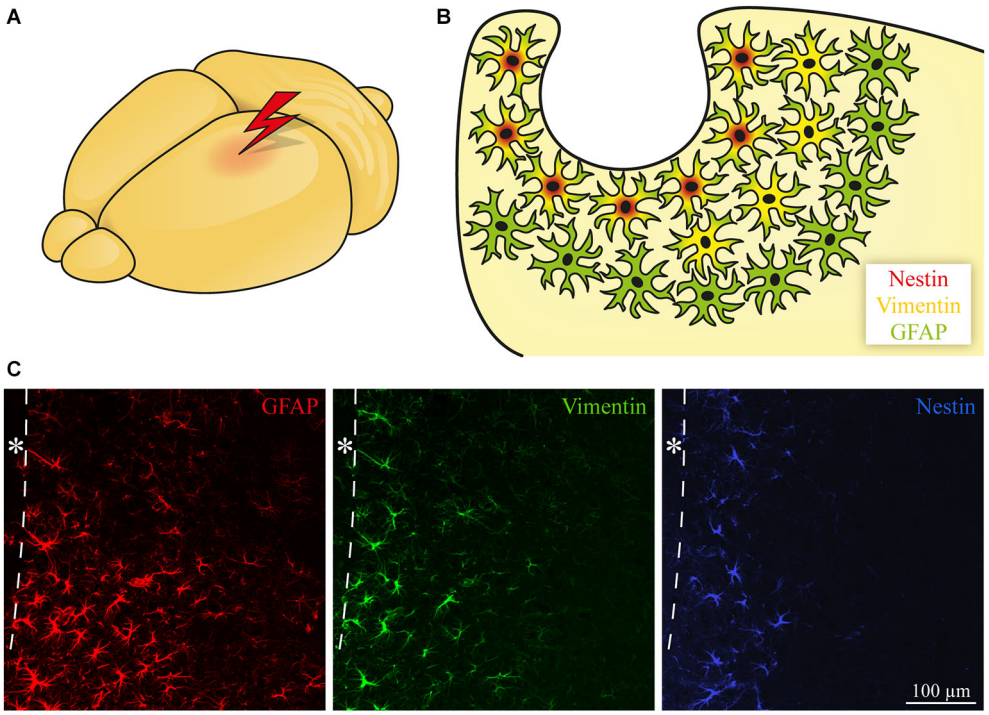

FIGURE 1 | Subtypes of reactive astroglia. (A, B) Astrocytes become reactive following CNS damage. In addition to GFAP upregulation, some of the cells start to express progenitor markers like Vimentin and Nestin. The position of the cells in relation to the lesion determines the expression pattern with Nestin near the lesion, Vimentin in a broader area and GFAP with a widespread upregulation. **(C)** Immunohistochemical staining of GFAP (red), Vimentin (green), and Nestin (blue, in an adjacent slice) expression after focal laser lesion of the mouse visual cortex. The dashed line depicts the border to the lesion core; * lesion core.

PROGENITOR MARKER EXPRESSION AFTER CNS DAMAGE

Astrocytes become reactive after CNS damage and typically express the intermediate filament Glial fibrillary acidic protein (GFAP). In addition, other markers like the intermediate filaments Vimentin and Nestin are coexpressed (Pekny and Nilsson, 2005). Vimentin is expressed in radial glia during development (Bignami et al., 1982), whereas Nestin is the prototypical neural stem/progenitor cell marker (Lendahl et al., 1990). Depending on the time point after lesion, additional markers for immature glia like Brain lipid-binding protein (BLBP), the DSD-1 epitope on members of the receptor protein tyrosine phosphatase (RPTP) β family, and Tenascin-C (TN-C) are expressed (Robel et al., 2011).

The function of GFAP and Vimentin in regeneration is discussed (Brenner, 2014). For example, knockout mice deficient for GFAP and Vimentin showed reduced hypertrophy of astrocytes, improved synaptic restoration after entorhinal cortex lesion (Wilhelmsson et al., 2004), and supported integration of cells transplanted into the retina (Kinouchi et al., 2003) or into the hippocampus (Widestrand et al., 2007). In contrast, an increased lesion volume was found in GFAP/Vimentin double knockout mice after stroke, whereas single knockouts had no effect on the lesion volume (Li et al., 2008). Different GFAP isoforms are described for human and mice, but especially for mice there is no clear correlation of certain isoforms with stem/progenitor cells (Kamphuis et al., 2012). The role of TN-C and other ECM components in regeneration is discussed in the second section.

ASTROGLIAL SUBTYPES ARE ARRANGED IN DISTINCT AREAS

The different markers mentioned above are not uniformly expressed by all astrocytes. Instead, subpopulations express different combinations. Here it was observed that the cells show a specific spatial distribution. For example, focal laser lesions in the adult mouse visual cortex (Roll et al., 2012) induce GFAP upregulation in a wide area, whereas the progenitor markers Vimentin and even more extremely Nestin are restricted to an area near the lesion core (**Figure 1**). Interestingly, astrocytes (type B cells) are the stem cells of the subventricular zone (SVZ) and are also positive for Nestin and Vimentin (Doetsch et al., 1997, 1999). Differences in marker expression may reflect the potential of the cells to form other neural cell types. It was described that multipotent neural stem/progenitor cells that give rise to neurons, astrocytes, and oligodendrocytes *in vitro* appear in the brain following stab wound (Buffo et al., 2008), laser lesion (Sirko et al., 2009), and in other lesion models (Sirko et al., 2013). As shown by differential marker expression, reactive astrocytes are a heterogeneous population with respect to the distance of a cell to the lesion. Additionally, astrocytes are also heterogeneous regarding morphology, function, CNS region, and severity of the lesion (reviewed by Anderson et al., 2014).

DIFFERENT ORIGINS OF MULTIPOTENT CELLS AFTER CNS DAMAGE

An obvious question regarding multipotent stem/progenitor cells in the damaged adult brain is the origin of those cells. Are adult stem cells attracted from the stem cells niches like the SVZ and migrate to the lesion site, or are local astrocytes induced to de-differentiate on-site? An argument for activation of local cells in focal laser lesions of the visual mouse cortex is the distinct spatial distribution of markers like GFAP, Vimentin, and Nestin. A similar finding of Nestin-expressing cells in a distinct pattern was made in the spinal cord after hemitransection and was also interpreted as local activation (Lang et al., 2004). Re-expression of the ECM molecule TN-C, which is expressed during development and later downregulated in the adult cortex, is also restricted to astrocytes located near the lesion (McKeon et al., 1991; Roll et al., 2012). It can be assumed that gradients of signaling molecules with high concentrations near the lesion and decreasing levels in the periphery influence the cell fate and result in the observed regional differences. Indeed, fate mapping studies by Buffo et al. (2008) showed that stab wounds activate local astrocytes in the cortex that are multipotent *in vitro*. Multipotent cells that give rise to neurons, astrocytes, or oligodendrocytes can be found in the developing nervous system. Different tripotential, but also subtypes of bipotential glial precursors have been described there. For example, glial restricted precursors (GRPs) that produce oligodendrocytes and astrocytes, O2A cells that give rise to oligodendrocytes and type-2 astrocytes, and others are distinguished according to their potential *in vivo* and *in vitro* and to their marker expression (Liu and Rao, 2004). The proteoglycan Neuron-glial antigen 2 (NG2) is associated with glial precursors during development, therefore the contribution of NG2-positive cells present in the adult CNS after damage is discussed (Han et al., 2004; Komitova et al., 2011). In the spinal cord, it has been shown that ependymal cells contribute significantly to newly formed astrocytes and show multilineage potential (Barnabé-Heider et al., 2010). To what extent cells after damage only share similarities or if they acquire a cell fate that is indeed identical to those developmental populations is hard to determine. Depending on the severity, in addition to a local response cells from the adult stem cell niches are activated (Shimada et al., 2010). A stem cell response in terms of an increased SVZ size (Thored et al., 2006) and attraction of neuroblasts from the SVZ to the striatum after stroke was reported (Arvidsson et al., 2002; Yamashita et al., 2006). Regional differences in the potential of SVZ cells are described, such as dorsolateral prevalence of oligodendroglial cells and neuronal and astroglial fates in the ventrolateral area (reviewed by Maki et al., 2013). In some cases, attraction of cells from the SVZ could not be shown by cell tracing experiments (Shimada et al., 2012) or fate mapping (Buffo et al., 2008). In contrast to the described promoting effects of stroke on the adult stem cell niche, chronic inflammation reduces proliferation and impairs migration of neuroblasts (Pluchino et al., 2008). So in general, local activation as well as an influence on the existing adult stem cell niches are conceivable and may take place in parallel. Certainly, this depends on the type, severity, and localization of the damage and further studies are needed to determine the contribution of both mechanisms in different lesion paradigms.

DIFFERENCES OF THE NEUROGENIC POTENTIAL *IN VIVO* AND *IN VITRO*

In many cases, the neurogenic potential of the cells *in vivo* is more restricted compared to the situation *in vitro*. So reactive astrocyte-derived cells appear to be multipotent in culture, but fail to form neurons after transplantation *in vivo* (Shimada et al., 2012). An approach to promote the neuronal fate of reactive astrocytes is retroviral expression of the proneural transcription factor NeuroD1, allowing astrocytes to differentiate into glutamatergic neurons (Guo et al., 2014). Another transcription factor, Sox2, was able to convert spinal cord astrocytes into neurons (Su et al., 2014). A further strategy is the administration of neurogenesis-promoting factors, as shown for Galectin-1 after stroke (Ishibashi et al., 2007). More strategies have been summarized by Obermair et al. (2008).

The main difference between endogenous stem/progenitor cells *in situ* and their isolated and cultured counterparts is the completely changed environment, where signals from other cell types are lost. Among them are several neurogenesis-inhibiting factors (Seidenfaden et al., 2006; Buddensiek et al., 2009), one of the candidates is Notch (Aruga et al., 2002). Stress during isolation, high concentrations of growth factors in the medium, and the oxygen and energy supply are additional factors that may influence the cells' potential. This shows that both, multipotent cells combined with a permissive environment, are necessary for the formation of neurons after lesion. The ECM contains a tremendous variety of signaling molecules and with regard to its importance for regeneration it is the topic of the next section.

EXTRACELLULAR SIGNALS INFLUENCE REGENERATION AND STEM/PROGENITOR CELLS

The ECM is a complex network of interacting molecules that are secreted by the cells into the extracellular space. Depending on the tissue, it functions as a scaffold for the cells and provides mechanical stability, for example in cartilage (Treilleux et al., 1992), but also in all other tissues. The ECM in the CNS is free of fibrillar elements, except after lesion (Heck et al., 2007). It contains glycoproteins like TN-C, TN-R, and proteoglycans (PG; Zimmermann and Dours-Zimmermann, 2008). PGs consist of a core protein and covalently attached carbohydrate (glycosaminoglycan, GAG) chains. Important Chondroitin sulfate proteoglycans (CSPGs) are molecules of the Lectican family and members of the RPTPβ family, whereas the Heparan sulfate proteoglycans (HSPGs) Syndecan and Glypican are other prominent constituents. Interactions of PGs with other molecules can be mediated by the core protein or by the carbohydrate structures. The ECM is able to bind growth factors and to present them to the cells (Clark, 2008; Brizzi et al., 2012). This allows the extracellular signals to regulate processes like cell survival, proliferation, and differentiation as well as migration or axon growth. According to changing requirements during development, the ECM composition is variable. After CNS lesions, the ECM is altered again. For example, reactive gliosis can lead to a glial scar that has a beneficial effect as it provides a barrier for healthy tissue from the environment (Pekny et al., 2014). At the same time, it is the major obstacle for axonal regrowth (Silver and Miller, 2004; Rolls et al., 2009). But the ECM is more than one constituent of

the glial scar. It is involved in synaptic plasticity in many ways, for example in long term potentiation (LTP; Dityatev and Schachner, 2003). As mentioned above, the ECM contains signals regulating processes that are critical for regeneration and therefore this second section of the review is dedicated to details regarding the ECM composition and associated factors after CNS damage.

EXTRACELLULAR MATRIX AFTER CNS DAMAGE

After lesion, several aspects of extracellular signals are critical: (i) barriers like the glial scar are important to protect the healthy tissue from the environment, but they also prevent axonal regrowth or cell migration; (ii) plasticity-limiting factors stabilize neuronal networks in the healthy brain but exacerbate reorganization in case of damage; and (iii) the balance between de-differentiation and re-differentiation. If cells are induced by the extracellular signals to de-differentiate, but get no signal for proper differentiation, they will stay in an undifferentiated state and cannot replace lost tissue. For example, TN-C, which is also present in the adult stem cell niche, may inhibit differentiation of an astrocyte-derived progenitor cell into a functional neuron or oligodendrocyte. This could be mediated by repression of the RNA-binding molecule Sam68 (Moritz et al., 2008; Czopka et al., 2010).

Extracellular matrix under different pathological conditions

Changes in the ECM composition differ depending on the type of CNS damage. As a disease-specific description is not the aim of this review, we recommend the following publications: stroke-induced effects were reviewed by Ellison et al. (1999), with focus on the blood-brain barrier by Baeten and Akassoglou (2011). Reports are also available for brain tumors (Gladson, 1999; Wade et al., 2013), spinal cord injuries (Condic and Lemons, 2002), neurodegenerative diseases like Alzheimer's (Morawski et al., 2012), and for autoimmune disorders like multiple sclerosis (Sobel, 1998; van Horssen et al., 2007).

SOURCES OF EXTRACELLULAR MATRIX AND ASSOCIATED SIGNALING MOLECULES

In response to damage, a number of cell types react and communicate via extracellular signals. The main sources of ECM and related important signaling molecules in the matrix are summarized in this paragraph. Important sources of extracellular molecules are listed in **Table 1**.

Table 1 | Important sources of extracellular signaling molecules after CNS lesion.

Cell type	Extracellular matrix-related molecule
Endothelial cells	Brain-derived neurotrophic factor (BDNF)
	Fibroblast growth factor 2 (FGF2)
	Leukaemia inhibitory factor (LIF)
	Pleiotrophin
	Vascular endothelial growth factor (VEGF)
	Fibrinogen [blood-derived]
Pericytes	Fibronectin
Astrocytes	Agrin
	Brevican
	Collagen IV, VIII
	Decorin
	Fibronectin
	Glypican
	Laminin
	Neurocan
	Phosphacan
	Syndecan
	Tenascin-C
	Thrombospondin
	Versican
Oligodendrocytes/ Precursors	Matrix metalloproteinase 9 (MMP-9)
	Neurocan
	Neuron-glial antigen 2 (NG2)
	Nogo-A
	Versican
Neurons	Chemokines
	Cytokines
	Sonic hedgehog (SHH)
Microglia/Macrophages	Cytokines
	Neuron-glial antigen 2 (NG2)
	Neurotrophic factors

As the origin of secreted molecules is often difficult to determine by immunological studies, more molecules are expressed that have not yet been allocated. References are given in the text.

Vasculature

Blood vessels are an important signal source, as reviewed by Gattazzo et al. (2014). As part of the neurovascular niche they are also thought to be crucial for the residing stem/progenitor cells in the adult stem cell niches (Shen et al., 2008). Endothelial cells produce a number of factors that act on neural cells. Among others, Pleiotrophin, Leukaemia inhibitory factor (LIF), Brain-derived neurotrophic factor (BDNF), and Fibroblast growth factor (FGF) 2 influence differentiation (Carmeliet, 2003; Dugas et al., 2008). Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis and neurogenesis (Jin et al., 2002; Nag et al., 2002). Angiogenesis is increased in the damaged CNS and therefore more cells get in contact with the perivascular niche (Arai et al., 2009). Hypoxia supports stemness during development, but also under pathological conditions like stroke (Panchision, 2009). This is mediated by Hypoxia-inducible factor (HIF) I α, which facilitates Notch signaling and inhibits factors responsible for differentiation, for example Bone morphogenetic proteins (BMPs). The blood-brain barrier is disrupted in many cases as result of mechanical damage or chemokines (Dimitrijevic et al., 2006), which allows cells like T lymphocytes and additional factors from the blood to enter the CNS that are retained under normal conditions. When Fibrinogen from the blood crosses the disturbed blood-brain barrier, it activates astrocytes by providing Transforming growth factor β (TGF-β; Schachtrup et al., 2010). Pericytes are involved in the production of the glial scar, including Fibronectin (FN; Göritz et al., 2011). In addition, vessels are used as guiding structures by migrating neural progenitors in the healthy and diseased brain (Massouh and Saghatelyan, 2010).

Astrocytes

Astrocytes are extremely heterogeneous in their expression profile, as described in the first section. Depending on the type of damage, the time point after lesion, and the position, reactive astrocytes are responsible for TN-C expression in many lesion

models (McKeon et al., 1991; Roll et al., 2012) and also express Brevican, Versican (Beggah et al., 2005), Neurocan (Haas et al., 1999), Phosphacan (McKeon et al., 1999), Decorin (Stichel et al., 1995), Laminin, and FN (Gris et al., 2007). HSPGs expressed by reactive astrocytes are Glypican, Syndecan, and Agrin (Iseki et al., 2002; Hagino et al., 2003; Falo et al., 2008). Collagen expression, which is involved in glial scar formation, was also shown for reactive astrocytes. The basement membrane-associated type IV (Liesi and Kauppila, 2002) and type VIII (Hirano et al., 2004) Collagens were detected. Astrocytes are also a source of Thrombospondins (TSPs; Lin et al., 2003) (reviewed by Sofroniew, 2009). Astrocytic expression of TN-C, Neurocan, and Phosphacan is increased by presence of meningeal fibroblasts *in vitro* (Wanner et al., 2008). Activation of astrocytes is mediated by pro-inflammatory cytokines like Interleukin (IL)-1β, IL-6, and Tumor necrosis factor α (TNF-α). They are secreted by microglia, in part by the astrocytes themselves, or by other cell types (Buffo et al., 2010).

Oligodendrocytes

Oligodendrocytes produce Nogo-A, an inhibitor of neurite outgrowth (Chen et al., 2000). Oligodendrocyte precursors express the CSPGs Neurocan and NG2 (Levine, 1994; Asher et al., 2000) and oligodendrocyte lineage cells secrete Versican under pathological conditions (Asher et al., 2002). Matrix metalloproteinase 9 (MMP-9) produced by oligodendrocyte precursors leads to blood-brain barrier leakage (Seo et al., 2013).

Neurons

Neurons start to express numerous chemokines like Chemokine (C-C motif) ligand 20 (CCL20), CCL21, CXC chemokine ligand 12 (CXCL12/SDF-1), and CXCL14/BRAK under pathological conditions (Das et al., 2012). They are primarily involved in immunomodulation, but additional functions as neuromodulators are assumed (Rostène et al., 2011).

Microglia

Microglia, the macrophages of the CNS, are activated by several mechanisms after injury. For example, they can detect reduced neuronal activity by alterations in neurotransmitter concentration and become activated (Biber et al., 2007; Kettenmann et al., 2011). Chemokines expressed after lesion can induce migration of microglia, for example Cysteine-cysteine (CC) chemokines (Carbonell et al., 2005). The consequence of microglial activation is the upregulation of cytokines and neurotrophic factors (Donnelly and Popovich, 2008). Increased levels of cytokines can induce remodeling of the ECM, for example by inducing the expression of MMPs (Gottschall and Yu, 1995) and of matrix molecules like TN-C by reactive astrocytes. Activated microglia also express ECM molecules themselves, for example NG2 (Sugimoto et al., 2014).

CLASSES OF EXTRACELLULAR MATRIX MOLECULES IN THE POSTLESIONAL CNS

Important classes of extracellular signaling molecules that are typically present after CNS damage are described below. In many cases, components typical of the stem cell niches are re-expressed and provide an environment that resembles—in part—a neural stem cell niche like the SVZ. That niche is formed by signals from the vasculature, cerebrospinal fluid, and a complex set of extracellular molecules secreted by the cells in the niche (reviewed by Kazanis and ffrench-Constant, 2011). The situation after lesion with upregulated ECM molecules and the niche-like environment is summarized in **Figure 2**. As described above, major sources of the signals are reactive astrocytes, microglia, and the vasculature. Important functions regulated by ECM molecules and associated factors are listed in **Table 2**.

Chondroitin sulfate proteoglycans

CSPGs act as barrier after CNS damage and contribute to the axonal growth-inhibitory environment including the glial scar (Brown et al., 2012). CSPGs are mainly expressed by reactive astrocytes. Their effect can be mediated by members of the Leukocyte common antigen-related (LAR) family of RPTPs that comprises for example LAR, RPTPδ, RPTPσ, and the Nogo receptors NgR1 and NgR3 (reviewed by Cregg et al., 2014). LARs are involved in β-Catenin recruitment, leading to changes in the Actin cytoskeleton, which in turn affects axon growth and synaptic organization (Um and Ko, 2013). The Actin cytoskeleton is also targeted by NgR1 and coreceptors, in this case via the small GTPase RhoA and Rho-associated kinase (ROCK; Fournier et al., 2003; Schwab and Strittmatter, 2014). In addition to the above mentioned receptors, CSPGs can bind to specific receptors like Integrins, a family of heterodimeric transmembrane receptors (see the detailed Integrin description below). CSPGs can also act indirectly, when they intensify growth factor-induced signaling by presenting a growth factor to its receptor, or conversely, block signaling by sequestering growth factors like Pleiotrophin (Deepa et al., 2002) from their receptors (reviewed by Carulli et al., 2005; Sharma et al., 2012b). Neurocan, Brevican, and Versican levels are increased after lesion (Jones et al., 2003; Beggah et al., 2005) as well as Biglycan, Decorin (Stichel et al., 1995), and NG2 (Levine, 1994). NG2 inhibits neurite outgrowth, consequently its neutralization improves recovery (Dou and Levine, 1994; Petrosyan et al., 2013). It is produced by oligodendrocyte progenitors, but not by GFAP-positive astrocytes. This is in line with reports that NG2-positive progenitors are not an important source of astrocytes after brain injury (Komitova et al., 2011). Macrophages and microglia are additional sources of NG2 (Jones et al., 2002; Sugimoto et al., 2014). Decorin antagonizes scar formation as it is able to reduce the expression of Neurocan, Brevican, NG2, and Phosphacan, probably by inhibiting TGF-β and antagonizing the Epidermal growth factor (EGF) receptor (Davies et al., 2004). The RPTPβ (or "RPTPβ/ζ") family comprises four isoforms. Alternative splicing of a single gene and proteolytic processing result in two transmembrane receptor forms (RPTPβ long and RPTPβ short) and two secreted molecules (Phosphacan and Phosphacan short isoform (PSI; Garwood et al., 2003; Chow et al., 2008)). The receptor forms contain two cytoplasmic tyrosine phosphatase domains, of which only one is catalytically active (Krueger and Saito, 1992). Substrates for this domain are β-Catenin, which is related to the Actin cytoskeleton and Wnt signaling (Meng et al., 2000), Fyn kinase (Pariser et al., 2005a), and others. Dimerization following

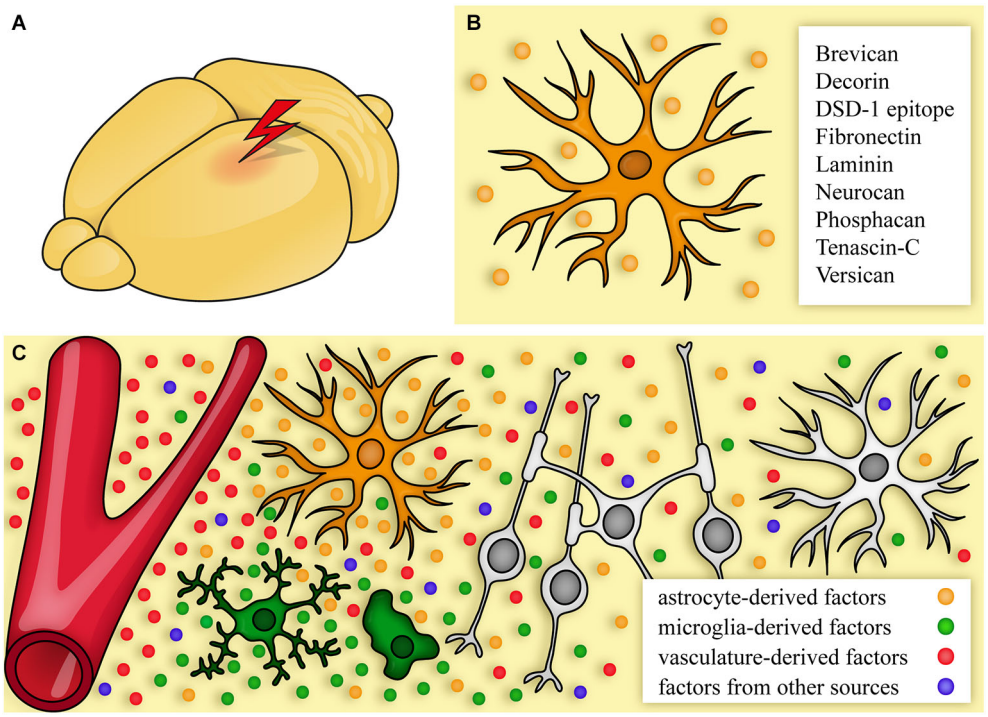

FIGURE 2 | The extracellular matrix after CNS damage. (A, B) After lesion, cells start to produce an altered extracellular matrix (ECM). One major source are reactive astrocytes **(B)** that express CSPGs and other ECM molecules. **(C)** Signals come from astrocytes (orange), the vasculature (red), immune cells/microglia (green), and additional sources (blue, for a detailed list see **Table 1**). This environment can promote, but also inhibit regeneration by affecting neurons, astrocytes, and oligodendrocytes.

Pleiotrophin binding inactivates the phosphatase domains and thereby increases the phosphorylation state of downstream factors like Adducin, a cytoskeletal protein (Pariser et al., 2005b). Other extracellular interaction partners are TN-C, Contactin/F3/F11, the cell adhesion molecules L1CAM and NCAM (Klausmeyer et al., 2007), and FGF (Milev et al., 1998b). RPTPβ isoforms are regulated after knife lesion of the cerebral cortex (Dobbertin et al., 2003), striatal stab wound (Barker et al., 1996), and after retinal laser lesion (Besser et al., 2009) and are critical for recovery (Harroch et al., 2002). PSI as the smallest member of the RPTPβ family promotes axon growth (Garwood et al., 2003). Two isoforms (RPTPβ long and Phosphacan) can be decorated with Chondroitin sulfate GAG chains. These chains regulate the affinity to binding partners, for example to Contactin/F3/F11 (Milev et al., 1998a). A specific Chondroitin sulfate structure, the "DSD-1" epitope, is recognized by the monoclonal antibody 473HD (Faissner et al., 1994; Gates et al., 1995). This epitope is expressed on neural stem cells during development (von Holst et al., 2006) and it has been shown that blocking of this epitope impairs neurosphere formation *in vitro*. Subpopulations of reactive astrocytes after laser lesion in the visual cortex of rats and mice express the DSD-1 epitope (Sirko et al., 2009; Roll et al., 2012). As mentioned above, this might reflect the immature characteristics of some reactive astrocytes and at the same time a neurogenic niche-like signal after CNS damage.

Collagens

Collagens comprise 28 members and can be divided into fibril-forming and network-forming Collagens (Ricard-Blum, 2011). In the CNS, Collagens are associated with the vasculature. Integrins are important Collagen receptors: the Integrin subunits α1, α2, α10, α11, and β1 interact with different Collagens. Additional receptors are members of the Discoidin domain receptors (DDRs), a subfamily of receptor tyrosine kinases (Leitinger and Hohenester, 2007). In addition to cell adhesion, Collagens are able to activate intracellular pathways and can induce proliferation (Pozzi et al., 1998). Collagens are expressed by astrocytes after lesion. Type IV and type VIII Collagens, both network-forming and associated with the basement membrane, have been found upregulated in the CNS (Liesi and Kauppila, 2002; Hirano et al., 2004). In general, Collagen expression is suppressed via EGF signaling (Heck et al., 2007).

Fibronectin

FN is a secreted glycoprotein that forms dimers via disulfide bonds near the C terminus. FN interacts with ECM molecules like Collagens, HSPGs, and TN-C and acts as ligand for Integrin receptors (Singh et al., 2010). FN is expressed by astrocytes and microglia/macrophages after CNS damage, also liver-derived plasma FN has a neuroprotective function in CNS repair (Tate et al., 2007a; Kim et al., 2013). FN activates microglia through

Table 2 | Parameters modulated by extracellular signals after lesion.

Parameter	Modulating factor in the extracellular matrix
Cell survival, proliferation	Neurotrophic factors*
	Cytokines*
	Growth factors*
Differentiation, axon growth, synaptic plasticity	Agrin
	Brevican
	Collagen
	Decorin
	Glypican
	Laminin
	Neurocan
	Neuron-glial antigen 2 (NG2)
	Nogo-A
	Phosphacan; with attached DSD-1 epitope
	Pleiotrophin
	Syndecan
	Tenascin-C
	Tenascin-R
	Thrombospondins
	Versican
Migration	CXC chemokine ligand 12 (CXCL12)
	Fibronectin
	Laminin
	Tenascin-C
	Thrombospondins

Many molecules regulate several aspects, only main functions are listed here. References are given in the text.

** signaling is modulated by the extracellular matrix.*

binding to Integrin α5β1 (Milner et al., 2007) and has a neurite growth-promoting effect *in vitro* (Tom et al., 2004).

Heparan sulfate proteoglycans

HSPGs are, in contrast to the inhibitory CSPGs, ascribed to the neurite outgrowth-promoting factors (Yamaguchi, 2001). It is assumed that HSPGs can interfere with CSPG signaling by interacting with the same receptors, for example RPTPσ (Coles et al., 2011). Accordingly, downstream factors like N-Cadherin (Siu et al., 2007) might be regulated. The HSPG Glypican is expressed by reactive astrocytes (Hagino et al., 2003). It is involved in Hedgehog signaling (Filmus and Capurro, 2014) and as a HSPG it also regulates FGF signaling (Gordon et al., 1989; Rapraeger et al., 1991; Yayon et al., 1991). Syndecan, which is also able to modulate FGF signaling (Filla et al., 1998), is produced by astrocytes after brain injury (Iseki et al., 2002). Important for axonal growth and cell migration could be the interaction of Glypican-1 with repellent Slit proteins (Ronca et al., 2001). Slit is expressed by reactive astrocytes (Hagino et al., 2003), treatment with specific inhibitors of this interaction is owing (Lau and Margolis, 2010). The *Drosophila* homolog of Syndecan is involved in Slit signaling (Steigemann et al., 2004), suggesting a similar effect in the mammalian CNS. The HSPG Agrin is also expressed by reactive astrocytes. It has functions in the immune system and in neuromuscular synapse formation and may additionally play a role in CNS synapse formation during regeneration (Falo et al., 2008). Perlecan, which is associated with the vascular basement membrane, is a secreted HSPG that is upregulated following damage. After stroke, a neuroprotective effect was shown after administration of Perlecan domain V. An Integrin-mediated upregulation of VEGF in endothelial cells was attributed to the beneficial function (Lee et al., 2011).

Laminins

Laminins are trimeric glycoproteins and a crucial component of the basement membrane. Different isoforms of α, β, and γ chains are combined to at least 16 Laminins and interaction with other extracellular molecules like Agrin, Collagen IV, Nidogen, and Perlecan leads to formation of complex networks (Domogatskaya et al., 2012). Laminins signal through different Integrins (Belkin and Stepp, 2000) and promote cell adhesion, migration, and axon growth during development (Calof et al., 1994). Laminins are upregulated in the glial scar by astrocytes (Liesi et al., 1984; McKeon et al., 1995) and in vessels after lesion (Tate et al., 2007b; Sarkar et al., 2012). CSPG-mediated inhibition of neurite growth in the scar interferes with Laminin-induced promoting effects (McKeon et al., 1995). Migration of activated neural stem cells along vessels after injury seems to involve Laminin (Kokovay et al., 2010). This could be mediated by α6β1 Integrin, which is a well-known Laminin receptor and is expressed on neural stem cells (Shen et al., 2008).

Tenascins

The TN family comprises four members in vertebrates, namely TN-C, TN-R, TN-W, and TN-X (Chiquet-Ehrismann et al., 2014). In the nervous system, TN-C and TN-R are expressed (Joester and Faissner, 2001). TN-C is a glycoprotein that is expressed by astrocytes during development and in adult stem cell niches (Garcion et al., 2004; von Holst, 2008). It has a modular structure including EGF-like repeats and, as a result of alternative splicing, a variable number of FN Type III-like domains (Joester and Faissner, 1999, 2001; von Holst et al., 2007). As each domain is responsible for specific interactions with other extracellular molecules, TN-C isoforms have diverse functions. Six TN-C monomers form a hexamer, called "hexabrachion". Expression of different TN-C isoforms is regulated by the transcription factor Pax6 (von Holst et al., 2007). TN-C interacts with CSPGs, for example with Neurocan (Rauch et al., 1997), members of the RPTPβ family (Barnea et al., 1994), as well as with Integrins (Yokosaki et al., 1998) and Contactin/F3/F11 (Rigato et al., 2002). Via its EGF-like repeats, TN-C also binds to the EGF receptor (Swindle et al., 2001). Interaction of TN-C has been shown for a number of Integrins, including αvβ3 (Jones and Jones, 2000a). TN-C-induced pathways include RhoA signaling and a Contactin-dependent inhibition of Fyn kinase. A model is proposed where inactive Fyn in turn prevents expression of the splicing factor Sam68 and thereby impairs oligodendrocyte differentiation (Czopka et al., 2010). TN-C blocks FN-dependent cell migration by interacting with Syndecan-4 (Chiquet-Ehrismann and Chiquet, 2003) and regulates axon growth (Faissner, 1997). During development, maintenance of oligodendrocyte precursors (Czopka et al., 2010) and astrocytic

lineage progression depend on TN-C (Karus et al., 2011). After lesion, TN-C is re-expressed and it contributes to the glial scar as barrier (McKeon et al., 1991; Deckner et al., 2000). TN-C is detected in different regions of the lesioned CNS, for example in the hippocampus (Niquet et al., 1995; Nakic et al., 1996), cerebellum, and cortex (Laywell et al., 1992). TN-C expression was also reported in the human brain after traumatic brain injury (Hausmann and Betz, 2001). TGF-β and FGF2 stimulate TN-C expression (Smith and Hale, 1997). After lesion, TN-C isoforms containing the FN Type III-like domains B and D are strongly upregulated (Dobbertin et al., 2010). After spinal cord injury, TN-C has a beneficial effect on spinal cord recovery (Chen et al., 2010). An overview of the different functions of TN-C is given by Chiquet-Ehrismann et al. (2014). TN-R has also a modular structure like TN-C, but forms trimers instead of hexamers (Joester and Faissner, 2001). TN-R interacts with Integrins, for example β1 Integrins (Xu et al., 2014), Contactin/F3/F11, Phosphacan (Jones and Jones, 2000a), and Myelin-associated glycoprotein (MAG; Yang et al., 1999). TN-R is expressed after spinal cord injury (Apostolova et al., 2006) and in the lesioned optic nerve by an increased number of cells (Becker et al., 2000). TN-R is part of perineuronal nets (PNNs) that limit synaptic plasticity in the adult. After damage, this property seems to inhibit synaptic remodeling and thereby affects regeneration.

Thrombospondins

TSPs are secreted glycoproteins that form trimers (members of subgroup A) or pentamers (subgroup B) (Adams and Lawler, 2011). Interaction partners include ECM molecules like Laminins, Collagens, and PGs, growth factors like FGF2, and Integrin receptors that eventually trigger intracellular signaling cascades (Resovi et al., 2014). Thrombospondins are also involved in Notch signaling, which regulates astrocytic differentiation after lesion (Benner et al., 2013). TSPs are expressed by reactive astrocytes and microglia (Möller et al., 1996; Lin et al., 2003), for example they are upregulated after spinal cord injury (Wang et al., 2009b). TSPs are involved in oligodendrocyte precursor migration (Scott-Drew and ffrench-Constant, 1997), synapse formation (Christopherson et al., 2005), and angiogenesis inhibition (Armstrong and Bornstein, 2003).

Cell adhesion molecules

Cell adhesion molecules of the Immunoglobulin superfamily (IgSF CAMs) are membrane-bound receptors that mediate contact to the ECM and cell-cell interactions. Axon guidance is one important function of CAMs. Among them, Neural CAM (NCAM) is upregulated after CNS lesion and promotes spinal cord recovery (Zhang et al., 2010). Accordingly, recovery in NCAM knockout mice is affected. The polysialylated form of NCAM (PSA-NCAM) is associated with plasticity and is expressed during development, in the adult stem cell niche, and after CNS damage (Emery et al., 2003). NCAM can modulate GDNF and BDNF signaling (Vutskits et al., 2001; Nielsen et al., 2009) and interacts with the CSPG Neurocan (Friedlander et al., 1994). NCAM can activate a number of intracellular cascades, for example the mitogen-activated protein (MAP) kinase pathway via a complex of Spectrin, RPTPα, Fyn kinase and Focal adhesion kinase (FAK; Budinich et al., 2012). L1CAM, another member of IgSF CAMs, is also an important regulator of axon growth during development. It is again upregulated after lesions, where it limits corticospinal tract sprouting (Jakeman et al., 2006). L1CAM activates, in part together with Integrins, MAP kinase signaling via Src kinase and Phosphoinositide 3-kinase (PI3K). It also modulates the Actin cytoskeleton through Ankyrin and Spectrin and can recruit microtubules via Doublecortin (Maness and Schachner, 2007).

Integrins

Integrins, a group of heterodimer transmembrane receptors for ECM molecules, are involved in cell adhesion, axon growth, and numerous other processes. Each Integrin heterodimer consists of one α and one β subunit. Eighteen α and eight β subunits are described, leading to 24 confirmed Integrin heterodimers. Integrins connect ECM and the cytoskeleton via adapters like Talin and activate intracellular pathways that regulate gene expression. The classical downstream cascade of Integrins leads to activation of FAK and subsequent activation of Akt and MAP kinases (Guan, 1997; Guo and Giancotti, 2004). A FAK-independent activation of Src kinase also exists (Arias-Salgado et al., 2003). Some Integrins, including the subunits αv, β1, β4, and β7, can crosstalk with growth factor receptors like EGFR (Brizzi et al., 2012). The β1 subunit is important in stem cell biology (Campos, 2005). As already mentioned, Integrins bind a huge number of ECM molecules. For example, Integrin α6β1 binds Laminin (Shen et al., 2008) and αvβ3 binds TN-C (Jones and Jones, 2000b). In addition to these outside-in signaling pathways, Integrins can transduce inside-out signals. They are mediated by the cytoskeletal protein Talin and increase the affinity of Integrin for extracellular ligands (Anthis et al., 2009). Like their ligands, Integrins are regulated under pathological conditions and contribute to postlesional changes (Ellison et al., 1999).

Matrix metalloproteinases

MMPs are able to modulate the ECM by specific proteolytic cleavage (Candelario-Jalil et al., 2009). This function is important in the damaged CNS when the cells' environment needs to be adjusted, for example to increase plasticity. MMP upregulation following injury is described for blood vessels, astrocytes, and microglia (Noble et al., 2002). MMPs degrade defined matrix components. So different TN-C isoforms are cleaved by specific MMPs (Siri et al., 1995), MMP-3 degrades the CSPGs Neurocan, Brevican, and Phosphacan (Muir et al., 2002). Effects of MMPs on regeneration have been described, for example of MMP-9 (Hsu et al., 2008). Here, migration of MMP-9-deficient astrocytes was reduced *in vitro*. MMP-9 is also involved in blood-brain barrier opening in the diseased CNS (Seo et al., 2013). The expression of MMPs in astrocytes can be regulated by the cytokines IL-1 and TNF-α (Gottschall and Yu, 1995). But not only migration is affected by MMPs: MMP-3 and MMP-9 play a role in neuronal differentiation in response to cytokines *in vitro* (Barkho et al., 2008).

Perineuronal nets

A specialized form of ECM are PNNs. They consist of Hyaluronan, TN-R, the CSPGs Aggrecan, Neurocan, and Brevican, and link proteins (Faissner et al., 2010; Ye and Miao, 2013). PNNs surround subpopulations of neurons, mostly Parvalbumin-positive GABAergic inhibitory interneurons, and are thought to stabilize synapses. Therefore their appearance is correlated with the end of a critical period during development. It has been shown that astrocytes play an important role in PNN formation, as they secrete components of the PNNs (Pyka et al., 2011). In line with this, the quadruple knockout of TN-C, TN-R, Neurocan, and Brevican either in astrocytes or hippocampal neurons leads to reduced PNN formation, accompanied by impaired synaptogenesis, synapse stability, and altered synaptic activity *in vitro* (Geissler et al., 2013). PNN degradation with the bacterial enzyme Chondroitinase ABC restored synaptic plasticity in the visual cortex after the critical period (Pizzorusso et al., 2002). Although single components like Neurocan can be upregulated after damage (Kwok et al., 2011), reduction of PNNs has been described, which is in line with the fact that plasticity is increased under this condition (Karetko-Sysa et al., 2011). This shows that not only the presence of molecules as such is important, but also the spatial distribution and the interaction of different factors.

GROWTH FACTORS AND OTHER SIGNALING MOLECULES IN THE MATRIX

Ephrins and Eph receptors

Ephrins and their counterparts, the Eph receptors, are potent regulators of neurite growth, as they are able to induce growth cone collapse. Activation of an Eph receptor tyrosine kinase following Ephrin binding leads to RhoA- and ROCK-induced changes of the Actin cytoskeleton (Wahl et al., 2000). Also bidirectional signaling is described, in this case Ephrins can activate RhoA and other pathways (Daar, 2012). Many Ephrins and Eph receptors are upregulated after CNS damage, as reviewed by Goldshmit et al. (2006). Ephrin-B2 and Eph-B2 seem to be involved in segregating invading fibroblasts at the glial scar (Bundesen et al., 2003).

Nogo-A

Nogo-A is a potent inhibitor of neurite outgrowth and interacts with a complex of receptors, including NgR1, that results in activation of ROCK signaling (reviewed by Schmandke et al., 2014). Nogo-A is produced by oligodendrocytes and is part of the myelin in the CNS. It is one important inhibitor of axonal regeneration (Chen et al., 2000). Interestingly, Nogo-A and NgR1 are also involved in the homeostasis of the adult SVZ. Here, proliferation and differentiation of neural stem cells are restricted by Nogo-A-expressing neuroblasts via NgR1. In addition, neuroblast migration is regulated by Nogo-A in an NgR1-independent mechanism that acts on ROCK (Rolando et al., 2012). Nogo-A and its receptor have been a target for several blocking experiments after stroke (Lee et al., 2004), spinal cord injury (Liebscher et al., 2005), and in other lesion models that showed beneficial effects of Nogo-A inactivation (reviewed by Overman and Carmichael, 2014; Schwab and Strittmatter, 2014). In intact animals, Nogo-A blocking showed no obvious side effects on cognitive function (Craveiro et al., 2013), although the Nogo-A receptor NgR1 is involved in memory formation (Karlén et al., 2009) and mice with downregulated Nogo-A show subtle cognitive deficits (Petrasek et al., 2014).

Semaphorins

Twenty Semaphorins are found in vertebrates, all of them contain a characteristic extracellular Sema domain. The most prominent function of Semaphorins is the regulation of axon growth and their ability to induce growth cone collapse. Semaphorins bind to Plexin and Neuropilin receptors. Different intracellular cascades eventually affect the cytoskeleton and, in many cases, induce growth cone collapse. Microtubule and Actin filament stability can be modified as well as gene expression (Neufeld and Kessler, 2008), pathways that depend on protein synthesis and independent mechanisms coexist (Manns et al., 2012). Sema3A is expressed by fibroblast-like cells after CNS injury (Pasterkamp et al., 1999) and in line with this, meningeal fibroblasts inhibit neurite outgrowth *in vitro* (Niclou et al., 2003). After optic nerve crush, short-term Sema3A upregulation was observed, whereas other class 3 Semaphorins were upregulated for days (Sharma et al., 2012a). L1CAMFc chimeric molecules can reverse the repellent effect of Sema3A, which shows that the combination of different factors is important rather than a single factor (Castellani et al., 2000).

Sonic hedgehog

Sonic hedgehog (SHH) is a signaling molecule that is able to regulate gene expression as well as the cytoskeleton. During development, it serves as morphogen and is involved in dorsoventral patterning. Binding of SHH to its receptor Patched relieves the repression of the seven-transmembrane receptor Smoothened. As a consequence, Gli transcription factors are activated and accumulate in the nucleus. This results in repression or activation of other transcription factors and altered gene expression (Fuccillo et al., 2006). Via a Gli-independent mechanism that involves a guanine nucleotide exchange factor (Tiam1) for the GTPase Rac1, SHH acts on the cytoskeleton during dendritic spine formation (Sasaki et al., 2010). SHH is secreted by neurons, endothelial cells (Sirko et al., 2013), and also by reactive astrocytes (Amankulor et al., 2009). This upregulation can only be observed under certain pathological conditions in the CNS. The expression differs depending on the type of damage. So it is detected after stab wound and stroke, but not in a murine Alzheimer's model (Sirko et al., 2013). In this study, it was shown that SHH expression is necessary for multipotency of endogenous neural stem/progenitor cells. The HSPG Glypican, itself upregulated after damage, is involved in Hedgehog signaling (Filmus and Capurro, 2014).

Wnts

Secreted Wnt proteins influence gene expression and the cytoskeleton. The canonical Wnt pathway starts with binding of Wnt to Frizzled receptor and to the coreceptor LRP. Subsequently, a β-Catenin-degrading complex is inactivated and β-Catenin accumulates in the cell. It enters the nucleus, where it interacts with transcription factors and activates Wnt target genes (Reya

and Clevers, 2005). Non-canonical Wnt pathways also affect the cytoskeleton (Semenov et al., 2007). Wnt proteins are upregulated after lesion and influence regeneration in different ways. It was shown that repulsive Wnts inhibit axonal growth in the spinal cord (Liu et al., 2008), on the other hand Wnt signaling is involved in symmetrical cell division of SVZ cells after stroke (Piccin and Morshead, 2011).

Notch

Notch is a transmembrane receptor that plays an important role in neural cell fate determination. After binding of its ligand, Jagged or Delta-like, the Notch intracellular domain (NICD) is cleaved by γ-Secretase. NICD translocates to the nucleus, where it interacts with transcription factors and allows target gene expression (Kopan and Ilagan, 2009). Notch is upregulated and activated after CNS lesion or stroke (Yamamoto et al., 2001; Xiao et al., 2009). Notch and its interaction with TSP 4 after cortical lesion are necessary for the differentiation of SVZ-derived astrocytes (Givogri et al., 2006; Benner et al., 2013). In another study, astrogliosis was promoted by the Notch ligand Jagged, produced by transplanted endothelial progenitor cells (Kamei et al., 2012).

Cytokines

Cytokines induce diverse cellular responses by activation of specific receptors. Examples for signaling cascades are reviewed by Leonard and Lin (2000) and Schlessinger and Ullrich (1992). Important cytokines expressed by activated microglia and macrophages are Interleukin-1 (IL-1), IL-6, Interferon, and TNF-α (Smith and Hale, 1997). TGF-β is increased in microglia under pathological conditions in the brain (Morgan et al., 1993) and the spinal cord (McTigue et al., 2000). They influence the expression levels of MMPs (see above) and activate astrocytes, IL-6 for example promotes EGF-induced astrocyte proliferation (Levison et al., 2000). Astrocytes are another major cytokine source (reviewed by Eddleston and Mucke, 1993; Wiese et al., 2012). BMPs and their inhibitor Noggin regulate neurogenesis in the adult stem cell niche (Lim et al., 2000). BMPs and Noggin are upregulated after brain and spinal cord injury (Hampton et al., 2007) and regulate astrocytic vs. neuronal and oligodendrocytic differentiation (Xiao et al., 2010). By driving the differentiation of astrocytes and CSPG production, BMPs are involved in glial scar formation (Fuller et al., 2007).

Neurotrophic factors

Neurotrophic factors and growth factors signal via specific transmembrane receptors. Depending on the class of growth factors, receptor tyrosine kinases (e.g., EGF receptor) or serine/threonine receptor kinases (e.g., TGFβ receptor) induce intracellular signaling cascades (Schlessinger and Ullrich, 1992). Neurotrophins like NT3 bind to Trk (Tropomyosin-related kinase) receptors (Hetman and Xia, 2000). Neurotrophic factors expressed by activated microglia and macrophages are BDNF, Ciliary neurotrophic factor (CNTF), Glial cell line-derived neurotrophic factor (GDNF), Hepatocyte growth factor (HGF), Insulin-like growth factor (IGF), Nerve growth factor (NGF), Neurotrophin (NT)-3 and Platelet-derived growth factor (PDGF; reviewed by Donnelly and Popovich, 2008). Microglia-produced neurotrophic factors can act in an autocrine way, for example BDNF and NT-3 increase microglia proliferation (Elkabes et al., 1996). In the adult spinal cord, high levels of CNTF are expressed following injury (Nakamura and Bregman, 2001). VEGF is secreted by endothelial cells and after damage also by astrocytes (Nag et al., 2002). It increases neurogenesis and plasticity (Jin et al., 2002; Licht et al., 2011). FGF and EGF increase levels of TGF-β (Lindholm et al., 1992), which is responsible for astrocyte activation and Neurocan expression (Asher et al., 2000). TGF-β increases NGF levels (Lindholm et al., 1990). NGF in turn induces microglial migration via the TrkA receptor, when combined with low concentrations of TGF-β (De Simone et al., 2007).

Chemokines

Chemokines are secreted signaling molecules that bind to G protein-coupled, seven-transmembrane receptors (Salanga et al., 2009). Activation of a receptor induces a cascade including second messengers like cAMP or IP_3 that can mediate diverse cellular responses (Patel et al., 2013). The chemokine stromal-derived factor 1 (SDF-1/CXCL12) is expressed by astrocytes after stroke and mediates attraction of cells by binding to its receptor, CXCR4. This system is involved in attraction of bone marrow stem cells (Hill et al., 2004), transplanted umbilical cord blood cells (Rosenkranz et al., 2010), and neural stem cells (Imitola et al., 2004). Two isoforms, SDF-1α and SDF-1β, have distinct expression patterns in the lesioned CNS. Neurons express SDF-1α, whereas SDF-1β is expressed by endothelial cells (Stumm et al., 2002). Hence, a dual function of SDF-1-mediated modulation of the immune response and neurotransmission is discussed.

REGENERATION IN ECM KNOCKOUT (KO) MODELS

The effect of ECM molecules on regeneration was examined in different knockout models. Double knockout of Neurocan and Brevican in mice does not obviously affect recovery after spinal cord injury (Quaglia et al., 2008). In a more sophisticated model with an additional priming lesion in the sensory nerve 7 days after spinal cord injury, only in KO animals axons crossed the dorsal root entry zone. This uncovered the growth-inhibiting effect of Neurocan and Brevican in the wild-type situation. TN-C KO mice have a mild phenotype, but recover worse after spinal cord injury (Chen et al., 2010). In contrast, deficiency of TN-R improves functional recovery of mice after spinal cord injury (Apostolova et al., 2006). TN-R seems to restrict synaptic reorganization after injury, which is in line with the role of TN-R in PNNs. EphA4 KO mice exhibit reduced gliosis and improved functional recovery after spinal cord injury (Goldshmit et al., 2004). Therefore, EphA4 seems to regulate two important aspects in regeneration.

EXTRACELLULAR MATRIX AS TARGET FOR IMPROVED RECOVERY

Inhibitory matrix was the target of different approaches so far, a number of studies have been reviewed by Soleman et al. (2013). The function can be blocked with antibodies, specific inhibitors, by knockdown, or already synthetized ECM can proteolytically be degraded, for example by ADAM proteases (reviewed by Burnside and Bradbury, 2014). Inhibition

of proteoglycan synthesis by β-D-Xylosides and sodium chlorate eliminates the inhibitory effect of astrocytic matrix on neurite growth *in vitro* (Smith-Thomas et al., 1995). Treatment with the Chondroitin sulfate-degrading enzyme Chondroitinase ABC (ChABC) revealed a recovery-promoting effect after spinal cord injury (Bradbury et al., 2002) and had neuroprotective effects (Chen et al., 2014). Although ChABC treatment is effective in many cases, ChABC-resistant inhibitory matrix also remains (Siddiqui et al., 2009) that needs to be addressed by other strategies. These inhibitory signals can be ascribed to the core protein part of PGs or to other molecules in the matrix. Treatment with antibodies directed against an extracellular factor relies on blocking its function. Studies using antibodies against Nogo-A (Zhao et al., 2013) and TN-R (You et al., 2012) have shown improved recovery from spinal cord injury. In addition to the aforementioned strategies that rely on the elimination of ECM function, delivery of regeneration-promoting ECM-related molecules is possible. For example, transplantation of L1CAM-overexpressing neural aggregates improved regeneration more effectively than those with normal L1CAM expression levels (Cui et al., 2011).

Possible mechanisms by which transplanted stem cells can promote regeneration in the context of ECM are discussed in the following third section.

POTENTIAL EFFECTS OF ENDOGENOUS AND TRANSPLANTED STEM CELLS ON REGENERATION

As described in the first section, endogenous progenitor-like cells appear in the damaged CNS. If a subset of astroglial cells starts to de-differentiate anyway, what could be the benefit of additional, transplanted stem cells? It is clear that in many cases positive effects are not due to cell replacement (Chopp et al., 2009). The last section of this review gives an overview of the different strategies behind stem cell therapies.

What are potential mechanisms by which transplanted stem cells support regeneration? Several strategies to improve recovery have been pursued so far (Burda and Sofroniew, 2014). Roughly the effects can be divided into three main aspects: (i) immune modulation; (ii) support of cell survival, differentiation, or axonal growth; and (iii) cell replacement (summarized in **Figure 3A**). Examples for factors that are produced by stem cells and their

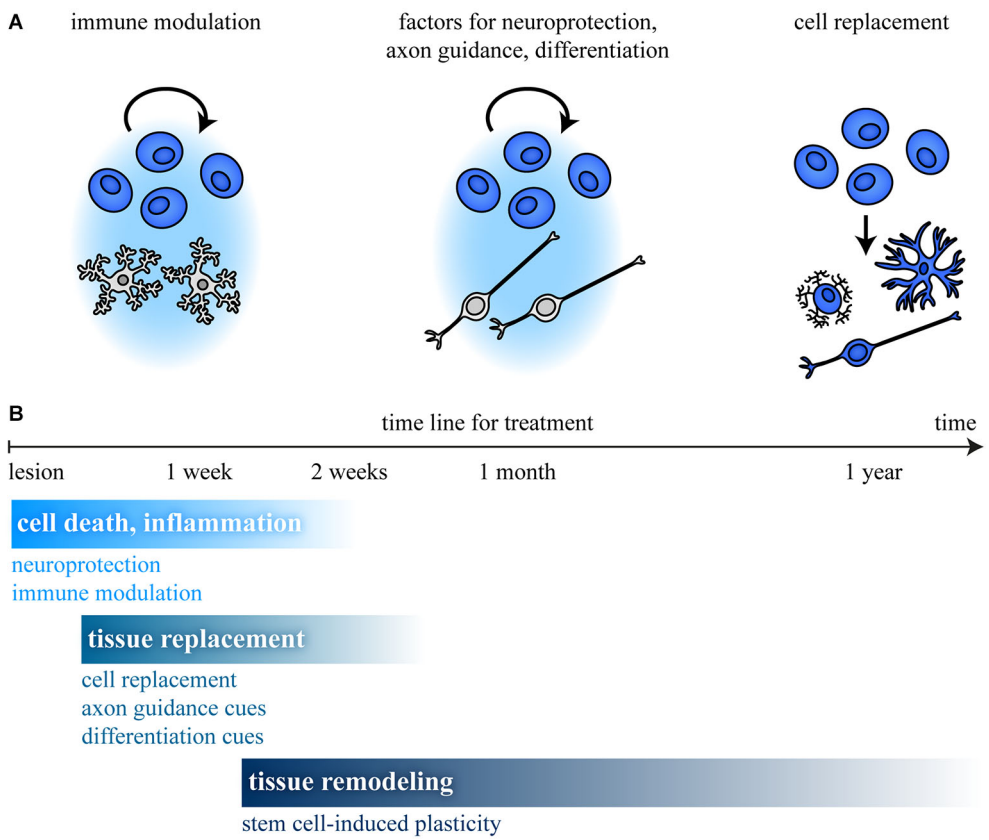

FIGURE 3 | Stem cell treatment and time line after CNS damage. (A) Stem cell transplantation can promote recovery by different mechanisms. Modulation of (i) the immune response; (ii) production of factors that regulate survival, differentiation, or axon growth; and (iii) cell replacement are possible mechanisms. **(B)** In a first phase, cell death and inflammation (*ca.* 2 weeks) predominate, followed by a phase of tissue replacement (days to weeks) and tissue remodeling (months to years). This limits the time frame for efficient stem cell treatment, as shown for different aspects involved in regeneration. **(B)** Modified from Burda and Sofroniew (2014).

Table 3 | Aspects potentially influenced by transplanted stem cells.

Parameter	Stem cell-released factor
Immune system modulation	Chondroitin sulfate proteoglycans (CSPGs) Prostaglandin E Tenascin-C
Neuroprotection	Glial cell line-derived neurotrophic factor (GDNF) Nerve growth factor (NGF)
Axon growth, differentiation, synaptic plasticity	Matrix metalloproteinases (MMPs) Neurocan Phosphacan; with attached DSD-1 epitope Tenascin-C Tissue inhibitors of metalloproteinase (TIMPs)

References are given in the text.

potential effects on regeneration are listed in **Table 3**. With regard to the phases of regeneration, where a first phase of cell death and inflammation (*ca.* 2 weeks) is followed by a phase of tissue replacement (days to weeks) and finally by tissue remodeling (months to years), at each time point different mechanisms have priority. This limits the time frame for efficient stem cell treatment (**Figure 3B**). Depending on the desired effect, stem cells of different origin and of different potential are the first choice.

The following aspects may account for differences of cultured and transplanted cells in comparison to the endogenous stem/progenitor pool: (i) the cell number of stem/progenitor cells may be higher when additional cells are transplanted; (ii) the potential of the cultured cells may differ from their endogenous counterparts due to the treatment (stress, addition of factors to the medium, etc.); (iii) not only the potential to differentiate may be changed during culture, but also the factors these cells secrete could differ depending on the pretreatment, the origin, or the differentiation state; (iv) transplantation of stem cells could modulate the immune system and therefore act indirectly on the CNS; and (v) stem cells can also be used as vehicle: for example, genetically modified stem cells stably deliver neurotrophic or other factors to damaged tissue, e.g., CNTF (Jung et al., 2013).

INTERACTION OF STEM CELLS AND THE IMMUNE SYSTEM

Immune cells can have both, positive and detrimental effects on regeneration, but also on the fate of endogenous and transplanted stem cells (reviewed by Martino et al., 2011; Kokaia et al., 2012). On the other hand, stem cells can modulate the immune response. For example, it has been shown that neurosphere-derived multipotent progenitors have a neuroprotective effect by immune modulation (Pluchino et al., 2005). Systemically transplanted NSCs improved recovery in a stroke model and reduced the expression of inflammation-associated genes (Bacigaluppi et al., 2009). Stem cells can also suppress T lymphocytes via increased nitric oxide and Prostaglandin E levels (Wang et al., 2009a).

In addition to the typical immune modulators mentioned above, molecules of the ECM can also influence the immune system: CSPGs do not only regulate axon growth, they are also able to modulate the immune response by binding to cytokines and Cluster of differentiation 44 (CD44; reviewed by Haylock-Jacobs et al., 2011). TN-C, a glycoprotein that is secreted by neural stem/progenitor cells *in vitro* (von Holst et al., 2007), can influence the immune system via Toll-like receptor (TLR) 4 (Midwood et al., 2009). TN-C and the immune system influence each other reciprocally: TN-C modulates the immune response, but is itself regulated by cytokines during inflammation. A detailed description is given in the review by Jakovcevski et al. (2013). Not only neural stem/progenitor cells can be utilized to improve recovery. For example, bone marrow stromal cells/mesenchymal stem cells (MSCs) reduce microglial activation (Yan et al., 2013). In addition to secreted factors, in the last years the role of exosomes in intercellular communication is being discussed (Ludwig and Giebel, 2012). These small vesicles loaded with lipids, proteins, and RNAs are released by MSCs and other cell types and might represent a mechanism by which transplanted cells can influence the immune system (Kordelas et al., 2014).

PRODUCTION OF NEUROTROPHIC FACTORS AND REGULATION OF PLASTICITY

As described, stem cells can support neuronal survival by modulating the immune response, but they can also have a direct neuroprotective effect. Stem cells produce neurotrophic factors like GDNF and NGF (Lladó et al., 2004). Furthermore, they express a number of ECM molecules that are present in the developing nervous system. Among them are CSPGs like Neurocan, Phosphacan carrying the DSD-1 epitope (Ida et al., 2006; von Holst et al., 2006) and, as already mentioned, the glycoprotein TN-C. Some of these factors are well-known for their impact on cell differentiation, migration, and axon growth. If these factors are able to influence regeneration after transplantation will mostly depend on the number of surviving cells. A positive effect of human neural progenitor cells on axonal transport and plasticity after stroke was in part attributed to VEGF, TSP and Slit expression (Andres et al., 2011). Transplanted cells that express MMPs or they counterparts, the Tissue inhibitors of metalloproteinases (TIMPs), can control the ECM composition. Also non-neural cells can affect plasticity. So expression of MMPs and TIMPs was shown in MSCs, with differences in the expression profile depending on the origin of the cells (Lozito et al., 2014). MSCs induce SHH expression in host astrocytes that increases plasticity in conjunction with tissue-type plasminogen activator (tPA; Samson and Medcalf, 2006; Ding et al., 2013).

Plasticity can be induced not only on the molecular level. Neural precursors transplanted into the visual cortex differentiated into inhibitory neurons and formed synapses to host cortical neurons, thereby promoting plasticity after the critical period (Southwell et al., 2010).

NEURAL DIFFERENTIATION AND CELL REPLACEMENT BY TRANSPLANTED STEM CELLS

Embryonic stem (ES) cells provide a source for the different cell types, because as pluripotent cells they can give rise to all cell types of the body, including neurons, astrocytes, and oligodendrocytes

(Fraichard et al., 1995). To avoid the ethically problematic use of the embryo-derived ES cells, alternative sources for pluripotent cells were investigated. A promising alternative was described in 2006, when fibroblasts were reprogrammed to induced pluripotent stem cells (iPSCs; Takahashi and Yamanaka, 2006). The "Yamanaka cocktail" contains the four factors Oct3/4, Sox2, c-Myc, and Klf4. Subsequently, the number of factors could be reduced to two when adult neural stem cells were reprogrammed to iPSCs (Kim et al., 2008). Meanwhile, direct conversion of fibroblasts to induced neural stem cells (iNSCs) without a pluripotent state has been described (Han et al., 2012; Thier et al., 2012), reducing the risk of tumor induction by undifferentiated cells.

Cell fate decisions can be regulated, so astrocytic differentiation of transplanted NSCs is reduced when the CNTF is neutralized (Ishii et al., 2006). Astrocytes can be driven to a glutamatergic, NG2 glia to a glutamatergic and GABAergic neuronal cell fate by retroviral expression of the transcription factor NeuroD1 (Guo et al., 2014). Procedures for the directed differentiation into distinct neuronal subtypes exist, where cells are exposed to defined soluble and surface-bound factors. For example, glutamatergic (Zeng et al., 2010) and GABAergic (Maroof et al., 2013) neurons can be derived from ESCs or iPSCs. Also regional identities like retinal progenitors (Lamba et al., 2006) and subsequently photoreceptors can be produced (Lamba et al., 2010). But not only neurons, also oligodendrocytes as the myelinating cells of the CNS are important for regeneration and protocols are described that promote this cell fate (Neman and de Vellis, 2012). Functional integration of such cells was already shown, also for human cells (Tornero et al., 2013). A combined approach of stem cell-based treatment and manipulation of the ECM is the injection of neural stem cells with Chondroitinase ABC into the lesioned spinal cord (Ikegami et al., 2005). Axonal regrowth was significantly increased by the combined application.

SUMMARY/OUTLOOK

As shown in this review, the CNS reacts to damage by upregulation of numerous extracellular signaling molecules that in part resemble the neurogenic stem cell niche. In line with this, endogenous stem/progenitor-like cells can be observed in many lesion models. The ECM contains important regulators of cell survival, differentiation, migration, or neurite outgrowth and is able to modulate signaling of associated molecules, for example of growth factors. These signals and the intrinsic properties of the cells present in the lesioned CNS are responsible for the outcome after regeneration. Stem cell transplantation now aims to influence this system or to add cells that replace lost tissue to a certain degree. Some approaches exploit neuroprotective effects by the stem cells, whereas others depend on cell replacement after differentiation and functional integration of the cells. The latter requires a carefully orchestrated sequence of complex actions and therefore seems to be harder to achieve. Stem cell transplantation experiments in animals will help to learn more about the molecular mechanisms that influence regeneration, as they allow to manipulate specific aspects in this complex interplay.

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REFERENCES

Adams, J. C., and Lawler, J. (2011). The thrombospondins. *Cold Spring Harb. Perspect. Biol.* 3:a009712. doi: 10.1101/cshperspect.a009712

Amankulor, N. M., Hambardzumyan, D., Pyonteck, S. M., Becher, O. J., Joyce, J. A., and Holland, E. C. (2009). Sonic hedgehog pathway activation is induced by acute brain injury and regulated by injury-related inflammation. *J. Neurosci.* 29, 10299–10308. doi: 10.1523/jneurosci.2500-09.2009

Anderson, M. A., Ao, Y., and Sofroniew, M. V. (2014). Heterogeneity of reactive astrocytes. *Neurosci. Lett.* 565C, 23–29. doi: 10.1016/j.neulet.2013.12.030

Andres, R. H., Horie, N., Slikker, W., Keren-Gill, H., Zhan, K., Sun, G., et al. (2011). Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain* 134, 1777–1789. doi: 10.1093/brain/awr094

Anthis, N. J., Wegener, K. L., Ye, F., Kim, C., Goult, B. T., Lowe, E. D., et al. (2009). The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J.* 28, 3623–3632. doi: 10.1038/emboj.2009.287

Apostolova, I., Irintchev, A., and Schachner, M. (2006). Tenascin-R restricts posttraumatic remodeling of motoneuron innervation and functional recovery after spinal cord injury in adult mice. *J. Neurosci.* 26, 7849–7859. doi: 10.1523/jneurosci.1526-06.2006

Arai, K., Jin, G., Navaratna, D., and Lo, E. H. (2009). Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. *FEBS J.* 276, 4644–4652. doi: 10.1111/j.1742-4658.2009.07176.x

Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc. Natl. Acad. Sci. U S A* 100, 13298–13302. doi: 10.1073/pnas.2336149100

Armstrong, L. C., and Bornstein, P. (2003). Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biol.* 22, 63–71. doi: 10.1016/s0945-053x(03)00005-2

Aruga, J., Tohmonda, T., Homma, S., and Mikoshiba, K. (2002). Zic1 promotes the expansion of dorsal neural progenitors in spinal cord by inhibiting neuronal differentiation. *Dev. Biol.* 244, 329–341. doi: 10.1006/dbio.2002.0598

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Asher, R. A., Morgenstern, D. A., Fidler, P. S., Adcock, K. H., Oohira, A., Braistead, J. E., et al. (2000). Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. *J. Neurosci.* 20, 2427–2438.

Asher, R. A., Morgenstern, D. A., Shearer, M. C., Adcock, K. H., Pesheva, P., and Fawcett, J. W. (2002). Versican is upregulated in CNS injury and is a product of oligodendrocyte lineage cells. *J. Neurosci.* 22, 2225–2236.

Bacigaluppi, M., Pluchino, S., Peruzzotti-Jametti, L., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251. doi: 10.1093/brain/awp174

Baeten, K. M., and Akassoglou, K. (2011). Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Dev. Neurobiol.* 71, 1018–1039. doi: 10.1002/dneu.20954

Barker, R. A., Dunnett, S. B., Faissner, A., and Fawcett, J. W. (1996). The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp. Neurol.* 141, 79–93. doi: 10.1006/exnr.1996.0141

Barkho, B. Z., Munoz, A. E., Li, X., Li, L., Cunningham, L. A., and Zhao, X. (2008). Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. *Stem Cells* 26, 3139–3149. doi: 10.1634/stemcells.2008-0519

Barnabé-Heider, F., Göritz, C., Sabelström, H., Takebayashi, H., Pfrieger, F. W., Meletis, K., et al. (2010). Origin of new glial cells in intact and injured adult spinal cord. *Cell Stem Cell* 7, 470–482. doi: 10.1016/j.stem.2010.07.014

Barnea, G., Grumet, M., Milev, P., Silvennoinen, O., Levy, J. B., Sap, J., et al. (1994). Receptor tyrosine phosphatase beta is expressed in the form of proteoglycan and binds to the extracellular matrix protein tenascin. *J. Biol. Chem.* 269, 14349–14352.

Becker, T., Anliker, B., Becker, C. G., Taylor, J., Schachner, M., Meyer, R. L., et al. (2000). Tenascin-R inhibits regrowth of optic fibers in vitro and persists in the optic nerve of mice after injury. *Glia* 29, 330–346. doi: 10.1002/(sici)1098-1136(20000215)29:4<330::aid-glia4>3.0.co;2-l

Beggah, A. T., Dours-Zimmermann, M. T., Barras, F. M., Brosius, A., Zimmermann, D. R., and Zurn, A. D. (2005). Lesion-induced differential expression and cell association of Neurocan, Brevican, Versican V1 and V2 in the mouse dorsal root entry zone. *Neuroscience* 133, 749–762. doi: 10.1016/j.neuroscience.2005.03.005

Belkin, A. M., and Stepp, M. A. (2000). Integrins as receptors for laminins. *Microsc. Res. Tech.* 51, 280–301. doi: 10.1002/1097-0029(20001101)51:3<280::aid-jemt7>3.0.co;2-o

Benner, E. J., Luciano, D., Jo, R., Abdi, K., Paez-Gonzalez, P., Sheng, H., et al. (2013). Protective astrogenesis from the SVZ niche after injury is controlled by notch modulator Thbs4. *Nature* 497, 369–373. doi: 10.1038/nature12069

Besser, M., Horvat-Bröcker, A., Eysel, U. T., and Faissner, A. (2009). Differential expression of receptor protein tyrosine phosphatases accompanies the reorganisation of the retina upon laser lesion. *Exp. Brain Res.* 198, 37–47. doi: 10.1007/s00221-009-1932-0

Biber, K., Neumann, H., Inoue, K., and Boddeke, H. W. (2007). Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci.* 30, 596–602. doi: 10.1016/j.tins.2007.08.007

Bignami, A., Raju, T., and Dahl, D. (1982). Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. In vivo and in vitro immunofluorescence study of the rat embryo with vimentin and neurofilament antisera. *Dev. Biol.* 91, 286–295. doi: 10.1016/0012-1606(82)90035-5

Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., et al. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416, 636–640. doi: 10.1038/416636a

Brenner, M. (2014). Role of GFAP in CNS injuries. *Neurosci. Lett.* 565, 7–13. doi: 10.1016/j.neulet.2014.01.055

Brizzi, M. F., Tarone, G., and Defilippi, P. (2012). Extracellular matrix, integrins and growth factors as tailors of the stem cell niche. *Curr. Opin. Cell Biol.* 24, 645–651. doi: 10.1016/j.ceb.2012.07.001

Brosius Lutz, A., and Barres, B. A. (2014). Contrasting the glial response to axon injury in the central and peripheral nervous systems. *Dev. Cell* 28, 7–17. doi: 10.1016/j.devcel.2013.12.002

Brown, J. M., Xia, J., Zhuang, B., Cho, K. S., Rogers, C. J., Gama, C. I., et al. (2012). A sulfated carbohydrate epitope inhibits axon regeneration after injury. *Proc. Natl. Acad. Sci. U S A* 109, 4768–4773. doi: 10.1073/pnas.1121318109

Buddensiek, J., Dressel, A., Kowalski, M., Storch, A., and Sabolek, M. (2009). Adult cerebrospinal fluid inhibits neurogenesis but facilitates gliogenesis from fetal rat neural stem cells. *J. Neurosci. Res.* 87, 3054–3066. doi: 10.1002/jnr.22150

Budinich, C. S., Chen, H., Lowe, D., Rosenberger, J. G., Bernstock, J. D., and McCabe, J. T. (2012). Mouse brain PSA-NCAM levels are altered by graded-controlled cortical impact injury. *Neural Plast.* 2012:378307. doi: 10.1155/2012/378307

Buffo, A., Rite, I., Tripathi, P., Lepier, A., Colak, D., Horn, A. P., et al. (2008). Origin and progeny of reactive gliosis: a source of multipotent cells in the injured brain. *Proc. Natl. Acad. Sci. U S A* 105, 3581–3586. doi: 10.1073/pnas.0709002105

Buffo, A., Rolando, C., and Ceruti, S. (2010). Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. *Biochem. Pharmacol.* 79, 77–89. doi: 10.1016/j.bcp.2009.09.014

Bundesen, L. Q., Scheel, T. A., Bregman, B. S., and Kromer, L. F. (2003). Ephrin-B2 and EphB2 regulation of astrocyte-meningeal fibroblast interactions in response to spinal cord lesions in adult rats. *J. Neurosci.* 23, 7789–7800.

Burda, J. E., and Sofroniew, M. V. (2014). Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron* 81, 229–248. doi: 10.1016/j.neuron.2013.12.034

Burnside, E. R., and Bradbury, E. J. (2014). Manipulating the extracellular matrix and its role in brain and spinal cord plasticity and repair. *Neuropathol. Appl. Neurobiol.* 40, 26–59. doi: 10.1111/nan.12114

Calof, A. L., Campanero, M. R., O'Rear, J. J., Yurchenco, P. D., and Lander, A. D. (1994). Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin. *Neuron* 13, 117–130. doi: 10.1016/0896-6273(94)90463-4

Campos, L. S. (2005). Beta1 integrins and neural stem cells: making sense of the extracellular environment. *Bioessays* 27, 698–707. doi: 10.1002/bies.20256

Candelario-Jalil, E., Yang, Y., and Rosenberg, G. A. (2009). Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia. *Neuroscience* 158, 983–994. doi: 10.1016/j.neuroscience.2008.06.025

Carbonell, W. S., Murase, S., Horwitz, A. F., and Mandell, J. W. (2005). Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *J. Neurosci.* 25, 7040–7047. doi: 10.1523/jneurosci.5171-04.2005

Carmeliet, P. (2003). Blood vessels and nerves: common signals, pathways and diseases. *Nat. Rev. Genet.* 4, 710–720. doi: 10.1038/nrg1158

Carulli, D., Laabs, T., Geller, H. M., and Fawcett, J. W. (2005). Chondroitin sulfate proteoglycans in neural development and regeneration. *Curr. Opin. Neurobiol.* 15, 116–120. doi: 10.1016/j.conb.2005.03.018

Castellani, V., Chédotal, A., Schachner, M., Faivre-Sarrailh, C., and Rougon, G. (2000). Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* 27, 237–249. doi: 10.1016/s0896-6273(00)00033-7

Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., and Spillmann, A. A. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434–439. doi: 10.1038/35000219

Chen, J., Joon Lee, H., Jakovcevski, I., Shah, R., Bhagat, N., Loers, G., et al. (2010). The extracellular matrix glycoprotein tenascin-C is beneficial for spinal cord regeneration. *Mol. Ther.* 18, 1769–1777. doi: 10.1038/mt.2010.133

Chen, X. R., Liao, S. J., Ye, L. X., Gong, Q., Ding, Q., Zeng, J. S., et al. (2014). Neuroprotective effect of chondroitinase ABC on primary and secondary brain injury after stroke in hypertensive rats. *Brain Res.* 1543, 324–333. doi: 10.1016/j.brainres.2013.12.002

Chiquet-Ehrismann, R., and Chiquet, M. (2003). Tenascins: regulation and putative functions during pathological stress. *J. Pathol.* 200, 488–499. doi: 10.1002/path.1415

Chiquet-Ehrismann, R., Orend, G., Chiquet, M., Tucker, R. P., and Midwood, K. S. (2014). Tenascins in stem cell niches. *Matrix Biol.* doi: 10.1016/j.matbio.2014.01.007. [Epub ahead of print].

Chopp, M., Li, Y., and Zhang, Z. G. (2009). Mechanisms underlying improved recovery of neurological function after stroke in the rodent after treatment with neurorestorative cell-based therapies. *Stroke* 40, S143–S145. doi: 10.1161/strokeaha.108.533141

Chow, J. P., Fujikawa, A., Shimizu, H., Suzuki, R., and Noda, M. (2008). Metalloproteinase- and gamma-secretase-mediated cleavage of protein-tyrosine phosphatase receptor type Z. *J. Biol. Chem.* 283, 30879–30889. doi: 10.1074/jbc.m802976200

Christopherson, K. S., Ullian, E. M., Stokes, C. C., Mullowney, C. E., Hell, J. W., Agah, A., et al. (2005). Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120, 421–433. doi: 10.1016/j.cell.2004.12.020

Clark, R. A. (2008). Synergistic signaling from extracellular matrix-growth factor complexes. *J. Invest. Dermatol.* 128, 1354–1355. doi: 10.1038/jid.2008.75

Coles, C. H., Shen, Y., Tenney, A. P., Siebold, C., Sutton, G. C., Lu, W., et al. (2011). Proteoglycan-specific molecular switch for RPTPsigma clustering and neuronal extension. *Science* 332, 484–488. doi: 10.1126/science.1200840

Condic, M. L., and Lemons, M. L. (2002). Extracellular matrix in spinal cord regeneration: getting beyond attraction and inhibition. *Neuroreport* 13, A37–A48. doi: 10.1097/00001756-200203040-00002

Craveiro, L. M., Weinmann, O., Roschitzki, B., Gonzenbach, R. R., Zörner, B., Montani, L., et al. (2013). Infusion of anti-Nogo-A antibodies in adult rats increases growth and synapse related proteins in the absence of behavioral alterations. *Exp. Neurol.* 250, 52–68. doi: 10.1016/j.expneurol.2013.09.015

Cregg, J. M., DePaul, M. A., Filous, A. R., Lang, B. T., Tran, A., and Silver, J. (2014). Functional regeneration beyond the glial scar. *Exp. Neurol.* 253, 197–207. doi: 10.1016/j.expneurol.2013.12.024

Cui, Y. F., Xu, J. C., Hargus, G., Jakovcevski, I., Schachner, M., and Bernreuther, C. (2011). Embryonic stem cell-derived L1 overexpressing neural aggregates enhance recovery after spinal cord injury in mice. *PLoS One* 6:e17126. doi: 10.1371/journal.pone.0017126

Czopka, T., von Holst, A., ffrench-Constant, C., and Faissner, A. (2010). Regulatory mechanisms that mediate tenascin C-dependent inhibition of oligodendrocyte precursor differentiation. *J. Neurosci.* 30, 12310–12322. doi: 10.1523/jneurosci.4957-09.2010

Daar, I. O. (2012). Non-SH2/PDZ reverse signaling by ephrins. *Semin. Cell Dev. Biol.* 23, 65–74. doi: 10.1016/j.semcdb.2011.10.012

Das, M., Mohapatra, S., and Mohapatra, S. S. (2012). New perspectives on central and peripheral immune responses to acute traumatic brain injury. *J. Neuroinflammation* 9:236. doi: 10.1186/1742-2094-9-236

Davies, J. E., Tang, X., Denning, J. W., Archibald, S. J., and Davies, S. J. (2004). Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. *Eur. J. Neurosci.* 19, 1226–1242. doi: 10.1111/j.1460-9568.2004.03184.x

Deckner, M., Lindholm, T., Cullheim, S., and Risling, M. (2000). Differential expression of tenascin-C, tenascin-R, tenascin/J1 and tenascin-X in spinal cord scar tissue and in the olfactory system. *Exp. Neurol.* 166, 350–362. doi: 10.1006/exnr.2000.7543

Deepa, S. S., Umehara, Y., Higashiyama, S., Itoh, N., and Sugahara, K. (2002). Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. *J. Biol. Chem.* 277, 43707–43716. doi: 10.1074/jbc.m207105200

De Simone, R., Ambrosini, E., Carnevale, D., Ajmone-Cat, M. A., and Minghetti, L. (2007). NGF promotes microglial migration through the activation of its high affinity receptor: modulation by TGF-beta. *J. Neuroimmunol.* 190, 53–60. doi: 10.1016/j.jneuroim.2007.07.020

Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., and Andjelkovic, A. V. (2006). Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. *J. Cereb. Blood Flow Metab.* 26, 797–810. doi: 10.1038/sj.jcbfm.9600229

Ding, X., Li, Y., Liu, Z., Zhang, J., Cui, Y., Chen, X., et al. (2013). The sonic hedgehog pathway mediates brain plasticity and subsequent functional recovery after bone marrow stromal cell treatment of stroke in mice. *J. Cereb. Blood Flow Metab.* 33, 1015–1024. doi: 10.1038/jcbfm.2013.50

Dityatev, A., and Schachner, M. (2003). Extracellular matrix molecules and synaptic plasticity. *Nat. Rev. Neurosci.* 4, 456–468. doi: 10.1038/nrn1115

Dobbertin, A., Czvitkovich, S., Theocharidis, U., Garwood, J., Andrews, M. R., Properzi, F., et al. (2010). Analysis of combinatorial variability reveals selective accumulation of the fibronectin type III domains B and D of tenascin-C in injured brain. *Exp. Neurol.* 225, 60–73. doi: 10.1016/j.expneurol.2010.04.019

Dobbertin, A., Rhodes, K. E., Garwood, J., Properzi, F., Heck, N., Rogers, J. H., et al. (2003). Regulation of RPTPbeta/phosphacan expression and glycosaminoglycan epitopes in injured brain and cytokine-treated glia. *Mol. Cell. Neurosci.* 24, 951–971. doi: 10.1016/s1044-7431(03)00257-4

Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716. doi: 10.1016/s0092-8674(00)80783-7

Doetsch, F., García-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.* 17, 5046–5061.

Domogatskaya, A., Rodin, S., and Tryggvason, K. (2012). Functional diversity of laminins. *Annu. Rev. Cell Dev. Biol.* 28, 523–553. doi: 10.1146/annurev-cellbio-101011-155750

Donnelly, D. J., and Popovich, P. G. (2008). Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp. Neurol.* 209, 378–388. doi: 10.1016/j.expneurol.2007.06.009

Dou, C. L., and Levine, J. M. (1994). Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. *J. Neurosci.* 14, 7616–7628.

Dugas, J. C., Mandemakers, W., Rogers, M., Ibrahim, A., Daneman, R., and Barres, B. A. (2008). A novel purification method for CNS projection neurons leads to the identification of brain vascular cells as a source of trophic support for corticospinal motor neurons. *J. Neurosci.* 28, 8294–8305. doi: 10.1523/JNEUROSCI.2010-08.2008

Eddleston, M., and Mucke, L. (1993). Molecular profile of reactive astrocytes—implications for their role in neurologic disease. *Neuroscience* 54, 15–36. doi: 10.1016/0306-4522(93)90380-x

Elkabes, S., DiCicco-Bloom, E. M., and Black, I. B. (1996). Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. *J. Neurosci.* 16, 2508–2521.

Ellison, J. A., Barone, F. C., and Feuerstein, G. Z. (1999). Matrix remodeling after stroke. De novo expression of matrix proteins and integrin receptors. *Ann. N Y Acad. Sci.* 890, 204–222. doi: 10.1111/j.1749-6632.1999.tb07996.x

Emery, D. L., Royo, N. C., Fischer, I., Saatman, K. E., and McIntosh, T. K. (2003). Plasticity following injury to the adult central nervous system: is recapitulation of a developmental state worth promoting? *J. Neurotrauma* 20, 1271–1292. doi: 10.1089/089771503322686085

Faissner, A. (1997). The tenascin gene family in axon growth and guidance. *Cell Tissue Res.* 290, 331–341. doi: 10.1007/978-3-642-60905-3_19

Faissner, A., Clement, A., Lochter, A., Streit, A., Mandl, C., and Schachner, M. (1994). Isolation of a neural chondroitin sulfate proteoglycan with neurite outgrowth promoting properties. *J. Cell Biol.* 126, 783–799. doi: 10.1083/jcb.126.3.783

Faissner, A., Pyka, M., Geissler, M., Sobik, T., Frischknecht, R., Gundelfinger, E. D., et al. (2010). Contributions of astrocytes to synapse formation and maturation—Potential functions of the perisynaptic extracellular matrix. *Brain Res. Rev.* 63, 26–38. doi: 10.1016/j.brainresrev.2010.01.001

Falo, M. C., Reeves, T. M., and Phillips, L. L. (2008). Agrin expression during synaptogenesis induced by traumatic brain injury. *J. Neurotrauma* 25, 769–783. doi: 10.1089/neu.2008.0511

Filla, M. S., Dam, P., and Rapraeger, A. C. (1998). The cell surface proteoglycan syndecan-1 mediates fibroblast growth factor-2 binding and activity. *J. Cell. Physiol.* 174, 310–321. doi: 10.1002/(sici)1097-4652(199803)174:3<310::aid-jcp5>3.0.co;2-r

Filmus, J., and Capurro, M. (2014). The role of glypicans in hedgehog signaling. *Matrix Biol.* 35, 248–252. doi: 10.1016/j.matbio.2013.12.007

Fournier, A. E., Takizawa, B. T., and Strittmatter, S. M. (2003). Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci.* 23, 1416–1423.

Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995). In vitro differentiation of embryonic stem cells into glial cells and functional neurons. *J. Cell Sci.* 108(Pt. 10), 3181–3188.

Friedlander, D. R., Milev, P., Karthikeyan, L., Margolis, R. K., Margolis, R. U., and Grumet, M. (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM and inhibits neuronal adhesion and neurite outgrowth. *J. Cell Biol.* 125, 669–680. doi: 10.1083/jcb.125.3.669

Fuccillo, M., Joyner, A. L., and Fishell, G. (2006). Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. *Nat. Rev. Neurosci.* 7, 772–783. doi: 10.1038/nrn1990

Fuller, M. L., DeChant, A. K., Rothstein, B., Caprariello, A., Wang, R., Hall, A. K., et al. (2007). Bone morphogenetic proteins promote gliosis in demyelinating spinal cord lesions. *Ann. Neurol.* 62, 288–300. doi: 10.1002/ana.21179

Garcion, E., Halilagic, A., Faissner, A., and ffrench-Constant, C. (2004). Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development* 131, 3423–3432. doi: 10.1242/dev.01202

Garwood, J., Heck, N., Reichardt, F., and Faissner, A. (2003). Phosphacan short isoform, a novel non-proteoglycan variant of phosphacan/receptor protein tyrosine phosphatase-beta, interacts with neuronal receptors and promotes neurite outgrowth. *J. Biol. Chem.* 278, 24164–24173. doi: 10.1074/jbc.m211721200

Gates, M. A., Thomas, L. B., Howard, E. M., Laywell, E. D., Sajin, B., Faissner, A., et al. (1995). Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. *J. Comp. Neurol.* 361, 249–266. doi: 10.1002/cne.903610205

Gattazzo, F., Urciuolo, A., and Bonaldo, P. (2014). Extracellular matrix: a dynamic microenvironment for stem cell niche. *Biochim. Biophys. Acta* 1840, 2506–2519. doi: 10.1016/j.bbagen.2014.01.010

Geissler, M., Gottschling, C., Aguado, A., Rauch, U., Wetzel, C. H., Hatt, H., et al. (2013). Primary hippocampal neurons, which lack four crucial extracellular matrix molecules, display abnormalities of synaptic structure and function and severe deficits in perineuronal net formation. *J. Neurosci.* 33, 7742–7755. doi: 10.1523/jneurosci.3275-12.2013

Givogri, M. I., de Planell, M., Galbiati, F., Superchi, D., Gritti, A., Vescovi, A., et al. (2006). Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Dev. Neurosci.* 28, 81–91. doi: 10.1159/000090755

Gladson, C. L. (1999). The extracellular matrix of gliomas: modulation of cell function. *J. Neuropathol. Exp. Neurol.* 58, 1029–1040. doi: 10.1097/00005072-199910000-00001

Goldshmit, Y., Galea, M. P., Wise, G., Bartlett, P. F., and Turnley, A. M. (2004). Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. *J. Neurosci.* 24, 10064–10073. doi: 10.1523/JNEUROSCI.2981-04.2004

Goldshmit, Y., McLenachan, S., and Turnley, A. (2006). Roles of Eph receptors and ephrins in the normal and damaged adult CNS. *Brain Res. Rev.* 52, 327–345. doi: 10.1016/j.brainresrev.2006.04.006

Gordon, P. B., Choi, H. U., Conn, G., Ahmed, A., Ehrmann, B., Rosenberg, L., et al. (1989). Extracellular matrix heparan sulfate proteoglycans modulate the mitogenic capacity of acidic fibroblast growth factor. *J. Cell. Physiol.* 140, 584–592. doi: 10.1002/jcp.1041400325

Göritz, C., Dias, D. O., Tomilin, N., Barbacid, M., Shupliakov, O., and Frisén, J. (2011). A pericyte origin of spinal cord scar tissue. *Science* 333, 238–242. doi: 10.1126/science.1203165

Gottschall, P. E., and Yu, X. (1995). Cytokines regulate gelatinase A and B (matrix metalloproteinase 2 and 9) activity in cultured rat astrocytes. *J. Neurochem.* 64, 1513–1520. doi: 10.1046/j.1471-4159.1995.64041513.x

Gris, P., Tighe, A., Levin, D., Sharma, R., and Brown, A. (2007). Transcriptional regulation of scar gene expression in primary astrocytes. *Glia* 55, 1145–1155. doi: 10.1002/glia.20537

Guan, J. L. (1997). Focal adhesion kinase in integrin signaling. *Matrix Biol.* 16, 195–200. doi: 10.1016/S0945-053X(97)90008-1

Guo, W., and Giancotti, F. G. (2004). Integrin signalling during tumour progression. *Nat. Rev. Mol. Cell Biol.* 5, 816–826. doi: 10.1038/nrm1490

Guo, Z., Zhang, L., Wu, Z., Chen, Y., Wang, F., and Chen, G. (2014). In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. *Cell Stem Cell* 14, 188–202. doi: 10.1016/j.stem.2013.12.001

Haas, C. A., Rauch, U., Thon, N., Merten, T., and Deller, T. (1999). Entorhinal cortex lesion in adult rats induces the expression of the neuronal chondroitin sulfate proteoglycan neurocan in reactive astrocytes. *J. Neurosci.* 19, 9953–9963.

Hagino, S., Iseki, K., Mori, T., Zhang, Y., Hikake, T., Yokoya, S., et al. (2003). Slit and glypican-1 mRNAs are coexpressed in the reactive astrocytes of the injured adult brain. *Glia* 42, 130–138. doi: 10.1002/glia.10207

Hampton, D. W., Asher, R. A., Kondo, T., Steeves, J. D., Ramer, M. S., and Fawcett, J. W. (2007). A potential role for bone morphogenetic protein signalling in glial cell fate determination following adult central nervous system injury in vivo. *Eur. J. Neurosci.* 26, 3024–3035. doi: 10.1111/j.1460-9568.2007.05940.x

Han, S. S., Liu, Y., Tyler-Polsz, C., Rao, M. S., and Fischer, I. (2004). Transplantation of glial-restricted precursor cells into the adult spinal cord: survival, glial-specific differentiation and preferential migration in white matter. *Glia* 45, 1–16. doi: 10.1002/glia.10282

Han, D. W., Tapia, N., Hermann, A., Hemmer, K., Höing, S., Araúzo-Bravo, M. J., et al. (2012). Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* 10, 465–472. doi: 10.1016/j.stem.2012.02.021

Harroch, S., Furtado, G. C., Brueck, W., Rosenbluth, J., Lafaille, J., Chao, M., et al. (2002). A critical role for the protein tyrosine phosphatase receptor type Z in functional recovery from demyelinating lesions. *Nat. Genet.* 32, 411–414. doi: 10.1038/ng1004

Hausmann, R., and Betz, P. (2001). Course of glial immunoreactivity for vimentin, tenascin and alpha1-antichymotrypsin after traumatic injury to human brain. *Int. J. Legal Med.* 114, 338–342. doi: 10.1007/s004140000199

Haylock-Jacobs, S., Keough, M. B., Lau, L., and Yong, V. W. (2011). Chondroitin sulphate proteoglycans: extracellular matrix proteins that regulate immunity of the central nervous system. *Autoimmun. Rev.* 10, 766–772. doi: 10.1016/j.autrev.2011.05.019

Heck, N., Garwood, J., Dobbertin, A., Calco, V., Sirko, S., Mittmann, T., et al. (2007). Evidence for distinct leptomeningeal cell-dependent paracrine and EGF-linked autocrine regulatory pathways for suppression of fibrillar collagens in astrocytes. *Mol. Cell. Neurosci.* 36, 71–85. doi: 10.1016/j.mcn.2007.06.002

Hetman, M., and Xia, Z. (2000). Signaling pathways mediating anti-apoptotic action of neurotrophins. *Acta Neurobiol. Exp. (Wars)* 60, 531–545.

Hill, W. D., Hess, D. C., Martin-Studdard, A., Carothers, J. J., Zheng, J., Hale, D., et al. (2004). SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke: association with bone marrow cell homing to injury. *J. Neuropathol. Exp. Neurol.* 63, 84–96.

Hirano, S., Yonezawa, T., Hasegawa, H., Hattori, S., Greenhill, N. S., Davis, P. F., et al. (2004). Astrocytes express type VIII collagen during the repair process of brain cold injury. *Biochem. Biophys. Res. Commun.* 317, 437–443. doi: 10.1016/j.bbrc.2004.03.049

Hsu, J. Y., Bourguignon, L. Y., Adams, C. M., Peyrollier, K., Zhang, H., Fandel, T., et al. (2008). Matrix metalloproteinase-9 facilitates glial scar formation in the injured spinal cord. *J. Neurosci.* 28, 13467–13477. doi: 10.1523/jneurosci.2287-08.2008

Ida, M., Shuo, T., Hirano, K., Tokita, Y., Nakanishi, K., Matsui, F., et al. (2006). Identification and functions of chondroitin sulfate in the milieu of neural stem cells. *J. Biol. Chem.* 281, 5982–5991. doi: 10.1074/jbc.m507130200

Ikegami, T., Nakamura, M., Yamane, J., Katoh, H., Okada, S., Iwanami, A., et al. (2005). Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth-associated protein-43-positive fibers after rat spinal cord injury. *Eur. J. Neurosci.* 22, 3036–3046. doi: 10.1111/j.1460-9568.2005.04492.x

Imitola, J., Raddassi, K., Park, K. I., Mueller, F. J., Nieto, M., Teng, Y. D., et al. (2004). Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc. Natl. Acad. Sci. U S A* 101, 18117–18122. doi: 10.1073/pnas.0408258102

Iseki, K., Hagino, S., Mori, T., Zhang, Y., Yokoya, S., Takaki, H., et al. (2002). Increased syndecan expression by pleiotrophin and FGF receptor-expressing astrocytes in injured brain tissue. *Glia* 39, 1–9. doi: 10.1002/glia.10078

Ishibashi, S., Kuroiwa, T., Sakaguchi, M., Sun, L., Kadoya, T., Okano, H., et al. (2007). Galectin-1 regulates neurogenesis in the subventricular zone and promotes functional recovery after stroke. *Exp. Neurol.* 207, 302–313. doi: 10.1016/j.expneurol.2007.06.024

Ishii, K., Nakamura, M., Dai, H., Finn, T. P., Okano, H., Toyama, Y., et al. (2006). Neutralization of ciliary neurotrophic factor reduces astrocyte production from transplanted neural stem cells and promotes regeneration of corticospinal tract fibers in spinal cord injury. *J. Neurosci. Res.* 84, 1669–1681. doi: 10.1002/jnr.21079

Jakeman, L. B., Chen, Y., Lucin, K. M., and McTigue, D. M. (2006). Mice lacking L1 cell adhesion molecule have deficits in locomotion and exhibit enhanced corticospinal tract sprouting following mild contusion injury to the spinal cord. *Eur. J. Neurosci.* 23, 1997–2011. doi: 10.1111/j.1460-9568.2006.04721.x

Jakovcevski, I., Miljkovic, D., Schachner, M., and Andjus, P. R. (2013). Tenascins and inflammation in disorders of the nervous system. *Amino Acids* 44, 1115–1127. doi: 10.1007/s00726-012-1446-0

Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L., and Greenberg, D. A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc. Natl. Acad. Sci. U S A* 99, 11946–11950. doi: 10.1073/pnas.182296499

Joester, A., and Faissner, A. (1999). Evidence for combinatorial variability of tenascin-C isoforms and developmental regulation in the mouse central nervous system. *J. Biol. Chem.* 274, 17144–17151. doi: 10.1074/jbc.274.24.17144

Joester, A., and Faissner, A. (2001). The structure and function of tenascins in the nervous system. *Matrix Biol.* 20, 13–22. doi: 10.1016/s0945-053x(00)00136-0

Jones, F. S., and Jones, P. L. (2000a). The tenascin family of ECM glycoproteins: structure, function and regulation during embryonic development and tissue remodeling. *Dev. Dyn.* 218, 235–259. doi: 10.1002/(sici)1097-0177(200006)218:2<235::aid-dvdy2>3.0.co;2-g

Jones, P. L., and Jones, F. S. (2000b). Tenascin-C in development and disease: gene regulation and cell function. *Matrix Biol.* 19, 581–596. doi: 10.1016/s0945-053x(00)00106-2

Jones, L. L., Margolis, R. U., and Tuszynski, M. H. (2003). The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan and versican are differentially

regulated following spinal cord injury. *Exp. Neurol.* 182, 399–411. doi: 10.1016/S0014-4886(03)00087-6

Jones, L. L., Yamaguchi, Y., Stallcup, W. B., and Tuszynski, M. H. (2002). NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J. Neurosci.* 22, 2792–2803.

Jung, G., Sun, J., Petrowitz, B., Riecken, K., Kruszewski, K., Jankowiak, W., et al. (2013). Genetically modified neural stem cells for a local and sustained delivery of neuroprotective factors to the dystrophic mouse retina. *Stem Cells Transl. Med.* 2, 1001–1010. doi: 10.5966/sctm.2013-0013

Kamei, N., Kwon, S. M., Ishikawa, M., Ii, M., Nakanishi, K., Yamada, K., et al. (2012). Endothelial progenitor cells promote astrogliosis following spinal cord injury through Jagged1-dependent notch signaling. *J. Neurotrauma* 29, 1758–1769. doi: 10.1089/neu.2011.2139

Kamphuis, W., Mamber, C., Moeton, M., Kooijman, L., Sluijs, J. A., Jansen, A. H., et al. (2012). GFAP isoforms in adult mouse brain with a focus on neurogenic astrocytes and reactive astrogliosis in mouse models of Alzheimer disease. *PLoS One* 7:e42823. doi: 10.1371/journal.pone.0042823

Karetko-Sysa, M., Skangiel-Kramska, J., and Nowicka, D. (2011). Disturbance of perineuronal nets in the perilesional area after photothrombosis is not associated with neuronal death. *Exp. Neurol.* 231, 113–126. doi: 10.1016/j.expneurol.2011.05.022

Karlén, A., Karlsson, T. E., Mattsson, A., Lundströmer, K., Codeluppi, S., Pham, T. M., et al. (2009). Nogo receptor 1 regulates formation of lasting memories. *Proc. Natl. Acad. Sci. U S A* 106, 20476–20481. doi: 10.1073/pnas.0905390106

Karus, M., Denecke, B., ffrench-Constant, C., Wiese, S., and Faissner, A. (2011). The extracellular matrix molecule tenascin C modulates expression levels and territories of key patterning genes during spinal cord astrocyte specification. *Development* 138, 5321–5331. doi: 10.1242/dev.067413

Kazanis, I., and ffrench-Constant, C. (2011). Extracellular matrix and the neural stem cell niche. *Dev. Neurobiol.* 71, 1006–1017. doi: 10.1002/dneu.20970

Kettenmann, H., Hanisch, U. K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol. Rev.* 91, 461–553. doi: 10.1152/physrev.00011.2010

Kim, H., Ahn, M., Choi, S., Kim, M., Sim, K. B., Kim, J., et al. (2013). Potential role of fibronectin in microglia/macrophage activation following cryoinjury in the rat brain: an immunohistochemical study. *Brain Res.* 1502, 11–19. doi: 10.1016/j.brainres.2013.01.043

Kim, J. B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., et al. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454, 646–650. doi: 10.1038/nature07061

Kinouchi, R., Takeda, M., Yang, L., Wilhelmsson, U., Lundkvist, A., Pekny, M., et al. (2003). Robust neural integration from retinal transplants in mice deficient in GFAP and vimentin. *Nat. Neurosci.* 6, 863–868. doi: 10.1038/nn1088

Klausmeyer, A., Garwood, J., and Faissner, A. (2007). Differential expression of phosphacan/RPTPbeta isoforms in the developing mouse visual system. *J. Comp. Neurol.* 504, 659–679. doi: 10.1002/cne.21479

Kokaia, Z., Martino, G., Schwartz, M., and Lindvall, O. (2012). Cross-talk between neural stem cells and immune cells: the key to better brain repair? *Nat. Neurosci.* 15, 1078–1087. doi: 10.1038/nn.3163

Kokovay, E., Goderie, S., Wang, Y., Lotz, S., Lin, G., Sun, Y., et al. (2010). Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. *Cell Stem Cell* 7, 163–173. doi: 10.1016/j.stem.2010.05.019

Komitova, M., Serwanski, D. R., Lu, Q. R., and Nishiyama, A. (2011). NG2 cells are not a major source of reactive astrocytes after neocortical stab wound injury. *Glia* 59, 800–809. doi: 10.1002/glia.21152

Kopan, R., and Ilagan, M. X. (2009). The canonical notch signaling pathway: unfolding the activation mechanism. *Cell* 137, 216–233. doi: 10.1016/j.cell.2009.03.045

Kordelas, L., Rebmann, V., Ludwig, A. K., Radtke, S., Ruesing, J., Doeppner, T. R., et al. (2014). MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28, 970–973. doi: 10.1038/leu.2014.41

Krueger, N. X., and Saito, H. (1992). A human transmembrane protein-tyrosine-phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. U S A* 89, 7417–7421. doi: 10.1073/pnas.89.16.7417

Kwok, J. C., Dick, G., Wang, D., and Fawcett, J. W. (2011). Extracellular matrix and perineuronal nets in CNS repair. *Dev. Neurobiol.* 71, 1073–1089. doi: 10.1002/dneu.20974

Lamba, D. A., Karl, M. O., Ware, C. B., and Reh, T. A. (2006). Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc. Natl. Acad. Sci. U S A* 103, 12769–12774. doi: 10.1073/pnas.0601990103

Lamba, D. A., McUsic, A., Hirata, R. K., Wang, P. R., Russell, D., and Reh, T. A. (2010). Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. *PLoS One* 5:e8763. doi: 10.1371/journal.pone.0008763

Lang, B., Liu, H. L., Liu, R., Feng, G. D., Jiao, X. Y., and Ju, G. (2004). Astrocytes in injured adult rat spinal cord may acquire the potential of neural stem cells. *Neuroscience* 128, 775–783. doi: 10.1016/j.neuroscience.2004.06.033

Lau, E., and Margolis, R. U. (2010). Inhibitors of slit protein interactions with the heparan sulphate proteoglycan glypican-1: potential agents for the treatment of spinal cord injury. *Clin. Exp. Pharmacol. Physiol.* 37, 417–421. doi: 10.1111/j.1440-1681.2009.05318.x

Laywell, E. D., Dörries, U., Bartsch, U., Faissner, A., Schachner, M., and Steindler, D. A. (1992). Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proc. Natl. Acad. Sci. U S A* 89, 2634–2638. doi: 10.1073/pnas.89.7.2634

Lee, B., Clarke, D., Al Ahmad, A., Kahle, M., Parham, C., Auckland, L., et al. (2011). Perlecan domain V is neuroprotective and proangiogenic following ischemic stroke in rodents. *J. Clin. Invest.* 121, 3005–3023. doi: 10.1172/JCI46358

Lee, J. K., Kim, J. E., Sivula, M., and Strittmatter, S. M. (2004). Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. *J. Neurosci.* 24, 6209–6217. doi: 10.1523/jneurosci.1643-04.2004

Leitinger, B., and Hohenester, E. (2007). Mammalian collagen receptors. *Matrix Biol.* 26, 146–155. doi: 10.1016/j.matbio.2006.10.007

Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* 60, 585–595. doi: 10.1016/0092-8674(90)90662-x

Leonard, W. J., and Lin, J. X. (2000). Cytokine receptor signaling pathways. *J. Allergy Clin. Immunol.* 105, 877–888. doi: 10.1067/mai.2000.106899

Levine, J. M. (1994). Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. *J. Neurosci.* 14, 4716–4730.

Levison, S. W., Jiang, F. J., Stoltzfus, O. K., and Ducceschi, M. H. (2000). IL-6-type cytokines enhance epidermal growth factor-stimulated astrocyte proliferation. *Glia* 32, 328–337. doi: 10.1002/1098-1136(200012)32:3<328::aid-glia110>3.0.co;2-7

Li, L., Lundkvist, A., Andersson, D., Wilhelmsson, U., Nagai, N., Pardo, A. C., et al. (2008). Protective role of reactive astrocytes in brain ischemia. *J. Cereb. Blood Flow Metab.* 28, 468–481. doi: 10.1038/sj.jcbfm.9600546

Licht, T., Goshen, I., Avital, A., Kreisel, T., Zubedat, S., Eavri, R., et al. (2011). Reversible modulations of neuronal plasticity by VEGF. *Proc. Natl. Acad. Sci. U S A* 108, 5081–5086. doi: 10.1073/pnas.1007640108

Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., et al. (2005). Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. *Ann. Neurol.* 58, 706–719. doi: 10.1002/ana.20627

Liesi, P., and Kauppila, T. (2002). Induction of type IV collagen and other basement-membrane-associated proteins after spinal cord injury of the adult rat may participate in formation of the glial scar. *Exp. Neurol.* 173, 31–45. doi: 10.1006/exnr.2001.7800

Liesi, P., Kaakkola, S., Dahl, D., and Vaheri, A. (1984). Laminin is induced in astrocytes of adult brain by injury. *EMBO J.* 3, 683–686.

Lim, D. A., Tramontin, A. D., Trevejo, J. M., Herrera, D. G., García-Verdugo, J. M., and Alvarez-Buylla, A. (2000). Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* 28, 713–726. doi: 10.1016/s0896-6273(00)00148-3

Lin, T. N., Kim, G. M., Chen, J. J., Cheung, W. M., He, Y. Y., and Hsu, C. Y. (2003). Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion. *Stroke* 34, 177–186. doi: 10.1161/01.str.0000047100.84604.ba

Lindholm, D., Castrén, E., Kiefer, R., Zafra, F., and Thoenen, H. (1992). Transforming growth factor-beta 1 in the rat brain: increase after injury and inhibition of astrocyte proliferation. *J. Cell Biol.* 117, 395–400. doi: 10.1083/jcb.117.2.395

Lindholm, D., Hengerer, B., Zafra, F., and Thoenen, H. (1990). Transforming growth factor-beta 1 stimulates expression of nerve growth factor in the rat CNS. *Neuroreport* 1, 9–12. doi: 10.1097/00001756-199009000-00003

Liu, Y., and Rao, M. S. (2004). Glial progenitors in the CNS and possible lineage relationships among them. *Biol. Cell* 96, 279–290. doi: 10.1111/j.1768-322x.2004.tb01416.x

Liu, Y., Wang, X., Lu, C. C., Kerman, R., Steward, O., Xu, X. M., et al. (2008). Repulsive Wnt signaling inhibits axon regeneration after CNS injury. *J. Neurosci.* 28, 8376–8382. doi: 10.1523/JNEUROSCI.1939-08.2008

Lladó, J., Haenggeli, C., Maragakis, N. J., Snyder, E. Y., and Rothstein, J. D. (2004). Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. *Mol. Cell. Neurosci.* 27, 322–331. doi: 10.1016/j.mcn.2004.07.010

Lozito, T. P., Jackson, W. M., Nesti, L. J., and Tuan, R. S. (2014). Human mesenchymal stem cells generate a distinct pericellular zone of MMP activities via binding of MMPs and secretion of high levels of TIMPs. *Matrix Biol.* 34, 132–143. doi: 10.1016/j.matbio.2013.10.003

Ludwig, A. K., and Giebel, B. (2012). Exosomes: small vesicles participating in intercellular communication. *Int. J. Biochem. Cell Biol.* 44, 11–15. doi: 10.1016/j.biocel.2011.10.005

Maki, T., Liang, A. C., Miyamoto, N., Lo, E. H., and Arai, K. (2013). Mechanisms of oligodendrocyte regeneration from ventricular-subventricular zone-derived progenitor cells in white matter diseases. *Front. Cell. Neurosci.* 7:275. doi: 10.3389/fncel.2013.00275

Maness, P. F., and Schachner, M. (2007). Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. *Nat. Neurosci.* 10, 19–26. doi: 10.1038/nn1827

Manns, R. P., Cook, G. M., Holt, C. E., and Keynes, R. J. (2012). Differing semaphorin 3A concentrations trigger distinct signaling mechanisms in growth cone collapse. *J. Neurosci.* 32, 8554–8559. doi: 10.1523/jneurosci.5964-11.2012

Maroof, A. M., Keros, S., Tyson, J. A., Ying, S. W., Ganat, Y. M., Merkle, F. T., et al. (2013). Directed differentiation and functional maturation of cortical interneurons from human embryonic stem cells. *Cell Stem Cell* 12, 559–572. doi: 10.1016/j.stem.2013.04.008

Martino, G., Pluchino, S., Bonfanti, L., and Schwartz, M. (2011). Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. *Physiol. Rev.* 91, 1281–1304. doi: 10.1152/physrev.00032.2010

Massouh, M., and Saghatelyan, A. (2010). De-routing neuronal precursors in the adult brain to sites of injury: role of the vasculature. *Neuropharmacology* 58, 877–883. doi: 10.1016/j.neuropharm.2009.12.021

McKeon, R. J., Höke, A., and Silver, J. (1995). Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp. Neurol.* 136, 32–43. doi: 10.1006/exnr.1995.1081

McKeon, R. J., Jurynec, M. J., and Buck, C. R. (1999). The chondroitin sulfate proteoglycans neurocan and phosphacan are expressed by reactive astrocytes in the chronic CNS glial scar. *J. Neurosci.* 19, 10778–10788.

McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* 11, 3398–3411.

McTigue, D. M., Popovich, P. G., Morgan, T. E., and Stokes, B. T. (2000). Localization of transforming growth factor-beta1 and receptor mRNA after experimental spinal cord injury. *Exp. Neurol.* 163, 220–230. doi: 10.1006/exnr.2000.7372

Meng, K., Rodriguez-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., et al. (2000). Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc. Natl. Acad. Sci. U S A* 97, 2603–2608. doi: 10.1073/pnas.020487997

Midwood, K., Sacre, S., Piccinini, A. M., Inglis, J., Trebaul, A., Chan, E., et al. (2009). Tenascin-C is an endogenous activator of toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat. Med.* 15, 774–780. doi: 10.1038/nm.1987

Milev, P., Chiba, A., Häring, M., Rauvala, H., Schachner, M., Ranscht, B., et al. (1998a). High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase-zeta/beta with tenascin-R, amphoterin and the heparin-binding growth-associated molecule. *J. Biol. Chem.* 273, 6998–7005. doi: 10.1074/jbc.273.12.6998

Milev, P., Monnerie, H., Popp, S., Margolis, R. K., and Margolis, R. U. (1998b). The core protein of the chondroitin sulfate proteoglycan phosphacan is a high-affinity ligand of fibroblast growth factor-2 and potentiates its mitogenic activity. *J. Biol. Chem.* 273, 21439–21442. doi: 10.1074/jbc.273.34.21439

Milner, R., Crocker, S. J., Hung, S., Wang, X., Frausto, R. F., and del Zoppo, G. J. (2007). Fibronectin- and vitronectin-induced microglial activation and matrix metalloproteinase-9 expression is mediated by integrins alpha5beta1 and alphavbeta5. *J. Immunol.* 178, 8158–8167. doi: 10.4049/jimmunol.178.12.8158

Möller, J. C., Klein, M. A., Haas, S., Jones, L. L., Kreutzberg, G. W., and Raivich, G. (1996). Regulation of thrombospondin in the regenerating mouse facial motor nucleus. *Glia* 17, 121–132. doi: 10.1002/(sici)1098-1136(199606)17:2<121::aid-glia4>3.3.co;2-b

Morawski, M., Brückner, G., Jager, C., Seeger, G., Matthews, R. T., and Arendt, T. (2012). Involvement of perineuronal and perisynaptic extracellular matrix in Alzheimer's disease neuropathology. *Brain Pathol.* 22, 547–561. doi: 10.1111/j.1750-3639.2011.00557.x

Morgan, T. E., Nichols, N. R., Pasinetti, G. M., and Finch, C. E. (1993). TGF-beta 1 mRNA increases in macrophage/microglial cells of the hippocampus in response to deafferentation and kainic acid-induced neurodegeneration. *Exp. Neurol.* 120, 291–301. doi: 10.1006/exnr.1993.1063

Moritz, S., Lehmann, S., Faissner, A., and von Holst, A. (2008). An induction gene trap screen in neural stem cells reveals an instructive function of the niche and identifies the splicing regulator sam68 as a tenascin-C-regulated target gene. *Stem Cells* 26, 2321–2331. doi: 10.1634/stemcells.2007-1095

Muir, E. M., Adcock, K. H., Morgenstern, D. A., Clayton, R., von Stillfried, N., Rhodes, K., et al. (2002). Matrix metalloproteases and their inhibitors are produced by overlapping populations of activated astrocytes. *Brain Res. Mol. Brain Res.* 100, 103–117. doi: 10.1016/s0169-328x(02)00132-8

Nag, S., Eskandarian, M. R., Davis, J., and Eubanks, J. H. (2002). Differential expression of vascular endothelial growth factor-A (VEGF-A) and VEGF-B after brain injury. *J. Neuropathol. Exp. Neurol.* 61, 778–788.

Nakamura, M., and Bregman, B. S. (2001). Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. *Exp. Neurol.* 169, 407–415. doi: 10.1006/exnr.2001.7670

Nakic, M., Mitrovic, N., Sperk, G., and Schachner, M. (1996). Kainic acid activates transient expression of tenascin-C in the adult rat hippocampus. *J. Neurosci. Res.* 44, 355–362. doi: 10.1002/(sici)1097-4547(19960515)44:4<355::aid-jnr7>3.3.co;2-h

Neman, J., and de Vellis, J. (2012). A method for deriving homogenous population of oligodendrocytes from mouse embryonic stem cells. *Dev. Neurobiol.* 72, 777–788. doi: 10.1002/dneu.22008

Neufeld, G., and Kessler, O. (2008). The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. *Nat. Rev. Cancer* 8, 632–645. doi: 10.1038/nrc2404

Niclou, S. P., Franssen, E. H., Ehlert, E. M., Taniguchi, M., and Verhaagen, J. (2003). Meningeal cell-derived semaphorin 3A inhibits neurite outgrowth. *Mol. Cell. Neurosci.* 24, 902–912. doi: 10.1016/s1044-7431(03)00243-4

Nielsen, J., Gotfryd, K., Li, S., Kulahin, N., Soroka, V., Rasmussen, K. K., et al. (2009). Role of glial cell line-derived neurotrophic factor (GDNF)-neural cell adhesion molecule (NCAM) interactions in induction of neurite outgrowth and identification of a binding site for NCAM in the heel region of GDNF. *J. Neurosci.* 29, 11360–11376. doi: 10.1523/JNEUROSCI.3239-09.2009

Niquet, J., Jorquera, I., Faissner, A., Ben-Ari, Y., and Represa, A. (1995). Gliosis and axonal sprouting in the hippocampus of epileptic rats are associated with an increase of tenascin-C immunoreactivity. *J. Neurocytol.* 24, 611–624. doi: 10.1007/bf01257376

Noble, L. J., Donovan, F., Igarashi, T., Goussev, S., and Werb, Z. (2002). Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J. Neurosci.* 22, 7526–7535.

Obermair, F. J., Schröter, A., and Thallmair, M. (2008). Endogenous neural progenitor cells as therapeutic target after spinal cord injury. *Physiology (Bethesda)* 23, 296–304. doi: 10.1152/physiol.00017.2008

Overman, J. J., and Carmichael, S. T. (2014). Plasticity in the injured brain: more than molecules matter. *Neuroscientist* 20, 15–28. doi: 10.1177/1073858413491146

Panchision, D. M. (2009). The role of oxygen in regulating neural stem cells in development and disease. *J. Cell. Physiol.* 220, 562–568. doi: 10.1002/jcp.21812

Pariser, H., Ezquerra, L., Herradon, G., Perez-Pinera, P., and Deuel, T. F. (2005a). Fyn is a downstream target of the pleiotrophin/receptor protein tyrosine phosphatase beta/zeta-signaling pathway: regulation of tyrosine phosphorylation of Fyn by pleiotrophin. *Biochem. Biophys. Res. Commun.* 332, 664–669. doi: 10.1016/j.bbrc.2005.05.007

Pariser, H., Perez-Pinera, P., Ezquerra, L., Herradon, G., and Deuel, T. F. (2005b). Pleiotrophin stimulates tyrosine phosphorylation of beta-adducin through inactivation of the transmembrane receptor protein tyrosine phosphatase beta/zeta. *Biochem. Biophys. Res. Commun.* 335, 232–239. doi: 10.1016/j.bbrc.2005.07.060

Pasterkamp, R. J., Giger, R. J., Ruitenberg, M. J., Holtmaat, A. J., De Wit, J., De Winter, F., et al. (1999). Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Mol. Cell. Neurosci.* 13, 143–166. doi: 10.1006/mcne.1999.0738

Patel, J., Channon, K. M., and McNeill, E. (2013). The downstream regulation of chemokine receptor signalling: implications for atherosclerosis. *Mediators Inflamm.* 2013:459520. doi: 10.1155/2013/459520

Pekny, M., and Nilsson, M. (2005). Astrocyte activation and reactive gliosis. *Glia* 50, 427–434. doi: 10.1002/glia.20207

Pekny, M., Wilhelmsson, U., and Pekna, M. (2014). The dual role of astrocyte activation and reactive gliosis. *Neurosci. Lett.* 565C, 30–38. doi: 10.1016/j.neulet.2013.12.071

Petrasek, T., Prokopova, I., Bahnik, S., Schonig, K., Berger, S., Vales, K., et al. (2014). Nogo-A downregulation impairs place avoidance in the Carousel maze but not spatial memory in the morris water maze. *Neurobiol. Learn. Mem.* 107, 42–49. doi: 10.1016/j.nlm.2013.10.015

Petrosyan, H. A., Hunanyan, A. S., Alessi, V., Schnell, L., Levine, J., and Arvanian, V. L. (2013). Neutralization of inhibitory molecule NG2 improves synaptic transmission, retrograde transport and locomotor function after spinal cord injury in adult rats. *J. Neurosci.* 33, 4032–4043. doi: 10.1523/JNEUROSCI.4702-12.2013

Piccin, D., and Morshead, C. M. (2011). Wnt signaling regulates symmetry of division of neural stem cells in the adult brain and in response to injury. *Stem Cells* 29, 528–538. doi: 10.1002/stem.589

Pizzorusso, T., Medini, P., Berardi, N., Chierzi, S., Fawcett, J. W., and Maffei, L. (2002). Reactivation of ocular dominance plasticity in the adult visual cortex. *Science* 298, 1248–1251. doi: 10.1126/science.1072699

Pluchino, S., Muzio, L., Imitola, J., Deleidi, M., Alfaro-Cervello, C., Salani, G., et al. (2008). Persistent inflammation alters the function of the endogenous brain stem cell compartment. *Brain* 131, 2564–2578. doi: 10.1093/brain/awn198

Pluchino, S., Zanotti, L., Rossi, B., Brambilla, E., Ottoboni, L., Salani, G., et al. (2005). Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 436, 266–271. doi: 10.1038/nature03889

Pozzi, A., Wary, K. K., Giancotti, F. G., and Gardner, H. A. (1998). Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo. *J. Cell Biol.* 142, 587–594. doi: 10.1083/jcb.142.2.587

Pyka, M., Wetzel, C., Aguado, A., Geissler, M., Hatt, H., and Faissner, A. (2011). Chondroitin sulfate proteoglycans regulate astrocyte-dependent synaptogenesis and modulate synaptic activity in primary embryonic hippocampal neurons. *Eur. J. Neurosci.* 33, 2187–2202. doi: 10.1111/j.1460-9568.2011.07690.x

Quaglia, X., Beggah, A. T., Seidenbecher, C., and Zurn, A. D. (2008). Delayed priming promotes CNS regeneration post-rhizotomy in Neurocan and Brevican-deficient mice. *Brain* 131, 240–249. doi: 10.1093/brain/awm279

Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* 252, 1705–1708. doi: 10.1126/science.1646484

Rauch, U., Clement, A., Retzler, C., Fröhlich, L., Fässler, R., Göhring, W., et al. (1997). Mapping of a defined neurocan binding site to distinct domains of tenascin-C. *J. Biol. Chem.* 272, 26905–26912. doi: 10.1074/jbc.272.43.26905

Resovi, A., Pinessi, D., Chiorino, G., and Taraboletti, G. (2014). Current understanding of the thrombospondin-1 interactome. *Matrix Biol.* doi: 10.1016/j.matbio.2014.01.012. [Epub ahead of print].

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* 434, 843–850. doi: 10.1038/nature03319

Ricard-Blum, S. (2011). The collagen family. *Cold Spring Harb. Perspect. Biol.* 3:a004978. doi: 10.1101/cshperspect.a004978

Rigato, F., Garwood, J., Calco, V., Heck, N., Faivre-Sarrailh, C., and Faissner, A. (2002). Tenascin-C promotes neurite outgrowth of embryonic hippocampal neurons through the alternatively spliced fibronectin type III BD domains via activation of the cell adhesion molecule F3/contactin. *J. Neurosci.* 22, 6596–6609.

Robel, S., Berninger, B., and Götz, M. (2011). The stem cell potential of glia: lessons from reactive gliosis. *Nat. Rev. Neurosci.* 12, 88–104. doi: 10.1038/nrn2978

Rolando, C., Parolisi, R., Boda, E., Schwab, M. E., Rossi, F., and Buffo, A. (2012). Distinct roles of nogo-a and nogo receptor 1 in the homeostatic regulation of adult neural stem cell function and neuroblast migration. *J. Neurosci.* 32, 17788–17799. doi: 10.1523/JNEUROSCI.3142-12.2012

Roll, L., Mittmann, T., Eysel, U. T., and Faissner, A. (2012). The laser lesion of the mouse visual cortex as a model to study neural extracellular matrix remodeling during degeneration, regeneration and plasticity of the CNS. *Cell Tissue Res.* 349, 133–145. doi: 10.1007/s00441-011-1313-4

Rolls, A., Shechter, R., and Schwartz, M. (2009). The bright side of the glial scar in CNS repair. *Nat. Rev. Neurosci.* 10, 235–241. doi: 10.1038/nrn2591

Ronca, F., Andersen, J. S., Paech, V., and Margolis, R. U. (2001). Characterization of slit protein interactions with glypican-1. *J. Biol. Chem.* 276, 29141–29147. doi: 10.1074/jbc.m100240200

Rosenkranz, K., Kumbruch, S., Lebermann, K., Marschner, K., Jensen, A., Dermietzel, R., et al. (2010). The chemokine SDF-1/CXCL12 contributes to the 'homing' of umbilical cord blood cells to a hypoxic-ischemic lesion in the rat brain. *J. Neurosci. Res.* 88, 1223–1233. doi: 10.1002/jnr.22292

Rostène, W., Dansereau, M. A., Godefroy, D., Van Steenwinckel, J., Reaux-Le Goazigo, A., Mélik-Parsadaniantz, S., et al. (2011). Neurochemokines: a menage a trois providing new insights on the functions of chemokines in the central nervous system. *J. Neurochem.* 118, 680–694. doi: 10.1111/j.1471-4159.2011.07371.x

Salanga, C. L., O'Hayre, M., and Handel, T. (2009). Modulation of chemokine receptor activity through dimerization and crosstalk. *Cell. Mol. Life Sci.* 66, 1370–1386. doi: 10.1007/s00018-008-8666-1

Samson, A. L., and Medcalf, R. L. (2006). Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity. *Neuron* 50, 673–678. doi: 10.1016/j.neuron.2006.04.013

Sarkar, S., Raymick, J., and Schmued, L. (2012). Temporal progression of kainic acid induced changes in vascular laminin expression in rat brain with neuronal and glial correlates. *Curr. Neurovasc. Res.* 9, 110–119. doi: 10.2174/156720212800410867

Sasaki, N., Kurisu, J., and Kengaku, M. (2010). Sonic hedgehog signaling regulates actin cytoskeleton via Tiam1-Rac1 cascade during spine formation. *Mol. Cell. Neurosci.* 45, 335–344. doi: 10.1016/j.mcn.2010.07.006

Savitz, S. I., Cramer, S. C., Wechsler, L., and Consortium, S. (2014). Stem cells as an emerging paradigm in stroke 3: enhancing the development of clinical trials. *Stroke* 45, 634–639. doi: 10.1161/STROKEAHA.113.003379

Schachtrup, C., Ryu, J. K., Helmrick, M. J., Vagena, E., Galanakis, D. K., Degen, J. L., et al. (2010). Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J. Neurosci.* 30, 5843–5854. doi: 10.1523/JNEUROSCI.0137-10.2010

Schlessinger, J., and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. *Neuron* 9, 383–391. doi: 10.1016/0896-6273(92)90177-F

Schmandke, A., Schmandke, A., and Schwab, M. E. (2014). Nogo-A: multiple roles in CNS development, maintenance and disease. *Neuroscientist* 20, 372–386. doi: 10.1177/1073858413516800

Schwab, M. E., and Strittmatter, S. M. (2014). Nogo limits neural plasticity and recovery from injury. *Curr. Opin. Neurobiol.* 27C, 53–60. doi: 10.1016/j.conb.2014.02.011

Scott-Drew, S., and ffrench-Constant, C. (1997). Expression and function of thrombospondin-1 in myelinating glial cells of the central nervous system. *J. Neurosci. Res.* 50, 202–214. doi: 10.1002/(sici)1097-4547(19971015)50:2<202::aid-jnr9>3.0.co;2-j

Seidenfaden, R., Desoeuvre, A., Bosio, A., Virard, I., and Cremer, H. (2006). Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. *Mol. Cell. Neurosci.* 32, 187–198. doi: 10.1016/j.mcn.2006.04.003

Semenov, M. V., Habas, R., Macdonald, B. T., and He, X. (2007). SnapShot: noncanonical Wnt signaling pathways. *Cell* 131, 1378.e1–1378.e2. doi: 10.1016/j.cell.2007.12.011

Seo, J. H., Miyamoto, N., Hayakawa, K., Pham, L. D., Maki, T., Ayata, C., et al. (2013). Oligodendrocyte precursors induce early blood-brain barrier opening after white matter injury. *J. Clin. Invest.* 123, 782–786. doi: 10.1172/JCI65863

Sharma, A., Pollett, M. A., Plant, G. W., and Harvey, A. R. (2012a). Changes in mRNA expression of class 3 semaphorins and their receptors in the adult rat retino-collicular system after unilateral optic nerve injury. *Invest. Ophthalmol. Vis. Sci.* 53, 8367–8377. doi: 10.1167/iovs.12-10799

Sharma, K., Selzer, M. E., and Li, S. (2012b). Scar-mediated inhibition and CSPG receptors in the CNS. *Exp. Neurol.* 237, 370–378. doi: 10.1016/j.expneurol.2012.07.009

Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S. M., Goderie, S. K., et al. (2008). Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell* 3, 289–300. doi: 10.1016/j.stem.2008.07.026

Shimada, I. S., LeComte, M. D., Granger, J. C., Quinlan, N. J., and Spees, J. L. (2012). Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke. *J. Neurosci.* 32, 7926–7940. doi: 10.1523/JNEUROSCI.4303-11.2012

Shimada, I. S., Peterson, B. M., and Spees, J. L. (2010). Isolation of locally derived stem/progenitor cells from the peri-infarct area that do not migrate from the lateral ventricle after cortical stroke. *Stroke* 41, e552–e560. doi: 10.1161/STROKEAHA.110.589010

Siddiqui, S., Horvat-Brœcker, A., and Faissner, A. (2009). Comparative screening of glial cell types reveals extracellular matrix that inhibits retinal axon growth in a chondroitinase ABC-resistant fashion. *Glia* 57, 1420–1438. doi: 10.1002/glia.20860

Silver, J., and Miller, J. H. (2004). Regeneration beyond the glial scar. *Nat. Rev. Neurosci.* 5, 146–156. doi: 10.1038/nrn1326

Singh, P., Carraher, C., and Schwarzbauer, J. E. (2010). Assembly of fibronectin extracellular matrix. *Annu. Rev. Cell Dev. Biol.* 26, 397–419. doi: 10.1146/annurev-cellbio-100109-104020

Siri, A., Knäuper, V., Veirana, N., Caocci, F., Murphy, G., and Zardi, L. (1995). Different susceptibility of small and large human tenascin-C isoforms to degradation by matrix metalloproteinases. *J. Biol. Chem.* 270, 8650–8654. doi: 10.1074/jbc.270.15.8650

Sirko, S., Behrendt, G., Johansson, P. A., Tripathi, P., Costa, M., Bek, S., et al. (2013). Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog. [corrected]. *Cell Stem Cell* 12, 426–439. doi: 10.1016/j.stem.2013.01.019

Sirko, S., Neitz, A., Mittmann, T., Horvat-Bröcker, A., von Holst, A., Eysel, U. T., et al. (2009). Focal laser-lesions activate an endogenous population of neural stem/progenitor cells in the adult visual cortex. *Brain* 132, 2252–2264. doi: 10.1093/brain/awp043

Siu, R., Fladd, C., and Rotin, D. (2007). N-cadherin is an in vivo substrate for protein tyrosine phosphatase sigma (PTPsigma) and participates in PTPsigma-mediated inhibition of axon growth. *Mol. Cell. Biol.* 27, 208–219. doi: 10.1128/mcb.00707-06

Smith, G. M., and Hale, J. H. (1997). Macrophage/Microglia regulation of astrocytic tenascin: synergistic action of transforming growth factor-beta and basic fibroblast growth factor. *J. Neurosci.* 17, 9624–9633.

Smith-Thomas, L. C., Stevens, J., Fok-Seang, J., Faissner, A., Rogers, J. H., and Fawcett, J. W. (1995). Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors. *J. Cell Sci.* 108(Pt. 3), 1307–1315.

Sobel, R. A. (1998). The extracellular matrix in multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* 57, 205–217. doi: 10.1097/00005072-199803000-00001

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* 32, 638–647. doi: 10.1016/j.tins.2009.08.002

Soleman, S., Filippov, M. A., Dityatev, A., and Fawcett, J. W. (2013). Targeting the neural extracellular matrix in neurological disorders. *Neuroscience* 253, 194–213. doi: 10.1016/j.neuroscience.2013.08.050

Southwell, D. G., Froemke, R. C., Alvarez-Buylla, A., Stryker, M. P., and Gandhi, S. P. (2010). Cortical plasticity induced by inhibitory neuron transplantation. *Science* 327, 1145–1148. doi: 10.1126/science.1183962

Steigemann, P., Molitor, A., Fellert, S., Jäckle, H., and Vorbrüggen, G. (2004). Heparan sulfate proteoglycan syndecan promotes axonal and myotube guidance by slit/robo signaling. *Curr. Biol.* 14, 225–230. doi: 10.1016/s0960-9822(04)00007-7

Stichel, C. C., Kappler, J., Junghans, U., Koops, A., Kresse, H., and Müller, H. W. (1995). Differential expression of the small chondroitin/dermatan sulfate proteoglycans decorin and biglycan after injury of the adult rat brain. *Brain Res.* 704, 263–274. doi: 10.1016/0006-8993(95)01131-5

Stumm, R. K., Rummel, J., Junker, V., Culmsee, C., Pfeiffer, M., Krieglstein, J., et al. (2002). A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. *J. Neurosci.* 22, 5865–5878.

Su, Z., Niu, W., Liu, M. L., Zou, Y., and Zhang, C. L. (2014). In vivo conversion of astrocytes to neurons in the injured adult spinal cord. *Nat. Commun.* 5:3338. doi: 10.1038/ncomms4338

Sugimoto, K., Nishioka, R., Ikeda, A., Mise, A., Takahashi, H., Yano, H., et al. (2014). Activated microglia in a rat stroke model express NG2 proteoglycan in peri-infarct tissue through the involvement of TGF-β1. *Glia* 62, 185–198. doi: 10.1002/glia.22598

Swindle, C. S., Tran, K. T., Johnson, T. D., Banerjee, P., Mayes, A. M., Griffith, L., et al. (2001). Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. *J. Cell Biol.* 154, 459–468. doi: 10.1083/jcb.200103103

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Tate, C. C., García, A. J., and LaPlaca, M. C. (2007a). Plasma fibronectin is neuroprotective following traumatic brain injury. *Exp. Neurol.* 207, 13–22. doi: 10.1016/j.expneurol.2007.05.008

Tate, C. C., Tate, M. C., and LaPlaca, M. C. (2007b). Fibronectin and laminin increase in the mouse brain after controlled cortical impact injury. *J. Neurotrauma* 24, 226–230. doi: 10.1089/neu.2006.0043

Thier, M., Wörsdörfer, P., Lakes, Y. B., Gorris, R., Herms, S., Opitz, T., et al. (2012). Direct conversion of fibroblasts into stably expandable neural stem cells. *Cell Stem Cell* 10, 473–479. doi: 10.1016/j.stem.2012.03.003

Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., et al. (2006). Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* 24, 739–747. doi: 10.1634/stemcells.2005-0281

Tom, V. J., Doller, C. M., Malouf, A. T., and Silver, J. (2004). Astrocyte-associated fibronectin is critical for axonal regeneration in adult white matter. *J. Neurosci.* 24, 9282–9290. doi: 10.1523/jneurosci.2120-04.2004

Tornero, D., Wattananit, S., Grønning Madsen, M., Koch, P., Wood, J., Tatarishvili, J., et al. (2013). Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain* 136, 3561–3577. doi: 10.1093/brain/awt278

Treilleux, I., Mallein-Gerin, F., le Guellec, D., and Herbage, D. (1992). Localization of the expression of type I, II, III collagen and aggrecan core protein genes in developing human articular cartilage. *Matrix* 12, 221–232. doi: 10.1016/s0934-8832(11)80065-x

Um, J. W., and Ko, J. (2013). LAR-RPTPs: synaptic adhesion molecules that shape synapse development. *Trends Cell Biol.* 23, 465–475. doi: 10.1016/j.tcb.2013.07.004

van Horssen, J., Dijkstra, C. D., and de Vries, H. E. (2007). The extracellular matrix in multiple sclerosis pathology. *J. Neurochem.* 103, 1293–1301. doi: 10.1111/j.1471-4159.2007.04897.x

von Holst, A. (2008). Tenascin C in stem cell niches: redundant, permissive or instructive? *Cells Tissues Organs* 188, 170–177. doi: 10.1159/000112848

von Holst, A., Egbers, U., Prochiantz, A., and Faissner, A. (2007). Neural stem/progenitor cells express 20 tenascin C isoforms that are differentially regulated by Pax6. *J. Biol. Chem.* 282, 9172–9181. doi: 10.1074/jbc.m608067200

von Holst, A., Sirko, S., and Faissner, A. (2006). The unique 473HD-Chondroitinsulfate epitope is expressed by radial glia and involved in neural precursor cell proliferation. *J. Neurosci.* 26, 4082–4094. doi: 10.1523/jneurosci.0422-06.2006

Vutskits, L., Djebbara-Hannas, Z., Zhang, H., Paccaud, J. P., Durbec, P., Rougon, G., et al. (2001). PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons. *Eur. J. Neurosci.* 13, 1391–1402. doi: 10.1046/j.0953-816x.2001.01516.x

Wade, A., Robinson, A. E., Engler, J. R., Petritsch, C., James, C. D., and Phillips, J. J. (2013). Proteoglycans and their roles in brain cancer. *FEBS J.* 280, 2399–2417. doi: 10.1111/febs.12109

Wahl, S., Barth, H., Ciossek, T., Aktories, K., and Mueller, B. K. (2000). Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J. Cell Biol.* 149, 263–270. doi: 10.1083/jcb.149.2.263

Wang, X., Chen, W., Liu, W., Wu, J., Shao, Y., and Zhang, X. (2009b). The role of thrombospondin-1 and transforming growth factor-beta after spinal cord injury in the rat. *J. Clin. Neurosci.* 16, 818–821. doi: 10.1016/j.jocn.2008.09.014

Wang, L., Shi, J., van Ginkel, F. W., Lan, L., Niemeyer, G., Martin, D. R., et al. (2009a). Neural stem/progenitor cells modulate immune responses by suppressing T lymphocytes with nitric oxide and prostaglandin E2. *Exp. Neurol.* 216, 177–183. doi: 10.1016/j.expneurol.2008.11.017

Wanner, I. B., Deik, A., Torres, M., Rosendahl, A., Neary, J. T., Lemmon, V. P., et al. (2008). A new in vitro model of the glial scar inhibits axon growth. *Glia* 56, 1691–1709. doi: 10.1002/glia.20721

Widestrand, A., Faijerson, J., Wilhelmsson, U., Smith, P. L., Li, L., Sihlbom, C., et al. (2007). Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of GFAP-/- Vim-/- mice. *Stem Cells* 25, 2619–2627. doi: 10.1634/stemcells.2007-0122

Wiese, S., Karus, M., and Faissner, A. (2012). Astrocytes as a source for extracellular matrix molecules and cytokines. *Front. Pharmacol.* 3:120. doi: 10.3389/fphar.2012.00120

Wilhelmsson, U., Li, L., Pekna, M., Berthold, C. H., Blom, S., Eliasson, C., et al. (2004). Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. *J. Neurosci.* 24, 5016–5021. doi: 10.1523/jneurosci.0820-04.2004

Xiao, Q., Du, Y., Wu, W., and Yip, H. K. (2010). Bone morphogenetic proteins mediate cellular response and, together with Noggin, regulate astrocyte differentiation after spinal cord injury. *Exp. Neurol.* 221, 353–366. doi: 10.1016/j.expneurol.2009.12.003

Xiao, M. J., Han, Z., Shao, B., and Jin, K. (2009). Notch signaling and neurogenesis in normal and stroke brain. *Int. J. Physiol. Pathophysiol. Pharmacol.* 1, 192–202.

Xu, J. C., Xiao, M. F., Jakovcevski, I., Sivukhina, E., Hargus, G., Cui, Y. F., et al. (2014). The extracellular matrix glycoprotein tenascin-R regulates neurogenesis during development and in the adult dentate gyrus of mice. *J. Cell Sci.* 127, 641–652. doi: 10.1242/jcs.137612

Yamaguchi, Y. (2001). Heparan sulfate proteoglycans in the nervous system: their diverse roles in neurogenesis, axon guidance and synaptogenesis. *Semin. Cell Dev. Biol.* 12, 99–106. doi: 10.1006/scdb.2000.0238

Yamamoto, S., Nagao, M., Sugimori, M., Kosako, H., Nakatomi, H., Yamamoto, N., et al. (2001). Transcription factor expression and notch-dependent regulation of neural progenitors in the adult rat spinal cord. *J. Neurosci.* 21, 9814–9823.

Yamashita, T., Ninomiya, M., Hernández Acosta, P., García-Verdugo, J. M., Sunabori, T., and Sakaguchi, M. (2006). Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J. Neurosci.* 26, 6627–6636. doi: 10.1523/jneurosci.0149-06.2006

Yan, K., Zhang, R., Sun, C., Chen, L., Li, P., Liu, Y., et al. (2013). Bone marrow-derived mesenchymal stem cells maintain the resting phenotype of microglia and inhibit microglial activation. *PLoS One* 8:e84116. doi: 10.1371/journal.pone.0084116

Yang, H., Xiao, Z. C., Becker, B., Hillenbrand, R., Rougon, G., and Schachner, M. (1999). Role for myelin-associated glycoprotein as a functional tenascin-R receptor. *J. Neurosci. Res.* 55, 687–701. doi: 10.1002/(sici)1097-4547(19990315)55:6<687::aid-jnr4>3.3.co;2-y

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 64, 841–848. doi: 10.1016/0092-8674(91)90512-w

Ye, Q., and Miao, Q. L. (2013). Experience-dependent development of perineuronal nets and chondroitin sulfate proteoglycan receptors in mouse visual cortex. *Matrix Biol.* 32, 352–363. doi: 10.1016/j.matbio.2013.04.001

Yokosaki, Y., Matsuura, N., Higashiyama, S., Murakami, I., Obara, M., Yamakido, M., et al. (1998). Identification of the ligand binding site for the integrin alpha9 beta1 in the third fibronectin type III repeat of tenascin-C. *J. Biol. Chem.* 273, 11423–11428. doi: 10.1074/jbc.273.19.11423

You, J., Hong, S. Q., Zhang, M. Y., Zhao, H. L., Liu, T. Z., Zhou, H. L., et al. (2012). Passive immunization with tenascin-R (TN-R) polyclonal antibody promotes axonal regeneration and functional recovery after spinal cord injury in rats. *Neurosci. Lett.* 525, 129–134. doi: 10.1016/j.neulet.2012.08.002

Zeng, H., Guo, M., Martins-Taylor, K., Wang, X., Zhang, Z., Park, J. W., et al. (2010). Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. *PLoS One* 5:e11853. doi: 10.1371/journal.pone.0011853

Zhang, S., Xia, Y. Y., Lim, H. C., Tang, F. R., and Feng, Z. W. (2010). NCAM-mediated locomotor recovery from spinal cord contusion injury involves neuroprotection, axon regeneration and synaptogenesis. *Neurochem. Int.* 56, 919–929. doi: 10.1016/j.neuint.2010.03.023

Zhao, R. R., Andrews, M. R., Wang, D., Warren, P., Gullo, M., Schnell, L., et al. (2013). Combination treatment with anti-Nogo-A and chondroitinase ABC is more effective than single treatments at enhancing functional recovery after spinal cord injury. *Eur. J. Neurosci.* 38, 2946–2961. doi: 10.1111/ejn.12276

Zimmermann, D. R., and Dours-Zimmermann, M. T. (2008). Extracellular matrix of the central nervous system: from neglect to challenge. *Histochem. Cell Biol.* 130, 635–653. doi: 10.1007/s00418-008-0485-9

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Fate of graft cells: what should be clarified for development of mesenchymal stem cell therapy for ischemic stroke?

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Mesenchymal stem cells (MSCs) are believed to be promising for cell administration therapy after ischemic stroke. Because of their advantageous characteristics, such as ability of differentiation into neurovascular lineages, avoidance of immunological problems, and abundance of graft cells in mesodermal tissues, studies regarding MSC therapy have increased recently. However, several controversies are yet to be resolved before a worldwide consensus regarding a standard protocol is obtained. In particular, the neuroprotective effects, the rate of cell migration to the lesion, and differentiation direction differ depending on preclinical observations. Analyses of these differences and application of recent developments in stem cell biology or engineering in imaging modality may contribute to identification of criteria for optimal stem cell therapy in which reliable protocols, which control cell quality and include safe administration procedures, are defined for each recovery phase after cerebral ischemia. In this mini review, we examine controversies regarding the fate of grafts and the prospects for advanced therapy that could be obtained through recent developments in stem cell research as direct conversion to neural cells.

Keywords: mesenchymal stem cell, ischemic stroke, stem cell therapy, translational research, neurovascular unit

DEVELOPMENT OF MESENCHYMAL STEM CELL THERAPY STUDY FOR ISCHEMIC STROKE

Ischemic stroke is a common central nervous system (CNS) disease. Despite continuous development in treatments, stroke is still a major cause of death or disability, and therefore, more effective therapies are required. In 1990s, clinical trials neuroprotective agents targeted single mechanism, i.e., glutamate-induced neurotoxicity revealed to become failure (Hoyte et al., 2004). In the lesion insulted by brain ischemia, multiple pathogenic mechanisms are activated. As the failures in the early neuroprotective drug development showed (Degraba and Pettigrew, 2000), a genuine effective therapy would be required to solve the pleiotropic pathology (Teng et al., 2008; Guo and Lo, 2009).

Another concept to treat lost function by ischemia is to supply cells or tissue for replacement of the damaged brain tissue. In the early days of stem cell research, stem cells were expected as a source of tissue regeneration. Since the publication of the earliest reports of attempted administration of embryonic or neonatal neural stem cells for regeneration of the CNS in the early 1990s (Renfranz et al., 1991; Snyder et al., 1992), diverse cell types have been investigated to identify an ideal cell line to generate tissue grafts for CNS. Candidate cells can be categorized into embryonic, fetal, neonatal, or adult by maturation of each origin tissue. When categorized by a stage of differentiation, the examined cells can be sourced from pluripotent cells (embryonic stem cells or induced pluripotent cells), ectodermal lineage (neural stem cells, olfactory neuroepithelial stem cells, or NT2 cell line derived from neuroteratocarcinoma), mesodermal lineage [mesenchymal stem cells (MSCs), CD34+ cells, endothelial progenitor cells, hematopoietic stem cells, or bone marrow mononuclear/stromal cells]. As discussed in published reviews on stem cell therapies (Locatelli et al., 2009; Bhasin et al., 2013; Kalladka and Muir, 2014), neural stem cells, and mesodermal lineage listed above have already been applied for ischemic stroke in clinical settings from subacute phase to chronic phase.

In this mini review, the advantages of MSCs, as a source for stem cell therapy, are summarized. Furthermore, controversial points in preclinical experimental studies and the developing field of MSC therapy resulting from the recent evolution in stem cell biology are discussed by focusing on the biological features of mesenchymal stem cells (MSCs).

Among stem cell therapies, the greatest numbers of clinical trial for MSC have been conducted (Rosado-De-Castro et al., 2013a), thus MSC therapy can be the most practical stroke treatments in cell-based therapies (Eckert et al., 2013). More than 30 years after when Friedenstein et al. (1966) isolated osteogenic cell population

from bone marrow, MSCs have been identified in bone marrow (Pittenger et al., 1999), adipose tissue (Zuk et al., 2002), umbilical cord (Erices et al., 2000), peripheral blood (Ukai et al., 2007), dental pulp (Gronthos et al., 2000), and a wide range of mesodermal tissues including perivascular site in brain (Kang et al., 2010; Paul et al., 2012). The criteria for identifying MSCs as proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy are (1) plastic adherence of isolated cells in culture; (2) in cell surface marker analysis, >95% of the culture positively expressing the cell surface markers CD105, CD73, and CD90, while being negative for CD34, CD45, CD14, or CD11b, CD79a, or CD19, and human leukocyte antigen-DR; and (3) *in vitro* differentiation into three mesodermal cell types, namely osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). Moreover, the characteristics of MSC present advantages. MSC have been shown their multipotency that is beneficial to differentiate into multiple lineages to repair neurovascular unit or neural network; they could demonstrate multiphasic actions to modify endogenous repairing process including reprogramming, harmful immune response, or chemical reactions via secretion abilities; they are easier to prepare for grafting due to their accessible cell source and proliferation potential for rapid cell expansion. (Doeppner and Hermann, 2010; Grande et al., 2013; Wan et al., 2013)

The first series of successful experiments for MSCs for the treatment of ischemic stroke was reported by Chopp's group (Chen et al., 2000; Li et al., 2000; Zhang et al., 2000). They have examined multiple protocols for bone marrow stromal-derived stem cells (BMSCs) such as administration route (intracerebral, transventricular, intra-arterial, transvenous), timing, or dose, as well as have analyzed mechanisms of functional recovery focused on restore or remodeling functional connectivity in neural circuits/tract. Subsequently, details required for the establishment of safe and effective therapy protocols (Borlongan, 2009; The STEPS Participants, 2009; Savitz et al., 2011) have been analyzed by a number of investigators. Most results in the preclinical studies have indicated that MSC administration is beneficial. In this context, clinical trials employing systemic administration via peripheral veins were initiated more recently (Lee et al., 2010; Honmou et al., 2011). So far, these trials have not demonstrated severe adverse results (Lalu et al., 2012), even during observation periods lasting longer than a few years, despite the prediction of risks, such as embolization (Ge et al., 2014; Yavagal et al., 2014), infection, and tumorigenesis (Coussens et al., 2000; Li et al., 2007), in experimental studies.

CONTROVERSIES IN PRECLINICAL STAGE

Overall, accumulated findings have indicated that MSC therapy is reliable for stroke treatment. However, several points must be clarified for achievement of consensus as a reliable protocol. As shown in **Table 1**, the conditions of some preclinical studies resulted in differing outcomes because of graft cell detection in the lesion, infarct volume reduction, functional recovery, marker expression (neuronal, glial, or vascular: direction of differentiation), and the type of MSCs considered to have more therapeutic effects, particularly BMSCs and adipose tissue-derived stem cells (ASCs).

MIGRATION TO THE LESION

A major discrepancy in the results of preclinical studies is whether graft cells have the ability to migrate to a cerebral lesion, although mechanisms of MSC transmigration across the blood–brain barrier (BBB) have been analyzed (Liu et al., 2013). The accumulation of graft cells in the lesion is expected to directly enhance neuroprotection and cell replacement in infarcted tissue. A comparison of different administration routes revealed that transarterial delivery was more successful in order to detect graft cells in the brain than transvenous delivery, although several studies reported a decrease in the number of detected cells in the later phase (Ishizaka et al., 2013; Mitkari et al., 2013). The transvenous route induced fewer side effects than intra-arterial infusion; however, physiologically, graft cells must pass through several traps, such as the lung and BBB. Although, the BBB can be disrupted by ischemic insult around the damaged areas, MSCs may have the basic ability to transmigrate the BBB as immune cells in response to homing signals to the lesion (Liu et al., 2013). Nonetheless, there are certainly successful examples demonstrating the integration of graft cells in the peri-infarct area even after transvenous infusion from a peripheral vessel (**Table 1**).

Classically, immunohistological analysis is a standard method to detect MSC migration, but recent imaging techniques, such as magnetic resonance imaging (MRI) with magnetic cell labeling (Detante et al., 2012; Canazza et al., 2013) and nuclear imaging using ^{99m}Tc-labeled graft (Detante et al., 2009; Vasconcelos-Dos-Santos et al., 2012), have been proposed to reveal the distribution of MSCs. Subsequently, a phase I clinical trial employing ^{99m}Tc – single photon emission computed tomography (SPECT) for assessment of biodistribution of the labeled grafts in subacute patients have safely conducted (Rosado-De-Castro et al., 2013b). The findings of these recent analytical methods may resolve the question of accurate distribution of graft cells.

FUNCTIONAL RECOVERY

Many preclinical studies have also reported differences in infarct volume reduction and functional recovery (Hao et al., 2014). Assessment methods of functional recovery vary, although there certainly are popular tests in animal studies, such as the treadmill test or Roger's test. Therefore, differences in functional assessment may simply be based on differences in the employed assessment methods. On the other hand, it is more difficult to elucidate discrepancies in infarct volume reduction. *In vivo* studies with rodents have been conducted to investigate the changes in infarct volume reduction by direct measurement of the brain tissue after decapitation. Regarding clinical applications, non-invasive methods, such as MRI, may be beneficial to translate the findings of *in vivo* studies to clinical settings. Although the availability of mechanical devices varies among laboratories, the development of alternative clinical methods is recommended for future *in vivo* experiments.

Another problem is whether MSCs isolated from different tissues also differ. MSCs are obtained from diverse mesodermal tissues, i.e., bone marrow, adipose tissue, dental pulp, or cord blood. MSCs from different sources show different characteristics *in vitro* (Kern et al., 2006; Hsiao et al., 2012). Therefore, comparative study for different cell sources as conducted by

Table 1 | Examles of precilinical reports present discrepancy in results.

	Graft cell detection in brain		Infarct volume reduction		Functional recovery		Differentiation in the lesion		
Administration route	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Neuronal**	**Oligodendro-glial**	**Vascular**
Intra-arterial	Shen et al. (2006; BM, A) Ishizaka et al. (2013; BM, A) Jiang et al. (2014; AT, S)		Ishizaka et al. (2013; BM, A)	Jiang et al. (2014; AT, S)	Shen et al. (2006; BM, A) Jiang et al. (2014; AT, S)		Jiang et al. (2014; AT, S)	Shen et al. (2006; BM, A) Jiang et al. (2014; AT, S)	
Intravenous	Chen et al. (2001; BM, A) Li et al. (2005; BM, S) Leu et al. (2010; AT, A) Wei et al. (2012; BM, A) Honmou et al. (2012; BM, A, S, C) Steiner et al. (2012; BM, A)	Ikegame et al. (2011; BM, AT, A) Gutierrez-Fernandez et al. (2013; BM, AT, A)	Leu et al. (2010; AT, A) Ikegame et al. (2011; BM, AT, A) Honmou et al. (2012; BM, A, S, C)	Steiner et al. (2012; BM, A) Gutierrez-Fernandez et al. (2013; BM, AT, A)	Chen et al. (2001; BM, A) Li et al. (2005; BM, S) Leu et al. (2010; AT, A) Ikegame et al. (2011; BM, AT, A) Wei et al. (2012; BM, A) Gutierrez-Fernandez et al. (2013; BM, AT, A); Honmou et al. (2012; BM, A, S, C)	Steiner et al. (2012; BM, A)	Chen et al. (2001; BM, A) Wei et al. (2012; BM, A) Honmou et al. (2012; BM, A, S, C)	Li et al. (2005; BM, S)	Leu et al. (2010; AT, A) Wei et al. (2012; BM, A)
Intracerebral	Chen et al. (2000; BM, A) Kubis et al. (2007; AT, A)				Chen et al. (2000; BM, A)		Chen et al. (2000; BM, A)		Kubis et al. (2007; AT, A)

BM, bone marrow-derived MSCs; AT, adipose tissue-derived MSCs; A, acute phase infusion; S, subasute phase infusion; C, choronic phase infusion.

Gutierrez-Fernandez's group is important, however, the therapeutic effects in similar experimental ischemic stroke models also differ in transvenous administration studies (Ikegame et al., 2011; Steiner et al., 2012; Gutierrez-Fernandez et al., 2013) compared to intra-arterial administration studies that have shown graft cells in the lesion. (**Table 1**)

On the other hand, nuclear imaging is another available method to assess the therapeutic effectiveness. Diffusion and perfusion-weighted imaging provide information of blood supply in the brain (Canazza et al., 2013). Furthermore, functional MRI is employed by experimental studies in rodents, which unable to assess functional recovery (Suzuki et al., 2013) and even neural network by analyses of resting state functional MRI (Canazza et al., 2013). The neural integrity has been investigated by ^{123}I – Iomazenil SPECT (Saito et al., 2013). A ^{18}F-FDG positron emission tomography study have measured glucose metabolism after MSC therapy in rats for cerebral ischemia (Miyamoto et al., 2013). For assessment of functional recovery, these methods from more biofunctional aspect would be practical in addition to observations of behavioral change.

DIRECTION OF DIFFERENTIATION

The direction of differentiation also remains controversial for *in vivo* experimental studies. Although MSCs are derived from mesenchymal tissue, they exhibit multipotency and transdifferentiation into ectodermal lineages, including neural cells, both *in vitro* and *in vivo* (Zuk, 2013). Previous *in vitro* immunochemistry studies have demonstrated the ability of MSCs to differentiate into cell types that comprise the neurovascular unit, including neurons, astrocytes (Wislet-Gendebien et al., 2004), and endothelial cells (Hess et al., 2002; Planat-Benard et al., 2004). Moreover, possible differentiation abilities toward oligodendrocyte lineage (NG2-positive cells; Shen et al., 2006), specific types of neurons, such as glutamatergic neurons (Yu et al., 2014), and smooth muscle cells of vessels (Kubis et al., 2007) have been demonstrated. *In vivo* studies have reported that graft cells detected in the lesion result from neuronal or glial differentiation (Guzman et al., 2008). However, one study demonstrated the vascular fate rather than differentiation to neural lineages (Kubis et al., 2007).

To ensure the practical differentiation, in addition to these morphological, immunohistochemical, or genetic assessments, cells should be further examined. With respect to neural differentiation, neurotransmitter responsiveness or electrophysiological recording is required to examine their function as a neuron (Yang et al., 2011). Moreover, when MSCs are employed, absence of cell fusion also should be excluded. Though the MSC's rate of spontaneous cell fusion is only 2–11 clones per million cells (Terada et al., 2002), and the mechanism may also participate in the tissue repair, nonetheless, biologically it should be distinguished from differentiation. BMSC and ASC are observed the neural differentiation that can show neural function in earlier studies. First, Ashjian et al. (2003) recorded K^+ current on neuronal cells induced from ASC. Cho et al. (2005) reported synaptic transmission, and Wislet-Gendebien et al. (2005) showed action potential of the neuron-like cells differentiated from BMSC.

AUTOLOGOUS OR ALLOGENIC?

With the exception of the acute phase after ischemic insult, both allogenic and autologous grafting of MSCs can be prepared. Although the efficacy of technologies has improved, besides the advantage of MSCs in immunomodulation, theoretically allogenic grafts cannot ameliorate all concerns regarding transinfection or immunological side effects. Autologous grafts can overcome the problems related to allogenic cells. Nonetheless, at the present stage, other than obtaining the major MSCs, the use of both BMSCs and ASCs requires invasive procedures. Bone marrow aspiration and harvesting of adipose tissue are considered safe and established techniques; however, because ischemic stroke patients usually take antiplatelet or anticoagulant agents, and in some case, the patient may be intolerant to other conditions, further less invasive methods, such as the use of peripheral blood, present alternative sources of cells. As mentioned in the previous section, each type of MSCs from different cell sources tend to exhibit original traits or abilities, although they meet the criteria of MSCs. Knowledge regarding defined factors/conditions for MSC-fate regulation could enable the preparation of homogenous MSCs, even from peripheral blood (Meng et al., 2013).

Autologous grafts may have an additional advantage over allogenic grafts. In preclinical observations, MSCs reportedly developed function following contact with a conditioned media (Egashira et al., 2013), serum (Honmou et al., 2011), or cerebrospinal fluid from patients (Orito et al., 2010), which is reflected in the biological responses to invasive stimulation. It is possible that MSCs may achieve proper function in reaction to insults (Kurozumi et al., 2005; Xin et al., 2013). Therefore, graft cells harvested from ischemic stroke patients may gain more favorable function than allogenic grafts from those who are not affected by ischemic insults. Strikingly, the first nonrandomized clinical trial for a protocol with autologous BMSCs and serum has been shown to be safe and effective (Bang et al., 2005; Lee et al., 2010; Honmou et al., 2011). A 5-year randomized trial also began in 2012, which will provide further information regarding autologous stem cell therapy (Kim et al., 2013).

POSSIBILITY OF ADVANCED MSC THERAPIES AS A SOLUTION OF QUESTIONS

MSC MODIFICATION AND IDENTIFICATION BY DEFINED FACTORS RELATED TO CELL FATE REGULATION

From a pharmacological viewpoint, the actions of agents should be confirmed after administration. If MSCs are regarded as a type of biological drug, then differences in differentiation ability should be better clarified.

Emerging induced pluripotent stem cells (iPSC) studies have shown promising benefits in the field of regenerative medicine that could have at least two major impacts on MSC studies. These findings may be useful to settle the controversies listed above, particularly those regarding the direction of differentiation of graft cells in the host and differences in the characteristics of MSCs originating from the cell source.

First, the appearance of iPSCs indicates the potential of multipotency in somatic cells (Takahashi and Yamanaka, 2006), which is supported by observations of differentiation into either neural or endothelial cells in MSCs. Although many reports

Table 2 | Suggested factors related to cell fate regulation for direct conversion.

	Transcriptional factors *(Yang et al., 2011; Abdullah et al., 2012; Kim et al., 2012; Lujan and Wernig, 2012; Shi and Jiao, 2012)	**microRNA** *(Feng and Feng, 2011; Pham and Gallicano, 2012; Bian et al., 2013)
NSC	Brn4/Sox2/Klf4/c-Myc/E47/Tcf3; Brn2/Ascl1/Myt1L; Sox2; Oct4/Sox2/Klf4/c-Myc	miR-134, miR-195, miR-184, miR-125, miR-137
NPC	Sox2/Oct4/Klf4/c-Myc; Brn2/Ascl1/Myt1L; Ascl1/Ngn2/Hes1/Id1/Pax6/Brn2/Sox2/Klf4/c-Myc; FoxG1/Sox2; Brn2/FoxG1/Sox2; Brn4/Sox2/Klf4/c-Myc	
Neuron	Brn2/Ascl1/Myt1L/miR-124; Brn2/Ascl1/Myt1L/NeuroD1; Ascl1/Myt1L/NeuroD2/miR-9/9*/miR-124; Brn2/Ascl1/Ngn2; Brn2/Ascl1/Myt1L/NeuroD1/Zic1	Let-7b, miR-125b, miR-9, miR-137, miR-124, miR-17, miR-92, miR-106
Dopaminergic neurons	Ascl1/Lmx1a/Nurr1; Ascl1/Lmx1a/Nurr1/Brn2/Mytl1L/FoxA2	miR-133b, miR-132, miR-7a
Spinal motor neurons	Brn2/Ascl1/Myt1L/NeuroD1/Lhx3/Hb9/Isl1/Ngn2	miR-17-3p, miR-9
Glia		
Astrocyte	FoxG1/Sox2; Sox2/Oct4/Klf4/c-Myc	miR-125b, miR-24, miR-29
Oligodendeoglial precursur cell	Sox2/Olg2/Zfp536 (Yang et al., 2013)	miR-7, miR-219, miR-23, miR-338

**Review articles.*

have demonstrated the ability MSCs of mesodermal origin to differentiate into other type of germ cells of ectodermal lineages (neural cells) and endodermal lineages (insulin-producing cells), which could indicate multipotency, the defined conditions for MSCs to differentiate into neural cells remain uncertain. In the infancy of stem cell research, cell fusion and contamination of neural crest cells were suggested as the mechanism of a graft cell to express neural markers in the host tissue after cell administration (Wrage et al., 2008; Maltman et al., 2011). If the postulates reveal to be the main mechanism, neural marker expression can't be called neural differentiation, which unable MSC to be called "stem cell." Therefore, until recently, the term "MSC" containing the term "stem cell" had its pros and cons, and thus, MSCs were called stromal cells. However, successful reprogramming of skin fibroblasts to the multipotent state has provided more information to support the multipotency of MSCs.

Second, induction techniques may contribute to further elucidate the quality control mechanisms for the use of MSCs. Protocols for chemical induction to neuron or glia had been developed recently (Safford and Rice, 2005; Franco Lambert et al., 2009; Yu et al., 2011). Following the publication of methods to harness and propagate iPSCs, other methods related to direct conversion from fibroblasts to neuronal cells by defined transcription factors have been reported (Vierbuchen et al., 2010; Yang et al., 2013). The neural lineage is composed of induced neuronal (iN) cells, induced neural progenitor cells (iNPCs), and induced NSCs (iNSCs; Yang et al., 2011; Abdullah et al., 2012; Corti et al., 2012; Shi and Jiao, 2012). Moreover, iPSC-derived MSCs (iPSC–MSCs) were identified (Jung et al., 2012). There are multiple pathways for neural induction. As listed in the **Table 2**, in addition to defined transcriptional factors for direct conversion, microRNA (Feng and Feng, 2011; Pham and Gallicano, 2012; Bian et al., 2013) or other epigenetic factors (Namihira and Nakashima, 2011) can contribute to differentiation. The definitive conditions to propagate/identify iN cells, iNSCs, iNPCs, or iPSC–MSCs may be useful to propose a standard protocol for the required type of MSCs.

ORGANOGENESIS FOR TISSUE REPLACEMENT

Lancaster et al.'s (2013) team developed a three-dimensional brain tissue from iPSCs by the floating culture method. To obtain functional recovery *in vivo*, several groups have shown that tissue regeneration or replacement of damaged tissue with *ex vivo* materials is not always necessary (**Table 1**). Particularly in the brain tissue, repair of the neural circuitry is required to improve function. Nonetheless, tissue engineering using scaffolds (Mahmood et al., 2013) or novel organogenesis methods present possible transplantation treatments to recover neurological deficits.

CONCLUSION

Since the first report of MSC (Pittenger et al., 1999), investigators have revealed favorable cell characteristics for cell therapies and have shown evidence for feasible stem cell therapy using MSCs in order to achieve safe applications in clinical settings. However, there are limited methods to ensure reliable treatment. Nevertheless, further studies combined with developments in other biological and/or engineering fields may solve these present problems, and establish an ideal stem cell therapy beyond categorization of MSCs.

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REFERENCES

Abdullah, A. I., Pollock, A., and Sun, T. (2012). The path from skin to brain: generation of functional neurons from fibroblasts. *Mol. Neurobiol.* 45, 586–595. doi: 10.1007/s12035-012-8277-6

Ashjian, P. H., Elbarbary, A. S., Edmonds, B., Deugarte, D., Zhu, M., Zuk, P. A., et al. (2003). In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. *Plast. Reconstr. Surg.* 111, 1922–1931. doi: 10.1097/01.PRS.0000055043.62589.05

Bang, O. Y., Lee, J. S., Lee, P. H., and Lee, G. (2005). Autologous mesenchymal stem cell transplantation in stroke patients. *Ann. Neurol.* 57, 874–882. doi: 10.1002/ana.20501

Bhasin, A., Srivastava, M. V., Mohanty, S., Bhatia, R., Kumaran, S. S., and Bose, S. (2013). Stem cell therapy: a clinical trial of stroke. *Clin. Neurol. Neurosurg.* 115, 1003–1008. doi: 10.1016/j.clineuro.2012.10.015

Bian, S., Xu, T. L., and Sun, T. (2013). Tuning the cell fate of neurons and glia by microRNAs. *Curr. Opin. Neurobiol.* 23, 928–934. doi: 10.1016/j.conb.2013.08.002

Borlongan, C. V. (2009). Cell therapy for stroke: remaining issues to address before embarking on clinical trials. *Stroke* 40, S146–S148. doi: 10.1161/STROKEAHA.108.533091

Canazza, A., Minati, L., Boffano, C., Parati, E., and Binks, S. (2013). Experimental models of brain ischemia: a review of techniques, magnetic resonance imaging, and investigational cell-based therapies. *Front. Neurol.* 5:19. doi: 10.3389/fneur.2014.00019

Chen, J., Li, Y., and Chopp, M. (2000). Intracerebral transplantation of bone marrow with BDNF after MCAo in rat. *Neuropharmacology* 39, 711–716. doi: 10.1016/S0028-3908(00)00006-X

Chen, J., Li, Y., Wang, L., Lu, M., Zhang, X., and Chopp, M. (2001). Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J. Neurol. Sci.* 189, 49–57. doi: 10.1002/jnr.10691

Cho, K. J., Trzaska, K. A., Greco, S. J., Mcardle, J., Wang, F. S., Ye, J. H., et al. (2005). Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1 alpha. *Stem Cells* 23, 383–391. doi: 10.1634/stemcells.2004-0251

Corti, S., Nizzardo, M., Simone, C., Falcone, M., Donadoni, C., Salani, S., et al. (2012). Direct reprogramming of human astrocytes into neural stem cells and neurons. *Exp. Cell Res.* 318, 1528–1541. doi: 10.1016/j.yexcr.2012.02.040

Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103, 481–490. doi: 10.1016/s0092-8674(00)00139-2

Degraba, T. J., and Pettigrew, L. C. (2000). Why do neuroprotective drugs work in animals but not humans? *Neurol. Clin.* 18, 475–493. doi: 10.1016/S0092-8674(00)00139-2

Detante, O., Moisan, A., Dimastromatteo, J., Richard, M. J., Riou, L., Grillon, E., et al. (2009). Intravenous administration of 99mTc-HMPAO-labeled human mesenchymal stem cells after stroke: in vivo imaging and biodistribution. *Cell Transplant.* 18, 1369–1379. doi: 10.3727/096368909X474230

Detante, O., Valable, S., De Fraipont, F., Grillon, E., Barbier, E. L., Moisan, A., et al. (2012). Magnetic resonance imaging and fluorescence labeling of clinical-grade mesenchymal stem cells without impacting their phenotype: study in a rat model of stroke. *Stem Cells Transl. Med.* 1, 333–341. doi: 10.5966/sctm.2011-0043

Doeppner, T. R., and Hermann, D. M. (2010). Mesenchymal stem cells in the treatment of ischemic stroke: progress and possibilities. *Stem Cells Cloning* 3, 157–163. doi: 10.2147/SCCAA.S7820

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317. doi: 10.1080/14653240600855905

Eckert, M. A., Vu, Q., Xie, K., Yu, J., Liao, W., Cramer, S. C., et al. (2013). Evidence for high translational potential of mesenchymal stromal cell therapy to improve recovery from ischemic stroke. *J. Cereb. Blood Flow Metab.* 33, 1322–1334. doi: 10.1038/jcbfm.2013.91

Egashira, Y., Suzuki, Y., Azuma, Y., Takagi, T., Mishiro, K., Sugitani, S., et al. (2013). The growth factor progranulin attenuates neuronal injury induced by cerebral ischemia-reperfusion through the suppression of neutrophil recruitment. *J. Neuroinflammation* 10, 105. doi: 10.1186/1742-2094-10-105

Erices, A., Conget, P., and Minguell, J. J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.* 109, 235–242. doi: 10.1046/j.1365-2141.2000.01986.x

Feng, W., and Feng, Y. (2011). MicroRNAs in neural cell development and brain diseases. *Sci. China Life Sci.* 54, 1103–1112. doi: 10.1007/s11427-011-4249-8

Franco Lambert, A. P., Fraga Zandonai, A., Bonatto, D., Cantarelli Machado, D., and Pegas Henriques, J. A. (2009). Differentiation of human adipose-derived adult stem cells into neuronal tissue: does it work? *Differentiation* 77, 221–228. doi: 10.1016/j.diff.2008.10.016

Friedenstein, A. J., Piatetzky-Shapiro, I. I., and Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. *J. Embryol. Exp. Morphol.* 16, 381–390.

Ge, J., Guo, L., Wang, S., Zhang, Y., Cai, T., Zhao, R. C., et al. (2014). The size of mesenchymal stem cells is a significant cause of vascular obstructions and stroke. *Stem Cell Rev.* 10, 295–303. doi: 10.1007/s12015-013-9492-x

Grande, A., Sumiyoshi, K., Lopez-Juarez, A., Howard, J., Sakthivel, B., Aronow, B., et al. (2013). Environmental impact on direct neuronal reprogramming in vivo in the adult brain. *Nat. Commun.* 4, 2373. doi: 10.1038/ncomms3373

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13625–13630. doi: 10.1073/pnas.240309797

Guo, S., and Lo, E. H. (2009). Dysfunctional cell-cell signaling in the neurovascular unit as a paradigm for central nervous system disease. *Stroke* 40, S4–S7. doi: 10.1161/STROKEAHA.108.534388

Gutierrez-Fernandez, M., Rodriguez-Frutos, B., Ramos-Cejudo, J., Teresa Vallejo-Cremades, M., Fuentes, B., Cerdan, S., et al. (2013). Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res. Ther.* 4, 11. doi: 10.1186/scrt159

Guzman, R., Choi, R., Gera, A., De Los Angeles, A., Andres, R. H., and Steinberg, G. K. (2008). Intravascular cell replacement therapy for stroke. *Neurosurg. Focus* 24, E15. doi: 10.3171/FOC/2008/24/3-4/E14

Hao, L., Zou, Z., Tian, H., Zhang, Y., Zhou, H., and Liu, L. (2014). Stem cell-based therapies for ischemic stroke. *Biomed. Res. Int.* 2014, 468748. doi: 10.1155/2014/468748

Hess, D. C., Hill, W. D., Martin-Studdard, A., Carroll, J., Brailer, J., and Carothers, J. (2002). Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. *Stroke* 33, 1362–1368. doi: 10.1161/01.STR.0000014925.09415.C3

Honmou, O., Houkin, K., Matsunaga, T., Niitsu, Y., Ishiai, S., Onodera, R., et al. (2011). Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 134, 1790–1807. doi: 10.1093/brain/awr063

Honmou, O., Onodera, R., Sasaki, M., Waxman, S. G., and Kocsis, J. D. (2012). Mesenchymal stem cells: therapeutic outlook for stroke. *Trends Mol. Med.* 18, 292–297. doi: 10.1016/j.molmed.2012.02.003

Hoyte, L., Barber, P. A., Buchan, A. M., and Hill, M. D. (2004). The rise and fall of NMDA antagonists for ischemic stroke. *Curr. Mol. Med.* 4, 131–136. doi: 10.2174/1566524043479248

Hsiao, S. T., Asgari, A., Lokmic, Z., Sinclair, R., Dusting, G. J., Lim, S. Y., et al. (2012). Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. *Stem Cells Dev.* 21, 2189–2203. doi: 10.1089/scd.2011.0674

Ikegame, Y., Yamashita, K., Hayashi, S., Mizuno, H., Tawada, M., You, F., et al. (2011). Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. *Cytotherapy* 13, 675–685. doi: 10.3109/14653249.2010.549122

Ishizaka, S., Horie, N., Satoh, K., Fukuda, Y., Nishida, N., and Nagata, I. (2013). Intra-arterial cell transplantation provides timing-dependent cell distribution and functional recovery after stroke. *Stroke* 44, 720–726. doi: 10.1161/STROKEAHA.112.677328

Jiang, W., Liang, G., Li, X., Li, Z., Gao, X., Feng, S., et al. (2014). Intracarotid transplantation of autologous adipose-derived mesenchymal stem cells significantly improves neurological deficits in rats after MCAo. *J. Mater. Sci. Mater. Med.* 25, 1357–1366. doi: 10.1007/s10856-014-5157-9

Jung, Y., Bauer, G., and Nolta, J. A. (2012). Concise review: induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. *Stem Cells* 30, 42–47. doi: 10.1002/stem.727

Kalladka, D., and Muir, K. W. (2014). Brain repair: cell therapy in stroke. *Stem Cells Cloning* 7, 31–44. doi: 10.2147/SCCAA.S38003

Kang, S. G., Shinojima, N., Hossain, A., Gumin, J., Yong, R. L., Colman, H., et al. (2010). Isolation and perivascular localization of mesenchymal stem cells from mouse brain. *Neurosurgery* 67, 711–720. doi: 10.1227/01.NEU.0000377859.06219.78

Kern, S., Eichler, H., Stoeve, J., Klueter, H., and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 24, 1294–1301. doi: 10.1634/stemcells.2005-0342

Kim, J., Ambasudhan, R., and Ding, S. (2012). Direct lineage reprogramming to neural cells. *Curr. Opin. Neurobiol* 22, 778–784. doi: 10.1016/j.conb.2012.05.001

Kim, S. J., Moon, G. J., Chang, W. H., Kim, Y. H., and Bang, O. Y. (2013). Intravenous transplantation of mesenchymal stem cells preconditioned with early phase stroke serum: current evidence and study protocol for a randomized trial. *Trials* 14, 317. doi: 10.1186/1745-6215-14-317

Kubis, N., Tomita, Y., Tran-Dinh, A., Planat-Benard, V., Andre, M., Karaszewski, B., et al. (2007). Vascular fate of adipose tissue-derived adult stromal cells in the ischemic murine brain: a combined imaging-histological study. *Neuroimage* 34, 1–11. doi: 10.1016/j.neuroimage.2006.09.014

Kurozumi, K., Nakamura, K., Tamiya, T., Kawano, Y., Ishii, K., Kobune, M., et al. (2005). Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol. Ther.* 11, 96–104. doi: 10.1016/j.ymthe.2004.09.020

Lalu, M. M., Mcintyre, L., Pugliese, C., Fergusson, D., Winston, B. W., Marshall, J. C., et al. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 7:e47559. doi: 10.1371/journal.pone.0047559

Lancaster, M. A., Renner, M., Martin, C. A., Wenzel, D., Bicknell, L. S., Hurles, M. E., et al. (2013). Cerebral organoids model human brain development and microcephaly. *Nature* 501, 373–379. doi: 10.1038/nature12517

Lee, J. S., Hong, J. M., Moon, G. J., Lee, P. H., Ahn, Y. H., and Bang, O. Y. (2010). A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 28, 1099–1106. doi: 10.1002/stem.430

Leu, S., Lin, Y. C., Yuen, C. M., Yen, C. H., Kao, Y. H., Sun, C. K., et al. (2010). Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. *J. Transl. Med.* 8, 63. doi: 10.1186/1479-5876-8-63

Li, H., Fan, X., Kovi, R. C., Jo, Y., Moquin, B., Konz, R., et al. (2007). Spontaneous expression of embryonic factors and p53 point mutations in aged mesenchymal stem cells: a model of age-related tumorigenesis in mice. *Cancer Res.* 67, 10889–10898. doi: 10.1158/0008-5472.can-07-2665

Li, Y., Chen, J., Zhang, C. L., Wang, L., Lu, D., Katakowski, M., et al. (2005). Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells. *Glia* 49, 407–417. doi: 10.1002/glia.20126

Li, Y., Chopp, M., Chen, J., Wang, L., Gautam, S. C., Xu, Y.-X., et al. (2000). Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. *J. Cereb. Blood Flow Metab.* 20, 1311–1319. doi: 10.1097/00004647-200009000-00006

Liu, L., Eckert, M. A., Riazifar, H., Kang, D. K., Agalliu, D., and Zhao, W. (2013). From blood to the brain: can systemically transplanted mesenchymal stem cells cross the blood-brain barrier? *Stem Cells Int.* 2013, 435093. doi: 10.1155/2013/435093

Locatelli, F., Bersano, A., Ballabio, E., Lanfranconi, S., Papadimitriou, D., Strazzer, S., et al. (2009). Stem cell therapy in stroke. *Cell. Mol. Life Sci.* 66, 757–772. doi: 10.1007/s00018-008-8346-1

Lujan, E., and Wernig, M. (2012). The many roads to Rome: induction of neural precursor cells from fibroblasts. *Curr. Opin. Genet. Dev.* 22, 517–522. doi: 10.1016/j.gde.2012.07.002

Mahmood, A., Wu, H., Qu, C., Xiong, Y., and Chopp, M. (2013). Effects of treating traumatic brain injury with collagen scaffolds and human bone marrow stromal cells on sprouting of corticospinal tract axons into the denervated side of the spinal cord. *J. Neurosurg.* 118, 381–389. doi: 10.3171/2012.11.JNS12753

Maltman, D. J., Hardy, S. A., and Przyborski, S. A. (2011). Role of mesenchymal stem cells in neurogenesis and nervous system repair. *Neurochem. Int.* 59, 347–356. doi: 10.1016/j.neuint.2011.06.008

Meng, X., Su, R. J., Baylink, D. J., Neises, A., Kiroyan, J. B., Lee, W. Y., et al. (2013). Rapid and efficient reprogramming of human fetal and adult blood CD34+ cells into mesenchymal stem cells with a single factor. *Cell Res.* 23, 658–672. doi: 10.1038/cr.2013.40

Mitkari, B., Kerkela, E., Nystedt, J., Korhonen, M., Mikkonen, V., Huhtala, T., et al. (2013). Intra-arterial infusion of human bone marrow-derived mesenchymal stem cells results in transient localization in the brain after cerebral ischemia in rats. *Exp. Neurol.* 239, 158–162. doi: 10.1016/j.expneurol.2012.09.018

Miyamoto, M., Kuroda, S., Zhao, S., Magota, K., Shichinohe, H., Houkin, K., et al. (2013). Bone marrow stromal cell transplantation enhances recovery of local glucose metabolism after cerebral infarction in rats: a serial 18F-FDG PET study. *J. Nucl. Med.* 54, 145–150. doi: 10.2967/jnumed.112.109017

Namihira, M., and Nakashima, K. (2011). "Fate specification of neural stem cells," in *Neurogenesis in the Adult Brain I*, eds T. Seki, K. Sawamoto, J. M. Parent, and A. Alvarez-Buylla (Tokyo: Springer Japan), 87–107. doi: 10.1007/978-4-431-53933-9_3

Orito, K., Harada, H., Hara, M., Yamashita, S., Kikuchi, K., and Shigemori, M. (2010). Cerebrospinal fluid following cerebral ischemia accelerates the proliferation of bone marrow stromal cells in vitro. *Kurume Med. J.* 57, 21–28. doi: 10.2739/kurumemedj.57.21

Paul, G., Ozen, I., Christophersen, N. S., Reinbothe, T., Bengzon, J., Visse, E., et al. (2012). The adult human brain harbors multipotent perivascular mesenchymal stem cells. *PLoS ONE* 7:e35577. doi: 10.1371/journal.pone.0035577

Pham, J. T., and Gallicano, G. I. (2012). Specification of neural cell fate and regulation of neural stem cell proliferation by microRNAs. *Am. J. Stem Cells* 1, 182–195.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147. doi: 10.1126/science.284.5411.143

Planat-Benard, V., Silvestre, J. S., Cousin, B., Andre, M., Nibbelink, M., Tamarat, R., et al. (2004). Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. *Circulation* 109, 656–663. doi: 10.1161/01.CIR.0000114522.38265.61

Renfranz, P. J., Cunningham, M. G., and Mckay, R. D. (1991). Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 66, 713–729. doi: 10.1016/0092-8674(91)90116-G

Rosado-De-Castro, P. H., Pimentel-Coelho, P. M., Da Fonseca, L. M., De Freitas, G. R., and Mendez-Otero, R. (2013a). The rise of cell therapy trials for stroke: review of published and registered studies. *Stem Cells Dev.* 22, 2095–2111. doi: 10.1089/scd.2013.0089

Rosado-De-Castro, P. H., Schmidt Fda, R., Battistella, V., Lopes De Souza, S. A., Gutfilen, B., Goldenberg, R. C., et al. (2013b). Biodistribution of bone marrow mononuclear cells after intra-arterial or intravenous transplantation in subacute stroke patients. *Regen. Med.* 8, 145–155. doi: 10.2217/rme.13.2

Safford, K. M., and Rice, H. E. (2005). Stem cell therapy for neurologic disorders: therapeutic potential of adipose-derived stem cells. *Curr. Drug Targets* 6, 57–62. doi: 10.2174/1389450053345028

Saito, H., Magota, K., Zhao, S., Kubo, N., Kuge, Y., Shichinohe, H., et al. (2013). ^{123}I-iomazenil single photon emission computed tomography visualizes recovery of neuronal integrity by bone marrow stromal cell therapy in rat infarct brain. *Stroke* 44, 2869–2874. doi: 10.1161/STROKEAHA.113.001612

Savitz, S. I., Chopp, M., Deans, R., Carmichael, T., Phinney, D., and Wechsler, L. (2011). Stem cell therapy as an emerging paradigm for stroke (STEPS) II. *Stroke* 42, 825–829. doi: 10.1161/STROKEAHA.110.601914

Shen, L. H., Li, Y., Chen, J., Zhang, J., Vanguri, P., Borneman, J., et al. (2006). Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. *Neuroscience* 137, 393–399. doi: 10.1016/j.neuroscience.2005.08.092

Shi, Z., and Jiao, J. (2012). Direct lineage conversion: induced neuronal cells and induced neural stem cells. *Protein Cell* 3, 826–833. doi: 10.1007/s13238-012-2068-8

Snyder, E. Y., Deitcher, D. L., Walsh, C., Arnold-Aldea, S., Hartwieg, E. A., and Cepko, C. L. (1992). Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* 68, 33–51. doi: 10.1016/0092-8674(92)90204-P

Steiner, B., Roch, M., Holtkamp, N., and Kurtz, A. (2012). Systemically administered human bone marrow-derived mesenchymal stem home into peripheral organs but do not induce neuroprotective effects in the MCAo-mouse model

for cerebral ischemia. *Neurosci. Lett.* 513, 25–30. doi: 10.1016/j.neulet.2012.01.078

Suzuki, J., Sasaki, M., Harada, K., Bando, M., Kataoka, Y., Onodera, R., et al. (2013). Bilateral cortical hyperactivity detected by fMRI associates with improved motor function following intravenous infusion of mesenchymal stem cells in a rat stroke model. *Brain Res.* 1497, 15–22. doi: 10.1016/j.brainres.2012.12.028

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Teng, H., Zhang, Z. G., Wang, L., Zhang, R. L., Zhang, L., Morris, D., et al. (2008). Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J. Cereb. Blood Flow Metab.* 28, 764–771. doi: 10.1038/sj.jcbfm.9600573

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., et al. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 416, 542–545. doi: 10.1038/nature730

The STEPS Participants. (2009). Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. *Stroke* 40, 510–515. doi: 10.1161/STROKEAHA.108.526863

Ukai, R., Honmou, O., Harada, K., Houkin, K., Hamada, H., and Kocsis, J. D. (2007). Mesenchymal stem cells derived from peripheral blood protects against ischemia. *J. Neurotrauma* 24, 508–520. doi: 10.1089/neu.2006.0161

Vasconcelos-Dos-Santos, A., Rosado-De-Castro, P. H., Lopes De Souza, S. A., Da Costa Silva, J., Ramos, A. B., Rodriguez De Freitas, G., et al. (2012). Intravenous and intra-arterial administration of bone marrow mononuclear cells after focal cerebral ischemia: is there a difference in biodistribution and efficacy? *Stem Cell Res.* 9, 1–8. doi: 10.1016/j.scr.2012.02.002

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Sudhof, T. C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035–1041. doi: 10.1038/nature08797

Wan, H., Li, F., Zhu, L., Wang, J., Yang, Z., and Pan, Y. (2013). Update on therapeutic mechanism for bone marrow stromal cells in ischemic stroke. *J. Mol. Neurosci.* 52, 177–185. doi: 10.1007/s12031-013-0119-0

Wei, L., Fraser, J. L., Lu, Z. Y., Hu, X., and Yu, S. P. (2012). Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiol. Dis.* 46, 635–645. doi: 10.1016/j.nbd.2012.03.002

Wislet-Gendebien, S., Bruyère, F., Hans, G., Leprince, P., Moonen, G., and Rogister, B. (2004). Nestin-positive mesenchymal stem cells favour the astroglial lineage in neural progenitors and stem cells by releasing active BMP4. *BMC Neurosci.* 5:33. doi: 10.1186/1471-2202-5-33

Wislet-Gendebien, S., Hans, G., Leprince, P., Rigo, J. M., Moonen, G., and Rogister, B. (2005). Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. *Stem Cells* 23, 392–402. doi: 10.1634/stemcells.2004-0149

Wrage, P. C., Tran, T., To, K., Keefer, E. W., Ruhn, K. A., Hong, J., et al. (2008). The neuro-glial properties of adipose-derived adult stromal (ADAS) cells are not regulated by Notch 1 and are not derived from neural crest lineage. *PLoS ONE* 3:e1453. doi: 10.1371/journal.pone.0001453

Xin, H., Li, Y., Cui, Y., Yang, J. J., Zhang, Z. G., and Chopp, M. (2013). Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J. Cereb. Blood Flow Metab.* 33, 1711–1715. doi: 10.1038/jcbfm.2013.152

Yang, N., Ng, Y. H., Pang, Z. P., Sudhof, T. C., and Wernig, M. (2011). Induced neuronal cells: how to make and define a neuron. *Cell Stem Cell* 9, 517–525. doi: 10.1016/j.stem.2011.11.015

Yang, N., Zuchero, J. B., Ahlenius, H., Marro, S., Ng, Y. H., Vierbuchen, T., et al. (2013). Generation of oligodendroglial cells by direct lineage conversion. *Nat. Biotechnol.* 31, 434–439. doi: 10.1038/nbt.2564

Yavagal, D. R., Lin, B., Raval, A. P., Garza, P. S., Dong, C., Zhao, W., et al. (2014). Efficacy and dose-dependent safety of intra-arterial delivery of mesenchymal stem cells in a rodent stroke model. *PLoS ONE* 9:e93735. doi: 10.1371/journal.pone.0093735

Yu, J. M., Bunnell, B. A., and Kang, S. K. (2011). Neural differentiation of human adipose tissue-derived stem cells. *Methods Mol. Biol.* 702, 219–231. doi: 10.1007/978-1-61737-960-4_16

Yu, Z., Wu, S., Liu, Z., Lin, H., Chen, L., Yuan, X., et al. (2014). Sonic hedgehog and retinoic acid induce bone marrow-derived stem cells to differentiate into glutamatergic neural cells. *J. Immunoassay Immunochem.* 36, 1–15. doi: 10.1080/15321819.2014.889025

Zhang, Z. G., Zhang, L., Jiang, Q., Zhang, R., Davies, K., Powers, C., et al. (2000). VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J. Clin. Invest.* 106, 829–838. doi: 10.1172/jci9369

Zuk, P. (2013). Adipose-derived stem cells in tissue regeneration: a review. *ISRN Stem Cells* 2013, 35. doi: 10.1155/2013/713959

Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., et al. (2002). Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13, 4279–4295. doi: 10.1091/mbc.E02-02-0105

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Granulocyte colony-stimulating factor (G-CSF) treatment in combination with transplantation of bone marrow cells is not superior to G-CSF treatment alone after cortical stroke in spontaneously hypertensive rats

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Granulocyte-colony stimulating factor (G-CSF) and bone marrow derived mononuclear cells (BM-MNCs) have both been shown to improve functional outcome following experimental stroke. These effects are associated with increased angiogenesis and neurogenesis. In the present study, we aimed to determine synergistic effects of G-CSF and BM-NMC treatment on long-term structural and functional recovery after photothrombotic stroke. To model the etiology of stroke more closely, we used spontaneously hypertensive (SH) rats in our experiment. Bone marrow derived mononuclear cells transplantation was initiated 1 h after the onset of photothrombotic stroke. Repeated G-CSF treatment commenced immediately after BM-MNC treatment followed by daily injections for five consecutive days. The primary endpoint was functional outcome after ischemia. Secondary endpoints included analysis of neurogenesis and angiogenesis as well as determination of infarct size. Granulocyte-colony stimulating factor treated rats, either in combination with BM-MNC or alone showed improved somatosensory but not gross motor function following ischemia. No beneficial effect of BM-MNC monotherapy was found. Infarct volumes were comparable in all groups. In contrast to previous studies, which used healthy animals, post-stroke neurogenesis and angiogenesis were not enhanced by G-CSF. In conclusion, the combination of G-CSF and BM-MNC was not more effective than G-CSF alone. The reduced efficacy of G-CSF treatment and the absence of any beneficial effect of BM-MNC transplantation might be attributed to hypertension-related morbidity.

Keywords: stroke, spontaneously hypertensive rat (SHR), bone marrow cells, G-CSF, neuroregeneration, neurogenesis, angiogenesis, functional recovery

INTRODUCTION

Stroke is a lethal disease, yet it disables more than it kills. Better controls of risk factors in preventing stroke and improved treatment options for those who have had a stroke are essential for diminishing its devastating consequences. There are substantial research efforts being made to develop treatments that improve the outcome following stroke. Both, the stimulation of endogenous bone marrow cells by the granulocyte colony-stimulating growth factor (G-CSF) and the transplantation of bone marrow mononuclear cells (BM-MNCs) were shown to enhance regeneration in a large number of animal stroke studies (Schneider et al., 2005; Giraldi-Guimarães et al., 2009; Diederich et al., 2012b). Mechanisms underlying G-CSF and BM-MNC induced functional recovery after stroke include the potentiation of endogenous neurogenesis and angiogenesis as well as an increased dendritic plasticity (Lee et al., 2005; Schneider et al., 2005). Granulocyte-colony stimulating factor mediates its actions on these mechanisms by the mobilization of bone marrow cells and by direct neuronal effects (Schneider et al., 2005). The BM-MNC mode of action is assumed to be based on their production and secretion of cytokines, which in turn modulate endogenous repair mechanisms. We hypothesized that the combination of G-CSF and BM-MNCs is more effective than either treatment alone because transplantation of exogenous BM-MNCs can bridge the gap until G-CSF mobilizes endogenous BM-MNCs into the blood. In addition, according to its genuine function, G-CSF might increase the survival of transplanted BM-MNCs and thereby improve their efficacy.

Besides its pro-regenerative effects G-CSF also exerts neuroprotective actions in animal stroke models (Minnerup et al., 2008; England et al., 2009; Sevimli et al., 2009; Strecker et al.,

2010). These neuroprotective modes of action, however, could not be confirmed in a recent clinical trial (Ringelstein et al., 2013). In our study we focused on G-CSFś effects on regeneration rather than on neuroprotective actions. We used the photothrombotic stroke model, which is characterized by small and highly reproducible infarct sizes and the reliable evocation of neuroregenerative events like neurogenesis, angiogenesis and dendritogenesis (Carmichael, 2005). We chose spontaneously hypertensive (SH) rats for our experiments for two reasons: first, the use of healthy animals without comorbidities was demonstrated to overstate the efficacy of candidate stroke treatments and second, SH rats are syngeneic, thus transplantation of BM-MNCs from one SH rat to another simulates autologous transplantation, which might be more practical from a translational perspective.

EXPERIMENTAL PROCEDURES

ANIMALS

All animal procedures were approved by the responsible ethics committee of the University of Münster and the appropriate authorities of the Federal State of North Rhine-Westphalia. The investigations were carried out in accordance with national and international animal welfare regulations and are reported in accordance with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines (Kilkenny et al., 2011). Surgery and evaluation of all read-outs were performed blinded to experimental groups. Experiments were performed on adult (12–13 weeks old) male SH rats weighing 260–290 g. Spontaneously hypertensive rats were shown to have an increased blood pressure starting from 5 to 6 weeks of age (Dickhout and Lee, 1998). All animals were randomly assigned to one of the following treatment groups: (1) placebo (n = 13); (2) G-CSF 50 μg/kg/day (n = 14); (3) 5 million BM-MNCs/rat (n = 14); or (4) 5 million BM-MNCs/rat and G-CSF 50 μg/kg/day (n = 14). One animal of the placebo group died during surgery. BM-MNC transplantation was initiated 1 h after the onset of the photothrombosis. Repeated G-CSF treatment started immediately after BM-MNC treatment followed by daily injections for five consecutive days.

The cell numbers used in our experiments were based on previous studies that investigated different intravenous cell therapies in animal stroke models (Iihoshi et al., 2004; Giraldi-Guimarães et al., 2009; Minnerup et al., 2014). The condition of animals was monitored at least every 8 h. Pre-defined termination criteria were: (1) a severe immobility; and (2) a persisting abnormal body position. The implementation of these criteria was required by the local ethics committee.

BONE MARROW MONONUCLEAR CELL PREPARATION

Bone marrow derived mononuclear cells were prepared as previously described (Minnerup et al., 2014). Briefly, syngeneic rat bone marrow was obtained from male SH rats at the age of 12 weeks. Femurs and tibias were aseptically opened and repeatedly flushed with phosphate buffered saline (PBS). After erythrocyte lysis by ammonium chloride-based buffer (0.155 M NH_4Cl, 10 mM $KHCO_3$ and 0.01 mM Na_2EDTA) cells were filtered by a 100 μm cell strainer, counted and prepared for immunomagnetic depletion of granulocytes: bone marrow cells were incubated with 10 ng/ml Phycoerythrin-conjugated anti-rat granulocyte antibody (clone RP1; BD Pharmingen, Heidelberg, Germany) for 15 min at 4°C. Subsequently, cells were washed with cold PBS plus 0.5% fetal calf serum (FCS) and incubated with 200 μl anti-Phycoerythrin MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) in 800 μl PBS plus 5% FCS for 15 min at 4°C. After incubation, non-adsorbed MicroBeads were removed by a further washing step. The cell suspension was then resuspended in 500 μl PBS plus 0.5% FCS and magnetically separated by a LD-column according to the manufacturer's instructions (Miltenyi). This procedure results in higher BM-MNC purity compared to standard density gradient centrifugation (Pösel et al., 2012). The obtained mononuclear cell fraction was collected, counted, cryopreserved in liquid nitrogen (25 million mononuclear cells in 1 ml FCS plus 8% DMSO) and stored at −80°C until further use. Vital cell numbers were determined by the trypan blue exclusion method using a hemocytometer (Pösel et al., 2012). Cellular composition of cell grafts was characterized by flow cytometry for B cells (CD45R+), T cells (CD3+) and myeloid cells (CD11b+ and RP1-).

STROKE MODEL AND THERAPY

The photothrombotic stroke model was utilized in this study and was executed as previously described (Schmidt et al., 2012). In brief, animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; Ketanest) and xylazine hydrochloride (8 mg/kg body weight). The left femoral vein was cannulated with a PE-50 tube for Bengal Rose infusion. The rectal temperature was maintained at 37°C by a thermostat-controlled heating pad (Föhr Medical Instruments). Photothrombotic ischemia was induced in the right frontal cortex. For illumination, a laser spot of 8 mm in diameter (G Laser Technologies) was placed stereotaxically onto the skull 0.5 mm anterior to the bregma and 3.5 mm lateral from the midline. The skull was illuminated for 20 min. During the first 2 min of illumination, the dye Bengal Rose (0.133 mL/kg body weight, 10 mg/mL saline) was injected intravenously. One hour after onset of the photothrombosis animals received BM-MNCs or vehicle intravenously. Animals of the respective treatment groups received treatment with G-CSF or saline daily for five consecutive days starting immediately after BM-NMC-treatment. To label dividing cells, each animal received a daily bromodeoxyuridine (BrdU) injection (50 mg/kg per day intraperitoneally) throughout the 5-day treatment period, 1 h before the respective G-CSF or saline injection.

FUNCTIONAL TESTING

Behavioral testing was conducted as previously described (Diederich et al., 2012a). In all animals, behavioral tests were performed before ischemia (baseline) as well as on days 1, 7, 14, 21, and 28 after ischemia by an investigator blinded to the experimental groups.

Motor deficits were examined by means of the cylinder test. For this purpose, the rats were placed into a transparent cylinder

and videotaped from below for 3 min. Spontaneous wall and ground touches of the forepaws were counted. An asymmetry score calculated for each animal was expressed by the following ratio: wall and ground touches of the ipsilateral forepaw—wall and ground touches of the contralateral forepaw/wall and ground touches of the ipsilateral forepaw+wall and ground touches of the contralateral forepaw.

Somatosensory deficits were measured using the adhesive removal test. Two small pieces of adhesive-backed paper dots of equal size used as bilateral tactile stimuli occupying the distal–radial region were placed at the wrist of each forelimb. The time to remove each stimulus was documented. An asymmetry score calculated for each animal was expressed by the following ratio: time to remove the ipsilateral dot—time to remove the contralateral dot/time to remove the ipsilateral dot+time to remove the contralateral dot.

TISSUE PROCESSING

Twenty-eight days after ischemia, animals were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were fixed in 4% paraformaldehyde at 4°C and then cryoprotected in 30% sucrose solution. Tissue was stored at −80°C until analysis.

DETERMINATION OF INFARCT SIZE

Lesion volumes were estimated by measurement of the maximum diameter and measurement of the maximum infarct areas on the slides, as previously described (Müller et al., 2008). Because infarct size and tissue loss do not always match, an additional analysis of the remaining cortical tissue was performed at the level of the largest infarct extension.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on coronal free-floating 40 μm sections with the following antibodies: anti-NeuN (1:200; Millipore, Darmstadt, Germany), rat anti-BrdU (1:500; Abcam, Cambridge, UK), mouse anti-neuronal nuclei and rat anti-CD31 (1:250; Abcam), anti-Iba1 (raised in goat, 1:200, Abcam, Cambridge, UK). Detection of anti-NeuN antibodies was done with a goat anti-mouse fluorescent dye (AlexaFluor488, 1:100, 45 min.; MolecularProbes, Leiden, Netherlands). Bromodeoxyuridine antibodies were detected using a biotin conjugated goat-anti-rat antibody (1:500; 45 min; Jackson Labs, West Grove, PA, USA); CD31-antibodies detection was done using a biotin conjugated goat-anti-rat antibody (1:100; 45 min; Jackson Labs). Detection of Iba1-antibodies was performed using a biotin conjugated donkey anti-goat antibody (1:200, 45 min, room temperature, Jackson Labs, West Grove, PA, USA).

For signal amplification of BrdU-, CD31- and Iba1-signal, sections were incubated with horseradish peroxidase/streptavidin (1:100, 45 min; DAKO, Glostrup, Denmark) and biotinyl tyramide (1:100). Bromodeoxyuridine, CD31- and Iba1-positive cells were visualized by a streptavidin/fluorescent dye (AlexaFluor594, Molecular Probes). Nuclei counterstain was done with a tissue preserving medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector, Burlingame, CA, USA). Immunoflurescence was computed and visualized with a Nikon Eclipse 80i fluorescence microscope (Nikon, Düsseldorf, Germany) equipped with proper filter sets for AlexaFluor594, AlexaFluor488 and DAPI.

ANALYSIS OF THE CELLULAR INFLAMMATORY RESPONSE

Quantification of Iba1-positive cells was performed by counting absolute cell amounts covering four random squares (200 × 200 μm) within the ipsilateral boundary zone of the infarct of four separate brain sections per animal.

ANALYSIS OF NEUROGENESIS

Quantification of neurogenesis was performed as described previously (Diederich et al., 2012a). Briefly, BrdU/NeuN-positive cells were analyzed in three brain regions: the dentate gyrus (DG), subventricular zone (SVZ), and peri-infarct area (PI). In the DG and SVZ, all BrdU-positive cells were counted on seven sections (every 12th section, 440-μm intervals) per hemisphere. For the analysis of BrdU/NeuN-positive cells in the PI, four squares (300 μm × 300 μm) adjacent to the PI were analyzed on four sections (bregma 1 mm to −0.5 mm). To determine the percentage of neurons among the newly generated cells, 50 randomly selected BrdU-positive cells within the DG, SVZ, and PI, respectively, were analyzed for BrdU/NeuN co-labeling. Multiplying the total number of BrdU-positive cells with the percentage of NeuN/BrdU, double-positive cells yielded the number of new neurons in the respective areas.

Analysis of angiogenesis

Vessel length was determined by calculating the area of CD31 staining using ImageJ v1.34 software program (NIH). Three areas (20× magnification) depicting the ischemic border zone from three sections per animal, respectively, were photographed and subsequently analyzed. Vessel cross-sectional surface area and perimeter were measured using Neurolucida (MicroBrightField). Two vessels featuring a minimum length of 300 μm for two sections were analyzed per animal.

Primary and secondary objectives of the study

According to the ARRIVE guidelines primary and secondary objectives were defined (Kilkenny et al., 2010). The primary endpoint was functional outcome after ischemia. Secondary endpoints were the structural outcome as analyzed by the generation of new neurons and angiogenesis as well as infarct size.

Statistical analysis

Randomization was carried out by the computer software "Research Randomizer" (Version 3.0; Urbaniak GC, Plous S, 2011, retrieved on March 23, 2011, from www.randomizer.org/). The values presented in this study are means ± SEM. Statistical analyses were calculated using the Statistical Package of Social Sciences (Version 15.0; SPSS Inc., Chicago, IL, USA). The normality distribution of the data was assessed by graphical examination of the histograms and verified by the Shapiro-Wilk test ($P > 0.05$). Behavioral measurements were analyzed by area under the curve analysis using analysis of variance (ANOVA) followed by the Fisher protected least significant difference test. Student t-test with Bonferroni correction was used to compare data between

two groups. An α error rate of 0.05 was taken as the criterion for significance.

RESULTS

All experiments were performed on a total number of 55 animals (placebo: $n = 13$, G-CSF: $n = 14$, BM-MNC: $n = 4$, BM-MNC and G-CSF: $n = 14$).

FUNCTIONAL OUTCOME

The photothrombotic stroke model causes distinct deficits in somatosensory and motor functions. Somatosensory recovery was evaluated by the adhesive removal test (**Figure 1A**) and motor recovery by means of the cylinder test (**Figure 1B**). A summarized analysis of the functional recovery was performed by means of the area under the curve. Baseline performance was comparable between all treatment groups. As expected, animals of all experimental groups exhibited notable deficits in somatosensory and motor function following photothrombotic stroke, which subsequently attenuate over the course of 28 days until the end of the experiment (**Figures 1A,B**).

The area under the curve analysis of the adhesive removal test revealed a statistically significant decrease of the somatosensory deficit in G-CSF ($P < 0.01$, Fisher protected least significant difference *post hoc* test after significant ANOVA, **Figure 1A**) and in the G-CSF+BM-MNC ($P < 0.05$) treated animals compared to vehicle treated animals. In addition, G-CSF treated animals also displayed a significantly improved somatosensory recovery compared to animals treated with BM-MNC alone ($P < 0.05$).

The area under the curve analysis of the cylinder test did not reveal any significant treatment effects on the recovery of motor functions ($P = 0.859$; one-way ANOVA, **Figure 1B**).

INFARCT VOLUMES

As expected after photothrombotic stroke, infarct volumes did not differ between the four groups ($P = 0.877$; ANOVA, **Figure 2A**).

ANALYSIS POSTISCHEMIC INFLAMMATION

To investigate treatment effects on postischemic inflammation in the chronic phase following ischemia we analyzed the extent of the inflammatory response 28 days after infarct induction.

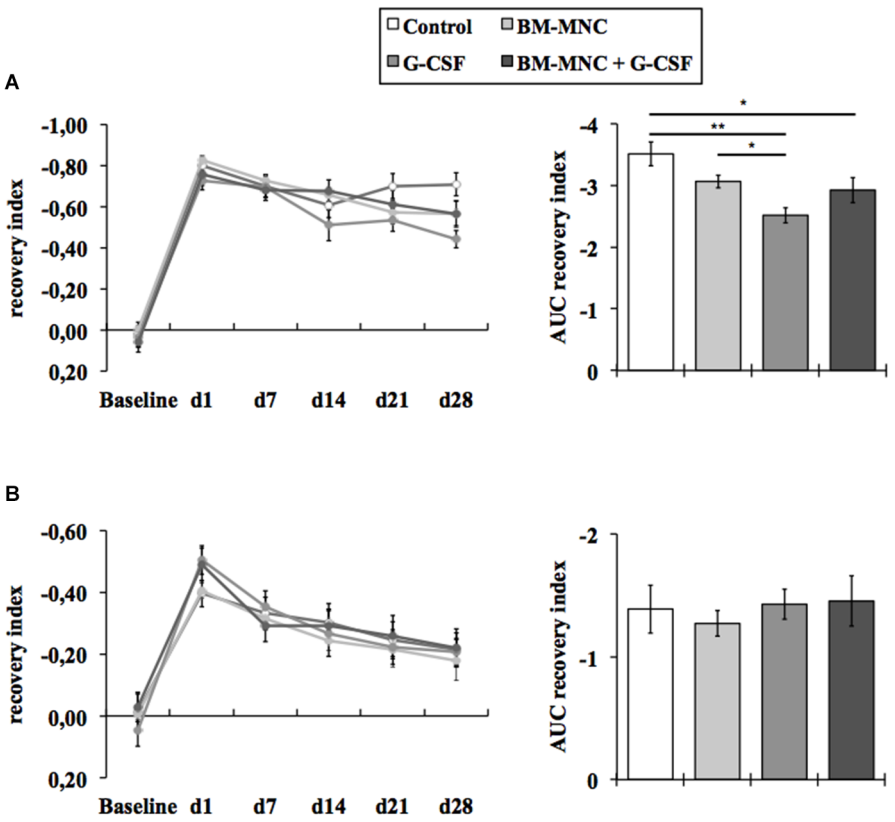

FIGURE 1 | Assessment of neurological deficits. Somatosensory recovery was assessed by the adhesive removal test **(A)**. Animals of all experimental groups exhibited notable deficits in somatosensory function following photothrombotic stroke, which subsequently attenuate over the course of 28 days until the end of the experiment. The area under the curve (AUC) analysis revealed a significantly improved functional recovery after G-CSF monotherapy and G-CSF + BM-MNC combination (*$P < 0.05$, **$p < 0.01$; Fisher protected least significant difference *post hoc* test after significant ANOVA). Motor recovery was assessed by the cylinder test **(B)**. Animals of all experimental groups showed deficits in motor function, which attenuate over the course of 28 days until the end of the experiment. The AUC analysis of the cylinder test did not reveal any significant treatment effects on the recovery of motor functions ($P = 0.859$; one-way ANOVA).

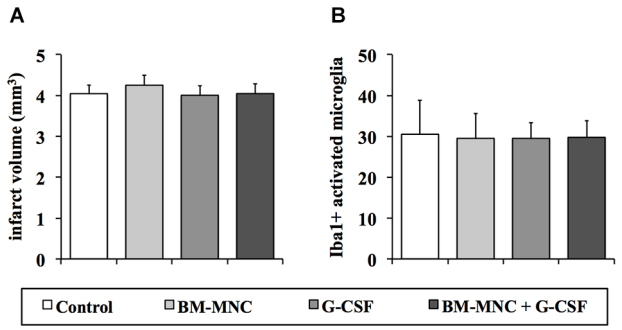

FIGURE 2 | Analyses of infarct volumes and the postischemic inflammatory response 28 days after photothrombotic stroke. Infarct volumes **(A)** did not differ between the treatment groups ($P = 0.877$; one-way ANOVA). There was no statistical difference between the groups regarding the number of activated microglia cells (Iba1-positive, **(B)**) in the boundary zone of the infarct ($P = 0.976$; one-way ANOVA).

The number of Iba1-positive cells with retracted processes and round cell bodies, representing activated microglia, did not differ between the different groups ($P = 0.976$; one-way ANOVA, **Figure 2B**).

QUANTIFICATION OF POSTISCHEMIC NEUROGENESIS

The analysis of postischemic neurogenesis revealed no significant treatment effects on the generation of new neurons in any of the analyzed regions in postischemic brains of SH animals (**Figures 3A–E**). As expected, the amount of BrdU/NeuN immunoreactive cells was significantly elevated in the SVZ of the ipsilateral compared to the contralateral hemisphere in animals of all treatment groups (ipsilateral vs. contralateral, control group: $P < 0.05$; BM-MNC group: $P < 0.001$; G-CSF group: $P < 0.01$; BM-MNC+G-CSF group: $P < 0.01$, Students *t*- test with Bonferroni correction, **Figure 3B**). A comparison of the treatment groups did not reveal significant treatment effects on the amount of newborn neurons in the SVZ in either of the two hemispheres (ipsilateral: $P = 0.101$, contralateral: $P = 0.108$; multivariate ANOVA, **Figure 3B**). Furthermore, no treatment effects on postischemic neurogenesis were detected in the DG and in the PI (DG ipsilateral: $P = 0.948$, contralateral: $P = 0.743$; ANOVA, **Figure 3A**/PI $P = 0.095$; one-way ANOVA, **Figure 3C**). In the DG, no differences were found between the contralateral and ipsilateral hemisphere (ipsilateral vs. contralateral control group: $P = 0.830$; BM-MNC group: $P = 0.637$; G-CSF group: $P = 0.349$; BM-MNC+G-CSF group: $P = 0.907$, Students *t*-test with Bonferroni correction, **Figure 3A**).

QUANTIFICATION OF POSTISCHEMIC ANGIOGENESIS

To investigate effects of the different treatment groups on angiogenesis (**Figures 4A–D**), blood vessel length was assessed at the ischemic border zone. There was no difference in vessel length between the groups ($P = 0.236$, one-way ANOVA, **Figure 4A**). In addition, analysis of individual vessels revealed no qualitative

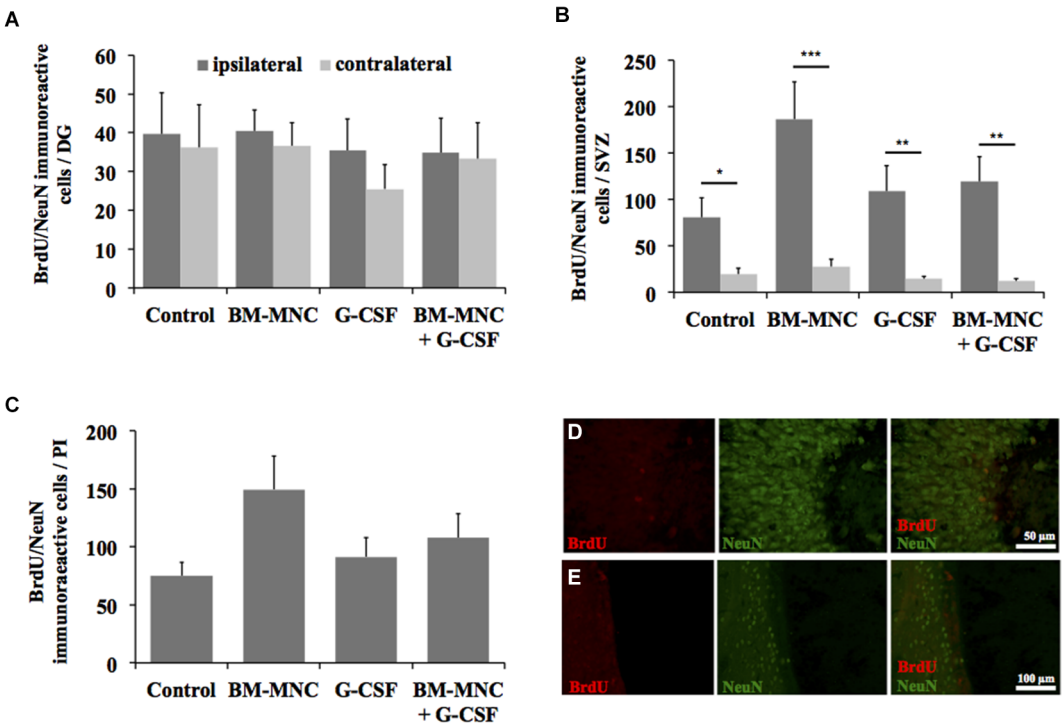

FIGURE 3 | Analysis of post-stroke neurogenesis. Quantification of neurogenesis by detection of BrdU/NeuN-expressing cells in the dentate gyrus (DG, **A**), subventicular zone (SVZ, **B**), and peri-infarct area (PI, **C**) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student *t*-test with Bonferroni correction). Representative photomicrographs from the DG **(D)** and SVZ **(E)** demonstrating BrdU/NeuN-expressing cells.

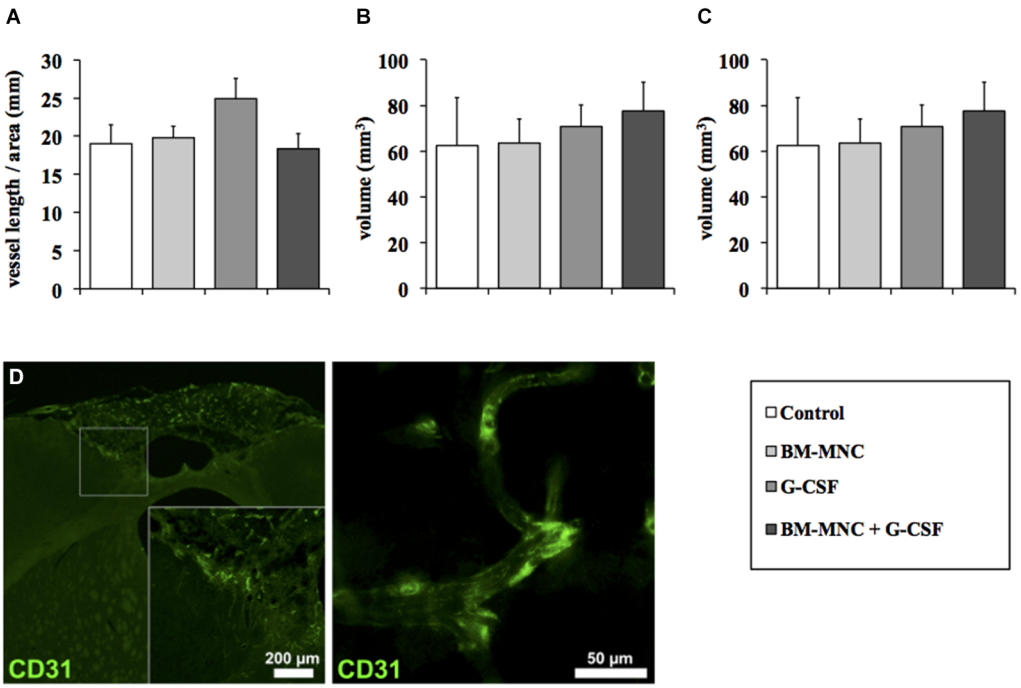

FIGURE 4 | Analysis of post-stroke angiogenesis. Quantification of total vascular length per area **(A)**. The vessel length per area did not differ between the groups (P = 0.236; ANOVA). Analysis of vessel surface area **(B)** and perimeter **(C)**. No qualitative changes in surface area (P = 0.949; ANOVA) and volume (P = 0.848; ANOVA) were detected. **(D)** Representative photomicrographs demonstrating blood vessels (CD31) within the peri-infarct region.

changes in surface area (P = 0.949, one-way ANOVA, **Figure 4B**) and volume (P = 0.848, one-way ANOVA, **Figure 4C**).

DISCUSSION

The present study investigated the hypothesis that the combination of G-CSF and BM-MNCs is more effective than either treatment alone. Granulocyte-colony stimulating factor treated rats, either in combination with BM-MNCs or alone, showed a significantly improved functional recovery compared to placebo rats measured by the adhesive removal test. In contrast, early BM-MNC monotherapy did not improve somatosensory rehabilitation. Regeneration of gross motor function as tested by the cylinder test remained unaffected in animals of all treatment groups. The observed improvement of functional recovery in G-CSF-treated animals was not due to infarct volume reduction, thus suggesting a true recovery-enhancing effect of G-CSF rather than neuroprotective actions. Post-stroke neurogenesis and angiogenesis were not significantly enhanced after G-CSF treatment. Furthermore, the extent of the local inflammatory response to cerebral ischemia were comparable in all treatment groups.

Initial reports indicated that treatment with BM-MNC might reduce neurological impairment in animal models of focal cerebral ischemia after application in acute and subacute phases (Iihoshi et al., 2004; Kamiya et al., 2008; Brenneman et al., 2009; Giraldi-Guimarães et al., 2009; Nakano-Doi et al., 2010; Kasam et al., 2012). However, there is cumulating evidence, that the efficacy and therapeutic applicability of BM-MNC-treatment might be more limited than initially anticipated. In contrast to previous studies which used healthy animals, a recently published study from our group could not demonstrate beneficial effects of early BM-MNC treatment following transient middle cerebral artery occlusion (MCAO) in SH animals. Infarct volumes, early behavioral outcomes, and the extent of the immediate inflammatory response to cerebral ischemia were not affected by BM-MNC treatment in co-morbid animals (Minnerup et al., 2014).

In addition, timing of cell administration may have limited the therapeutic impact of BM-MNC transplantation as a therapeutic efficacy of BM-MNC application was previously shown for a time window between 3 h and 7 days only (Iihoshi et al., 2004; Vasconcelos-dos-Santos et al., 2012). Since we found that BM-MNC in combination with G-CSF treatment time-dependently abolished the beneficial effect of G-CSF on long-term functional rehabilitation after MCAO in co-morbid SH animals (Pösel et al., 2014), we chose 1 h as the time point for BM-MNC application to reduce the likeliness for such detrimental interaction. Whether this very early timing has contributed to the lack of therapeutic impact remains for further investigation. However, the most important difference in our study design is the use of co-morbid animals as opposed to healthy animals. In fact, divergent results on effect size with greater infarct size reductions in healthy animals compared to animals with comorbidities were previously reported (Crossley et al., 2008). The predominant use of young and healthy animals in preclinical

studies is assumed to contribute considerably to the translational failure in experimental stroke research (Crossley et al., 2008).

While the efficacy of BM-MNC treatment on post-stroke regeneration is diminished by the occurrence of co-morbidities, the rehabilitative effects of G-CSF are still ascertainable, albeit seemingly less pronounced. Zhao et al. (2007b) reported limited and unstable long-term functional improvement following MCAO in SH rats when G-CSF was administered daily, 3 h to 7 days after ischemia. When G-CSF treatment was delayed until 3.5 month after MCAO no functional benefit could be ascertained (Zhao et al., 2007a). In accordance with these findings, we detected a limited efficacy of G-CSF treatment following photothrombotic stroke in SH rats. While long-term somatosensory function was improved, as revealed by the adhesive removal test, the cylinder test did not detect any differences in motor function. In contrast, we recently demonstrated, that G-CSF monotherapy led to a robust improvement of both qualities following photothrombotic stroke in normotensive rats of the same age (Diederich et al., 2012b).

Granulocyte-colony stimulating factor treatment as well as transplantation of BM-MNC both potentiate angiogenesis and neurogenesis (Lee et al., 2005; Schneider et al., 2005; Giraldi-Guimarães et al., 2009; Nakano-Doi et al., 2010) after ischemia in normotensive animals. These mechanisms are suggested to be primarily mediating long-term neurorehabilitation in non-comorbid animals (Kojima et al., 2010; Osman et al., 2011). Increased neurogenesis (Kronenberg et al., 2007) and angiogenesis (Yang et al., 2011) occur in SH animals, regarded as part of a counter-regulatory repair process against hypertension-induced brain damage. Our results demonstrate, that in SH animals, these repair mechanisms are not enhanced by G-CSF-treatment following stroke and therefore do not mediate G-CSF-induced functional improvement. These findings indicate, that therapeutic modulation of angiogenesis and neurogenesis may be crucially limited by comorbid disease, which might result in reduced effectiveness. A potential explanation therefore might be an exhausted angiogenic and neurogenic reserve, as suggested for neurodegenerative diseases (Kempermann, 2008).

Transplanted BM-MNCs (Brenneman et al., 2009) as well as G-CSF (Sehara et al., 2007; Solaroglu et al., 2009; Dietel et al., 2012) exert neuroprotective anti-inflammatory actions after cerebral ischemia in normotensive animals. In SH animals, however, BM-MNC treatment did not affect the local microglial immune response after MCAO (Minnerup et al., 2014). In the current study, we investigated treatment effects on postischemic local inflammation in the chronic phase 28 days after photothrombotic stroke. Neither the respective monotherapies with BM-MNCs and G-CSF, nor the combination of both resulted in an altered local microglial response. This result indicates, that local inflammation might not be directly responsible for improved long-term functional recovery following G-CSF treatment. However, hypertension has been shown to entail systemic vascular inflammation including chronic activation of microglia (Zubcevic et al., 2011) and may therefore influence poststroke immune responses (Möller et al., 2014) and might also interfere with the therapeutic potential of G-CSF and BM-MNC.

Our study has strength and limitations. The experiments were conducted in concordance with recommendations for good preclinical stroke research (Sutherland et al., 2012) and rigorously adhered to stringent quality criteria in experimental stroke research such as randomization, surgery and evaluations performed in a blinded fashion, and controlled physiological parameters. Furthermore, the use of animals with a relevant comorbidity may increase the predictive value of the presented findings regarding a human stroke patient population. While we could demonstrate G-CSF-induced improvements in long-term functional recovery following cortical ischemia in co-morbid animals, the underlying mechanism remains to be determined. Analyses of post-stroke angiogenesis might be confounded by hypertension-related endothelial abnormalities. Further research is needed to determine how co-morbidities like hypertension affect post-stroke repair mechanisms. The results of the present study cannot be attributed to the co-morbid condition of the animals with absolute certainty, since no control groups of healthy animals were included in the present study. Future preclinical stroke studies on co-morbid animals should also include groups of healthy animals in order to determine whether the obtained results can be directly attributed to the comorbid condition. In the present study, we focused on syngenic BM-MNC transplantation as it simulates autologous transplantation. Bone marrow derived mononuclear cells derived from normotensive rats might differ in their phenotype and thus in their therapeutic capacity as compared to BM-MNC derived from hypertensive rats used in the present study. Further research is needed to determine the therapeutic potential of BM-MNCs and their limitations, including analysis of their cytokine profiles. The cell numbers used in our experiments were based on previous studies that investigated different intravenous cell therapies in animal stroke models (Minnerup et al., 2011). However, we cannot rule out that the use of a higher BM-MNC dose might have yielded different results.

Our study confirms the beneficial effect of G-CSF treatment on long-term functional recovery following cortical stroke in hypertensive animals. Angiogenesis and neurogenesis, which are regarded as decisive mechanisms of G-CSF-mediated regeneration in normotensive animals, remained unaffected by G-CSF treatment in hypertensive animals. Contrary to our hypothesis, the combination of G-CSF and BM-MNC was not more effective than G-CSF alone and the monotherapy with BM-MNC was without any effect on functional recovery. The reduced efficacy of G-CSF treatment and the absence of any beneficial effect of BM-MNC transplantation might be attributed to hypertension-related morbidity. The findings of our study further corroborate the importance of evaluating the efficacy of treatments and determining the underlying neuroregenerative mechanisms in animal models more adequately reflecting the characteristic pathophysiological state of stroke patients.

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REFERENCES

Brenneman, M., Sharma, S., Harting, M., Strong, R., Cox, C. S., Aronowski, J., et al. (2009). Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J. Cereb. Blood Flow Metab.* 30, 140–149. doi: 10.1038/jcbfm.2009.198

Carmichael, S. T. (2005). Rodent models of focal stroke: size, mechanism and purpose. *NeuroRx* 2, 396–409. doi: 10.1602/neurorx.2.3.396

Crossley, N. A., Sena, E., Goehler, J., Horn, J., van der Worp, B., Bath, P. M. W., et al. (2008). Empirical evidence of bias in the design of experimental stroke studies: a metaepidemiologic approach. *Stroke* 39, 929–934. doi: 10.1161/strokeaha.107.498725

Dickhout, J. G., and Lee, R. M. (1998). Blood pressure and heart rate development in young spontaneously hypertensive rats. *Am. J. Physiol.* 274, H794–H800.

Diederich, K., Frauenknecht, K., Minnerup, J., Schneider, B. K., Schmidt, A., Altach, E., et al. (2012a). Citicoline enhances neuroregenerative processes after experimental stroke in rats. *Stroke* 43, 1931–1940. doi: 10.1161/strokeaha.112.654806

Diederich, K., Quennet, V., Bauer, H., Müller, H. D., Wersching, H., Schäbitz, W.-R., et al. (2012b). Successful regeneration after experimental stroke by granulocyte-colony stimulating factor is not further enhanced by constraint-induced movement therapy either in concurrent or in sequential combination therapy. *Stroke* 43, 185–192. doi: 10.1161/strokeaha.111.622159

Dietel, B., Cicha, I., Kallmünzer, B., Tauchi, M., Yilmaz, A., Daniel, W. G., et al. (2012). Suppression of dendritic cell functions contributes to the anti-inflammatory action of granulocyte-colony stimulating factor in experimental stroke. *Exp. Neurol.* 237, 379–387. doi: 10.1016/j.expneurol.2012.06.019

England, T. J., Gibson, C. L., and Bath, P. M. W. (2009). Granulocyte-colony stimulating factor in experimental stroke and its effects on infarct size and functional outcome: a systematic review. *Brain Res. Rev.* 62, 71–82. doi: 10.1016/j.brainresrev.2009.09.002

Giraldi-Guimarães, A., Rezende-Lima, M., Bruno, F. P., and Mendez-Otero, R. (2009). Treatment with bone marrow mononuclear cells induces functional recovery and decreases neurodegeneration after sensorimotor cortical ischemia in rats. *Brain Res.* 1266, 108–120. doi: 10.1016/j.brainres.2009.01.062

Iihoshi, S., Honmou, O., Houkin, K., Hashi, K., and Kocsis, J. D. (2004). A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. *Brain Res.* 1007, 1–9. doi: 10.1016/j.brainres.2003.09.084

Kamiya, N., Ueda, M., Igarashi, H., Nishiyama, Y., Suda, S., Inaba, T., et al. (2008). Intra-arterial transplantation of bone marrow mononuclear cells immediately after reperfusion decreases brain injury after focal ischemia in rats. *Life Sci.* 83, 433–437. doi: 10.1016/j.lfs.2008.07.018

Kasam, M., Yang, B., Strong, R., Schaar, K., Misra, V., Xi, X., et al. (2012). Nitric oxide facilitates delivery and mediates improved outcome of autologous bone marrow mononuclear cells in a rodent stroke model. *PLoS One* 7:e32793. doi: 10.1371/journal.pone.0032793

Kempermann, G. (2008). The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for?. *Trends in Neurosci.* 31, 163–169. doi: 10.1016/j.tins.2008.01.002

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., and Altman, D. G. (2010). Improving bioscience research reporting: the arrive guidelines for reporting animal research. *PLoS Biol.* 8:e1000412. doi: 10.1371/journal.pbio.1000412

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., Altman, D. G., and National Centre for the Replacement, Refinement and Reduction of Amimals in Research (2011). Animal research: reporting in vivo experiments–the ARRIVE guidelines. *J. Cereb. Blood Flow Metab.* 31, 991–993. doi: 10.1038/jcbfm.2010.220

Kojima, T., Hirota, Y., Ema, M., Takahashi, S., Miyoshi, I., Okano, H., et al. (2010). Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells* 28, 545–554. doi: 10.1002/stem.306

Kronenberg, G., Lippoldt, A., and Kempermann, G. (2007). Two genetic rat models of arterial hypertension show different mechanisms by which adult hippocampal neurogenesis is increased. *Dev. Neurosci.* 29, 124–133. doi: 10.1159/000096217

Lee, S.-T., Chu, K., Jung, K.-H., Ko, S.-Y., Kim, E.-H., Sinn, D. I., et al. (2005). Granulocyte colony-stimulating factor enhances angiogenesis after focal cerebral ischemia. *Brain Res.* 1058, 120–128. doi: 10.1016/j.brainres.2005.07.076

Minnerup, J., Heidrich, J., Wellmann, J., Rogalewski, A., Schneider, A., and Schäbitz, W.-R. (2008). Meta-analysis of the efficacy of granulocyte-colony stimulating factor in animal models of focal cerebral ischemia. *Stroke* 39, 1855–1861. doi: 10.1161/strokeaha.107.506816

Minnerup, J., Kim, J. B., Schmidt, A., Diederich, K., Bauer, H., Schilling, M., et al. (2011). Effects of neural progenitor cells on sensorimotor recovery and endogenous repair mechanisms after photothrombotic stroke. *Stroke* 42, 1757–1763. doi: 10.1161/STROKEAHA.110.599282

Minnerup, J., Wagner, D.-C., Strecker, J.-K., Pösel, C., Sevimli-Abdis, S., Schmidt, A., et al. (2014). Bone marrow-derived mononuclear cells do not exert acute neuroprotection after stroke in spontaneously hypertensive rats. *Front. Cell. Neurosci.* 7:288. doi: 10.3389/fncel.2013.00288

Möller, K., Boltze, J., Pösel, C., Seeger, J., Stahl, T., and Wagner, D.-C. (2014). Sterile inflammation after permanent distal MCA occlusion in hypertensive rats. *J. Cereb. Blood Flow Metab.* 34, 307–315. doi: 10.1038/jcbfm.2013.199

Müller, H. D., Hanumanthiah, K. M., Diederich, K., Schwab, S., Schäbitz, W.-R., and Sommer, C. (2008). Brain-derived neurotrophic factor but not forced arm use improves long-term outcome after photothrombotic stroke and transiently upregulates binding densities of excitatory glutamate receptors in the rat brain. *Stroke* 39, 1012–1021. doi: 10.1161/strokeaha.107.495069

Nakano-Doi, A., Nakagomi, T., Fujikawa, M., Nakagomi, N., Kubo, S., Lu, S., et al. (2010). Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 28, 1292–1302. doi: 10.1002/stem.454

Osman, A. M., Porritt, M. J., Nilsson, M., and Kuhn, H. G. (2011). Long-Term stimulation of neural progenitor cell migration after cortical ischemia in mice. *Stroke* 42, 3559–3565. doi: 10.1161/strokeaha.111.627802

Pösel, C., Möller, K., Fröhlich, W., Schulz, I., Boltze, J., and Wagner, D.-C. (2012). Density gradient centrifugation compromises bone marrow mononuclear cell yield. *PLoS One* 7:e50293. doi: 10.1371/journal.pone.0050293

Pösel, C., Scheibe, J., Kranz, A., Bothe, V., Quente, E., Fröhlich, W., et al. (2014). Bone marrow cell transplantation time-dependently abolishes efficacy of granulocyte colony-stimulating factor after stroke in hypertensive rats. *Stroke* 45, 2431–2437. doi: 10.1161/strokeaha.113.004460

Ringelstein, E. B., Thijs, V., Norrving, B., Chamorro, A., Aichner, F., Grond, M., et al. (2013). Granulocyte colony-stimulating factor in patients with acute ischemic stroke: results of the AX200 for ischemic stroke trial. *Stroke* 44, 2681–2687. doi: 10.1161/STROKEAHA.113.001531

Schmidt, A., Hoppen, M., Strecker, J.-K., Diederich, K., Schäbitz, W.-R., Schilling, M., et al. (2012). Photochemically induced ischemic stroke in rats. *Exp. Transl. Stroke Med.* 4:13. doi: 10.1186/2040-7378-4-13

Schneider, A., Krüger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., et al. (2005). The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J. Clin. Invest.* 115, 2083–2098. doi: 10.1172/jci23559

Sehara, Y., Hayashi, T., Deguchi, K., Zhang, H., Tsuchiya, A., Yamashita, T., et al. (2007). Decreased focal inflammatory response by G-CSF may improve stroke outcome after transient middle cerebral artery occlusion in rats. *J. Neurosci. Res.* 85, 2167–2174. doi: 10.1002/jnr.21341

Sevimli, S., Diederich, K., Strecker, J.-K., Schilling, M., Klocke, R., Nikol, S., et al. (2009). Endogenous brain protection by granulocyte-colony stimulating factor after ischemic stroke. *Exp. Neurol.* 217, 328–335. doi: 10.1016/j.expneurol.2009.03.018

Solaroglu, I., Cahill, J., Tsubokawa, T., Beskonakli, E., and Zhang, J. H. (2009). Granulocyte colony-stimulating factor protects the brain against experimental stroke via inhibition of apoptosis and inflammation. *Neurol. Res.* 31, 167–172. doi: 10.1179/174313209x393582

Strecker, J.-K., Sevimli, S., Schilling, M., Klocke, R., Nikol, S., Schneider, A., et al. (2010). Effects of G-CSF treatment on neutrophil mobilization and neurological outcome after transient focal ischemia. *Exp. Neurol.* 222, 108–113. doi: 10.1016/j.expneurol.2009.12.012

Sutherland, B. A., Minnerup, J., Balami, J. S., Arba, F., Buchan, A. M., and Kleinschnitz, C. (2012). Neuroprotection for ischaemic stroke: translation from the bench to the bedside. *Int. J. Stroke* 7, 407–418. doi: 10.1111/j.1747-4949.2012.00770.x

Vasconcelos-dos-Santos, A., Rosado-de-Castro, P. H., Lopes de Souza, S. A., da Costa Silva, J., Ramos, A. B., Rodriguez de Freitas, G., et al. (2012). Intravenous and intra-arterial administration of bone marrow mononuclear cells after focal cerebral ischemia: is there a difference in biodistribution and efficacy? *Stem Cell Res.* 9, 1–8. doi: 10.1016/j.scr.2012.02.002

Yang, M., Aragon, M., and Murfee, W. L. (2011). Angiogenesis in mesenteric microvascular networks from spontaneously hypertensive versus normotensive rats. *Microcirculation* 18, 574–582. doi: 10.1111/j.1549-8719.2011.00117.x

Zhao, L.-R., Berra, H. H., Duan, W.-M., Singhal, S., Mehta, J., Apkarian, A. V., et al. (2007a). Beneficial effects of hematopoietic growth factor therapy in chronic ischemic stroke in rats. *Stroke* 38, 2804–2811. doi: 10.1161/strokeaha.107.486217

Zhao, L.-R., Singhal, S., Duan, W.-M., Mehta, J., and Kessler, J. A. (2007b). Brain repair by hematopoietic growth factors in a rat model of stroke. *Stroke* 38, 2584–2591. doi: 10.1161/strokeaha.106.476457

Zubcevic, J., Waki, H., Raizada, M. K., and Paton, J. F. R. (2011). Autonomic-immune-vascular interaction: an emerging concept for neurogenic hypertension. *Hypertension* 57, 1026–1033. doi: 10.1161/hypertensionaha.111.169748

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Bone marrow-derived mononuclear cells do not exert acute neuroprotection after stroke in spontaneously hypertensive rats

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Bone marrow-derived mononuclear cells (BM-MNCs) were shown to improve the outcome in animal stroke models and clinical pilot studies on BM-MNCs for stroke patients were already conducted. However, relevant aspects of pre-clinical evaluation, such as the use of animals with comorbidities and dose-response studies, were not thoroughly addressed so far. We therefore investigated different BM-MNC doses in the clinical meaningful stroke model of spontaneously hypertensive (SH) rats. Three hours after the onset of transient middle cerebral artery occlusion (MCAO) animals received either one of three syngeneic BM-MNC doses or placebo intravenously. The primary endpoint was the infarct size. Secondary endpoints included functional outcome, mortality, inflammatory processes, and the dose-response relationship. In contrast to previous studies which used healthy animals no beneficial effect of BM-MNCs was found. Infarct volumes, mortality, behavioral outcomes, and the extent of the inflammatory response to cerebral ischemia were comparable in all groups. In conclusion, we could not demonstrate that early BM-MNC treatment improves the outcome after stroke in SH rats. Whether BM-MNCs improve neurological recovery after delayed treatment initiation was not investigated in the present study, but our data indicates that this should be determined in co-morbid animal stroke models before moving to large-scale clinical studies. Future preclinical stroke studies on co-morbid animals should also include groups of healthy animals in order to determine whether negative results can be attributed to the comorbid condition.

Keywords: bone marrow-derived mononuclear cells, middle cerebral artery occlusion, stroke, dose-response, spontaneously hypertensive rats

INTRODUCTION

Cell based therapies have been proposed as means of treating stroke. Among the different cell types under investigation for stroke, bone marrow-derived mononuclear cells (BM-MNCs) represent a particularly attractive treatment option. BM-MNCs can be easily obtained by bone marrow aspiration, do not require extensive preparation or cultivation, and permit autologous intravenous transplantation (Savitz et al., 2011b). Within the last several years, a number of reports showed that BM-MNCs reduce neurological impairment in animal models of focal cerebral ischemia after application in the acute and subacute phases (Iihoshi et al., 2004; Kamiya et al., 2008; Giraldi-Guimarães et al., 2009; Brenneman et al., 2010; Nakano-Doi et al., 2010). BM-MNC mediated neurological improvements including reduction of the final lesion volume, attenuated microglial activation, and promoted recovery after the initial ischemic event (Brenneman et al., 2010; Nakano-Doi et al., 2010; Sharma et al., 2010). Recent evidence suggests that BM-MNCs mediate their actions by the production and secretion of cytokines (Sharma et al., 2010). These in turn modulate the post-ischemic inflammatory response and attenuate neuronal cell death (Brenneman et al., 2010; Sharma et al., 2010; Wagner et al., 2012). Giving results from previous investigations reporting highest efficacy after BM-MNC transplantation within 3 h, early neuroprotective effects may be responsible for the majority of observed benefits (Iihoshi et al., 2004; Brenneman et al., 2010).

Recently, an open-label prospective study showed that intravenous BM-MNC treatment is safe and feasible in acute stroke patients (Savitz et al., 2011b). However, the pre-clinical package that is recommended to work up before proceeding to larger clinical efficacy studies has not been completed yet. For example, the *Stroke Therapy Academic Industry Roundtable* (STAIR)

and *The Stem Cell Therapies as an Emerging Paradigm in Stroke* (STEPS) recommendations stress the importance of testing candidate cell therapies in animals with comorbidities (Fisher et al., 2009; Stem Cell Therapies as an Emerging Paradigm in Stroke Participants, 2009; Savitz et al., 2011a). Such studies are lacking for BM-MNC so far. This is remarkable given that the majority of stroke patients have comorbidities, such as hypertension. Moreover, analyzes of previous animal stroke studies revealed that experiments with healthy animals compared to those using animals with comorbidities overstated the efficacy of a given treatment (Crossley et al., 2008). Pre-clinical cell dose-response investigations are not only requested by the STAIR and STEPS guidelines but also recommended by the US Food and Drug Administration (FDA) to be performed prior to initiating a clinical trial.[1]

Here, we investigated the neuroprotective properties of systemically transplanted BM-MNCs on infarct size, functional outcome, and glial inflammatory processes as well as their dose-response relationship in spontaneously hypertensive (SH) rats with focal cerebral ischemia at 3 h following stroke, for which best efficacy was reported in a pervious study (Iihoshi et al., 2004).

EXPERIMENTAL PROCEDURES

ANIMALS

All animal procedures were approved by the responsible ethics committee of the University of Münster and the appropriate authorities of the Federal State of North Rhine-Westphalia. The investigations were carried out in accordance with national and international animal welfare regulations and are reported in accordance with the *Animal Research: Reporting In Vivo Experiments* (ARRIVE) guidelines (Kilkenny et al., 2011). Surgery and evaluation of all read-outs were performed blinded to experimental groups. Experiments were performed on adult (12–13 weeks old) male SH rats weighing 260–290 g. SH rats were shown to have an increased blood pressure starting from 5 to 6 weeks of age (Dickhout and Lee, 1998). All animals were randomly assigned to one of the following treatment groups: (1) placebo ($n = 19$), (2) 1 million BM-MNCs/rat ($n = 18$), (3) 5 million BM-MNCs/rat ($n = 20$), or (4) 20 million BM-MNCs/rat ($n = 17$). The cell numbers used in our experiments were based on previous studies that investigated different intravenous cell therapies in animal stroke models (Iihoshi et al., 2004; Giraldi-Guimarães et al., 2009; Minnerup et al., 2011). The condition of animals was monitored at least every 8 h. Pre-defined termination criteria were: (1) a severe immobility and (2) a persisting abnormal body position. The implementation of these criteria was required by the local ethics committee.

BONE MARROW MONONUCLEAR CELL PREPARATION

Syngeneic rat bone marrow was obtained from male SH rats at the age of 12 weeks. Femurs and tibias were aseptically opened and repeatedly flushed with phosphate buffered saline (PBS). After erythrocyte lysis by ammonium chloride-based buffer (0.155 M NH_4Cl, 10 mM $KHCO_3$ and 0.01 mM Na_2EDTA) cells were filtered by a 100 μm cell strainer, counted and prepared for immunomagnetic depletion of granulocytes: 100 million bone marrow cells were incubated with 10 ng/ml Phycoerythrin-conjugated anti-rat granulocyte antibody (clone RP1; BD Pharmingen, Heidelberg, Germany) for 15 min at 4°C. Subsequently, cells were washed with cold PBS plus 0.5% fetal calf serum (FCS) and incubated with 200 μl anti-Phycoerythrin MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) in 800 μl PBS plus 5% FCS for 15 min at 4°C. After incubation, non-adsorbed MicroBeads were removed by a further washing step. The cell suspension was then resuspended in 500 μl PBS plus 0.5% FCS and magnetically separated by a LD-column according to the manufacturer's instructions (Miltenyi). This procedure results in higher BM-MNC purity compared to standard density gradient centrifugation (Pösel et al., 2012). The obtained mononuclear cell fraction was collected, counted, cryopreserved in liquid nitrogen (25 million mononuclear cells in 1 ml FCS plus 8% DMSO) and stored at −80°C until further use. BM-MNCs were labeled by PKH26 before transplantation (Zhang et al., 2011).

STROKE MODEL AND CELL THERAPY

Animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg/kg body weight; Ketanest) and xylazine hydrochloride (8 mg/kg body weight). The rectal temperature was maintained at 37°C by a thermostatically controlled heating pad (Föhr Medical Instruments). Transient middle cerebral artery occlusion (tMCAO) was induced as described previously (Longa et al., 1989; Schäbitz et al., 2003). Briefly, following a midline neck incision, a 3–0 nylon filament (Ethicon) with a tip heated to form a bulb shape was inserted into the right common carotid artery and advanced via the internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). Cerebral blood flow was monitored continuously to verify MCA occlusion and reperfusion using Laser-Doppler flowmetry (Periflux 5001; Perimed, Stockholm, Sweden) with a probe positioned over the MCA territory. After 60 min middle cerebral artery occlusion (MCAO), the filament was withdrawn to allow reperfusion. Three hours after onset of occlusion animals received the different cell doses or vehicle intravenously.

BEHAVIORAL TESTING

After a 3 day training period, behavioral tests were performed during the light cycle 1 day before MCAO (baseline), and at 24 and 72 h after ischemia. Functional outcome was studied using the neurological score as described by Menzies (Menzies et al., 1992). This score ranges from 0, indicating no deficit, to 4, indicating a severe neurological deficit (spontaneous circling). For Rotarod tests, rats were placed on an accelerating Rotarod cylinder, and the time the animals remained on this cylinder was measured (Minnerup et al., 2010). Speed was increased from 4 to 40 rpm within 5 min. The trial ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rungs. An arbitrary time limit of 300 s was set for rats on the Rotarod cylinder in training and testing procedures. The mean duration (seconds) on the device was recorded with three measurements 1 day before

[1] http://www.fda.gov/cber/guidelines.htm

surgery. For the adhesive-removal test, the somatosensory deficit was measured both before and after ischemia (Minnerup et al., 2010). Two small pieces of adhesive-backed paper dots of equal size were used as bilateral tactile stimuli occupying the palmar surface of each forepaw. The rat was then returned to a cage. The time to remove each stimulus from forelimbs was documented by three trials per day for each forepaw (Schneider et al., 2005). For the cylinder test, the rats were placed in a transparent cylinder (16 cm diameter, 21 cm height) and videotaped from underneath for 2 min (Minnerup et al., 2010). Spontaneous wall and ground touches of the impaired contralateral forelimb were counted.

DETERMINATION OF INFARCT SIZE AND IMMUNOHISTOCHEMISTRY

Three days after ischemia, animals were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were fixed in 4% paraformaldehyde at 4°C and then cryoprotected in 30% sucrose solution. Tissue was stored at −80°C until analysis. For infarct size calculation, 10 μm serial coronal brain section were cut in a cryostat (Leica, Nussloch, Germany), collected at 800 μm intervals and stained with toluidine blue (Sigma, St. Louis, USA). Infarct volumes were quantified with a standard computer assisted image analysis technique. To correct for the effect of brain edema, a correction method was used as described previously (Sevimli et al., 2009). For histological evaluation of inflammation, immunohistochemistry was performed on glass mounted coronal brain sections (10 μm, approx. bregma −1 mm). After blocking of non-specific proteins (using blocking reagent, 15 min, Roche Diagnostics, Mannheim, Germany) the following primary antibodies were applied (overnight, 4°C): anti-GFAP (raised in mouse, 1:500, Dako, Hamburg, Germany) and anti-Iba1 (raised in goat, 1:200, Abcam, Cambridge, UK). To detect anti-GFAP-antibodies we used a biotinylated goat anti-mouse antibody (1:100, Jackson Labs, West Grove, PA, USA) for 45 min at room temperature. Astrocytes were visualized by a streptavidin conjugated fluorescent dye (AlexaFluor594, Molecular Probes, Leiden, the Netherlands). For signal amplification of the Iba1 signal, brain slices were pre-treated with 3% H_2O_2/Methanol for 10 min in order to block endogenous peroxidase. Detection of Iba1-antibodies was performed using a biotin conjugated donkey anti-goat antibody (1:200, 45 min, room temperature, Jackson Labs, West Grove, PA, USA), followed by incubation with horseradish peroxidase/streptavidin (1:100, 45 min, DAKO, Glostrup, Denmark) and biotinyl tyramide, (1:100) for 10 min at room temperature. Iba1-positive cells were visualized using AlexaFluor594. A fluorescent-preserving mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining (Vector, Burlingame, CA, USA). The immunofluorescence signal was visualized with a fluorescent microscope (Nikon Eclipse 80i microscope, Nikon GmbH, Duesseldorf, Germany) with appropriate filter sets for AlexaFluor594 and DAPI. Digitizing was done with a Stereo Investigator Software (MicroBrightField Inc., Williston, VT, USA). To determine whether administered bone marrow-derived mononuclear cells have an influence on the post-ischemic reactive astrogliosis, GFAP-stained sections (three randomly chosen animals per group) were analyzed with respect to differences in morphology and immunoreactivity between the investigated groups. Quantification of Iba1-posive cells was performed by counting and averaging absolute cell amounts covering four random fields (0.25 mm^2 each) within the ipsilateral ischemic core and the boundary zone of the infarct of two separate brain sections per animal.

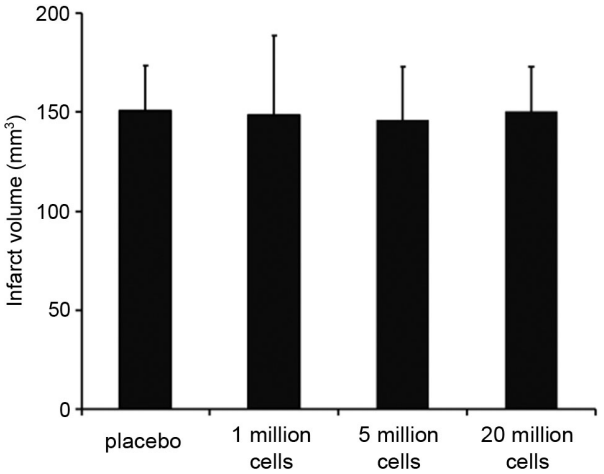

FIGURE 1 | Infarct volumes 3 days after transient MCAO. Infarct volumes did not differ between the four groups (n = 15–16/group). P = 0.595.

PRIMARY AND SECONDARY OBJECTIVES OF THE STUDY

According to the ARRIVE guidelines primary and secondary objectives were defined.[2] The primary endpoint was infarct size measured on day 3 after ischemia. Secondary endpoints were the functional outcome, mortality between treatment and day 3 (when animals were killed), inflammatory processes, and the dose-response relationship.

STATISTICAL ANALYSIS

Values are presented as mean ± SD. The sample size (number of animals per group) was calculated *a priori* with the following assumptions: (1) infarct size reductions of ≥20% should be detected, (2) a power of 0.8, (3) an α of 0.05, and (4) a SD 15% of the mean. A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare of infarct volumes, inflammatory processes, and physiological measures. Mortality was compared using the chi-square test. Behavioral tests were analyzed with two-way repeated-measures ANOVA. Statistical significance was determined as an α error <0.05. Statistical analyses were carried out using the Statistical Package of Social Sciences (version 18).

RESULTS

MORTALITY AND PHYSIOLOGICAL MEASUREMENTS

There was no statistically significant difference in mortality between the four groups. A total of 11 animals died until day 3: Three of the placebo group, 2 of the 1 million BM-MNCs/rat group, 4 of the 5 million BM-MNCs/rat

[2]http://www.nc3rs.org/ARRIVE

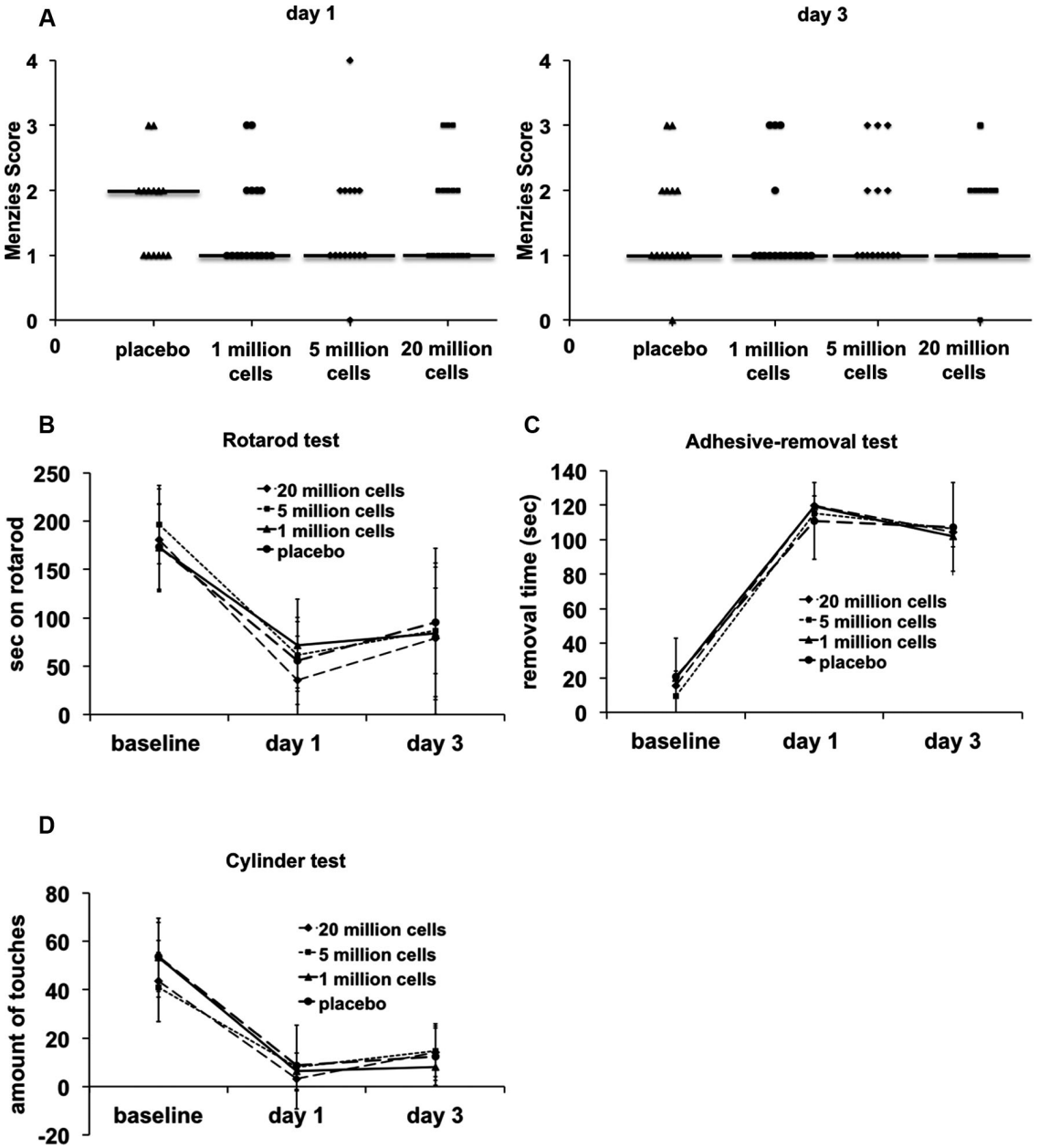

FIGURE 2 | Behavioral testing until day 3 after transient MCAO. No significant differences between the groups were detected in any of the functional tests (n = 15–16/group). **(A)** Menzies score, P = 0.980. **(B)** Rotarod test, P = 0.670. **(C)** Adhesive-removal test, P = 0.922. **(D)** Cylinder test, P = 0.350.

group, and 2 of the 20 million BM-MNCs/rat group (P = 0.859). None of the animals met any of the termination criteria. The animals died spontaneously due to the cerebral ischemia. Rectal temperature, body weight, and cerebral blood flow measurements were not different between groups (all P-values > 0.05).

INFARCT VOLUMES

Infarct volumes did not differ between the four groups (placebo: 150.55 mm^3 ± 23.11 mm^3, 1 million BM-MNCs/rat: 148.74 mm^3 ± 39.90 mm^3, 5 million BM-MNCs/rat: 145.59 ± 27.52 mm^3, 20 million BM-MNCs/rat: 150.55 mm^3 ± 23.11 mm^3; P = 0.595; **Figure 1**).

FUNCTIONAL OUTCOME

Functional outcomes as assessed by a behavioral test battery were not improved in any of the BM-MNC treated groups (**Figure 2**) (Menzies score: P = 0.980, Rotarod test: P = 0.670, adhesive-removal test: P = 0.922, cylinder test: P = 0.350).

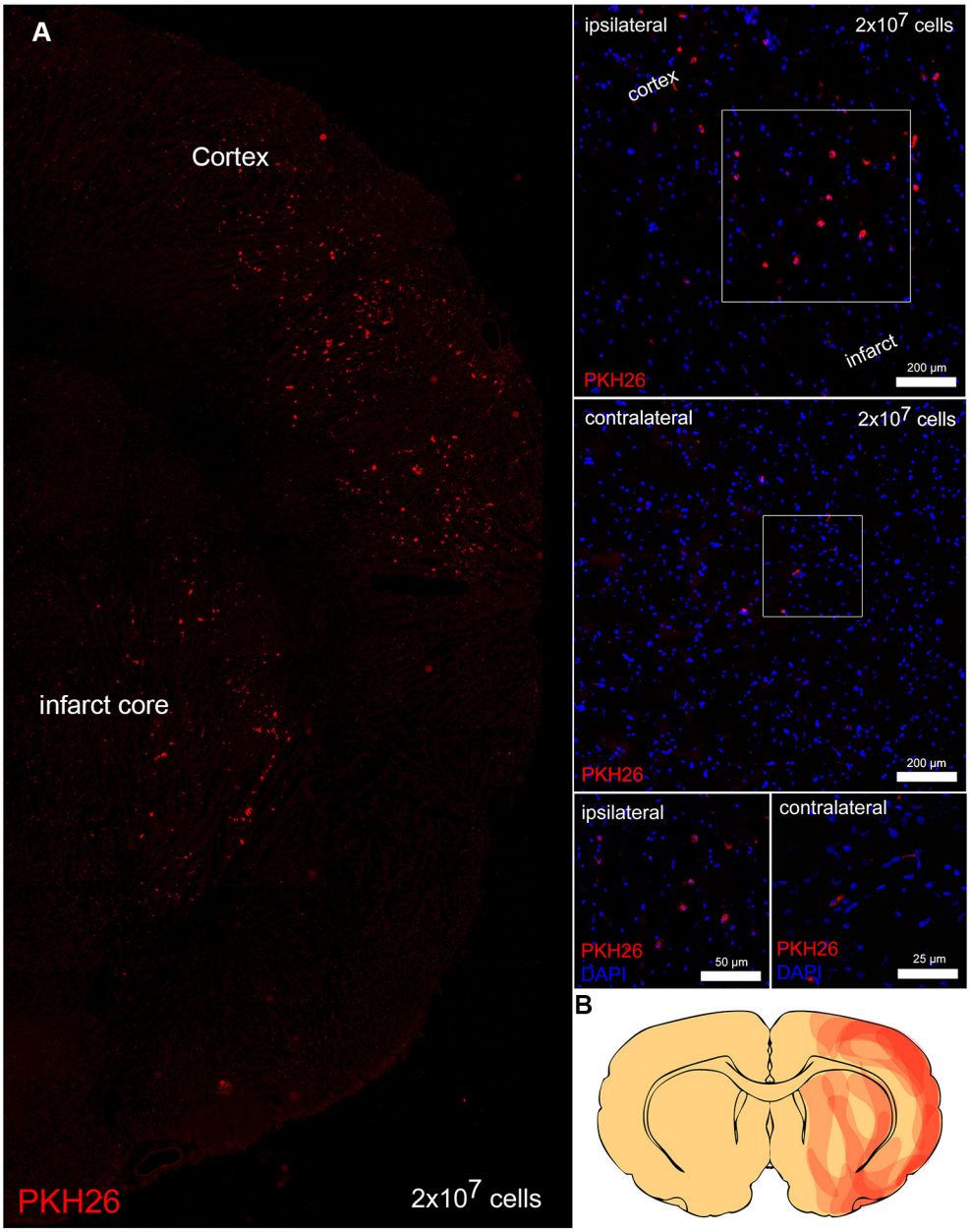

FIGURE 3 | Transplanted cells in the brain. (A) Transplanted PKH26 positive (red) BM-MNCs in the boundary zone of the infarct and in the infarct core. Ipsilateral to the infarct BM-MNCs were located in the parenchyma, whereas contralateral PKH26 positive cells were only detected in vessels. Overall, only a few PKH26 positive cells were detected contralateral. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, blue). **(B)** The map depicts immigrated PKH26 positive cells within the infarcted hemisphere (obtained from five animals).

ANALYSIS OF TRANSPLANTED BONE MARROW-DERIVED MONONUCLEAR CELLS (BM-MNCs) AND POST-SCHEMIC INFLAMMATION

Transplanted, PKH26 labeled cells were predominantly located on the ischemic side of the brain and were sparsely detected at the contralateral side. On the ischemic side, PKH26 positive cells were located within the boundary zone of the ischemic lesion (**Figure 3**). On the contralateral side BM-MNCs were only detected in vessels.

Inflammation contributes to ischemic brain damage and BM-MNCs were assumed to exert neuroprotective effects via anti-inflammatory mechanisms (Dirnagl et al., 1999). We therefore analyzed the extent of the glial inflammatory response. The number of Iba1-positive cells with retracted processes and round cell bodies, representing activated microglia, did not significantly differ within the infarct core ($P = 0.122$) and within the boundary zone of the infarct ($P = 0.476$) between the different groups (**Figure 4A**). Pattern of astrocyte activation visualized by

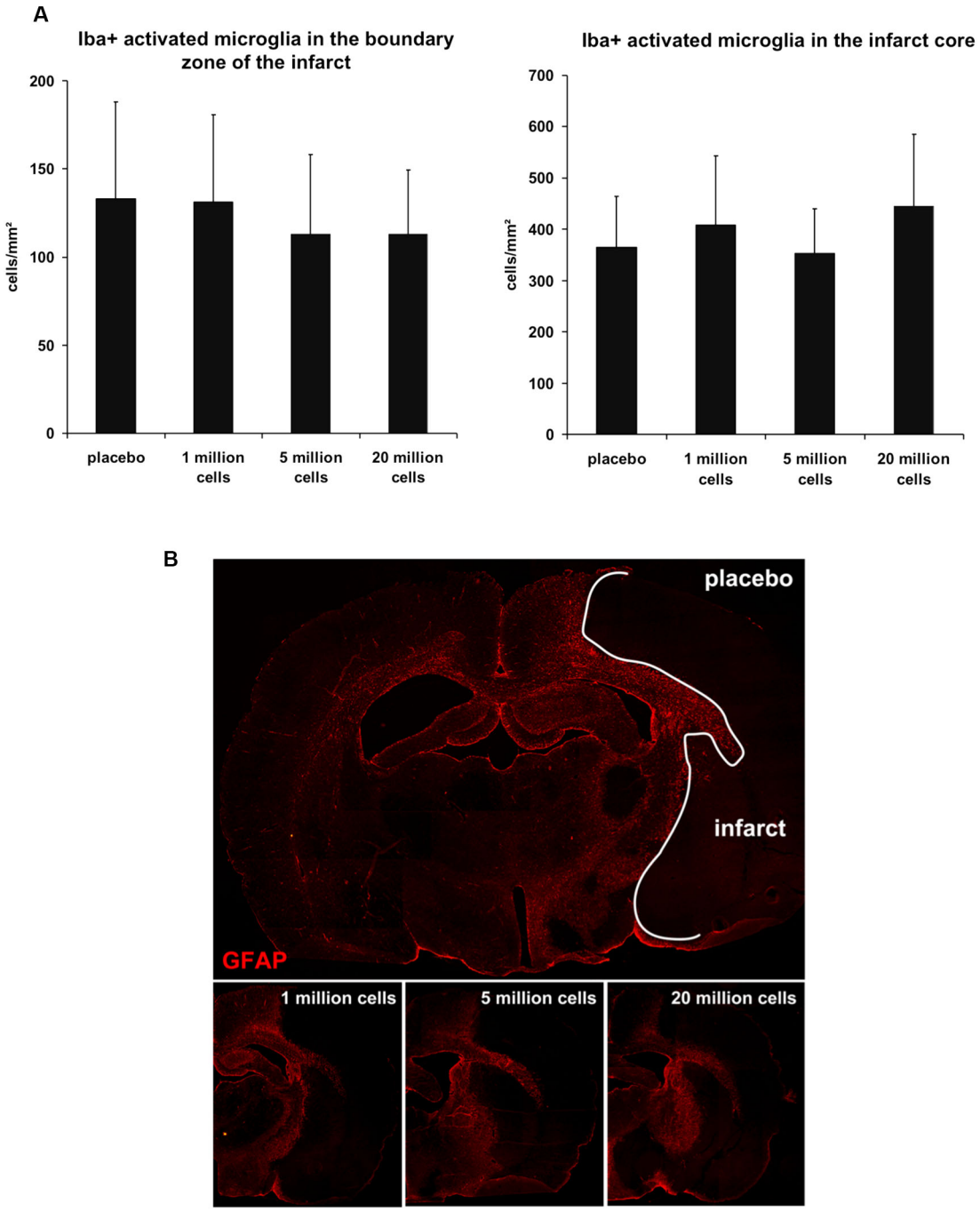

FIGURE 4 | Cellular inflammatory response 3 days after MCAO. (A) There was no statistical difference between the groups regarding the number of activated microglia cells (Iba1-positive) within the infarct core ($P = 0.122$) and in the boundary zone ($P = 0.476$) of the infarct ($n = 15$–16/group). **(B)** GFAP staining visualized the common distribution of astrogliosis surrounding the infarcted tissue. No differences with respect to GFAP-immunoreactivity and morphology between the BM-MNC and the placebo treated groups were observed ($n = 3$/group).

GFAP-immunoreactivity was also comparable among all groups (**Figure 4B**).

DISCUSSION

The present study did not show a beneficial effect for either dose of BM-MNCs given intravenously 3 h after the onset of focal cerebral ischemia in SH rats. There were no significant effects on the primary and secondary end points. Infarct volumes, mortality, behavioral outcomes, and the extent of the inflammatory response to cerebral ischemia were comparable in the placebo and in all BM-MNC groups. Particularly, no dose-response relationship of BM-MNC treatment was found. Our

results indicate that BM-MNCs do not exert acute neuroprotective properties in SH rats although transplanted BM-MNCs reached the brain.

The efficacy of early BM-MNC treatment initiation within 6 h after stroke onset was investigated by a number of studies (Iihoshi et al., 2004; Baker et al., 2007; Kamiya et al., 2008). For intravenous cell administration, a higher efficacy of early BM-MNC therapy at 3 h following stroke was shown as compared to later treatment (Iihoshi et al., 2004). Moreover, recombinant tissue-type plasminogen activator (rt-PA) treatment was approved until 3 h after the onset of ischemia at the time when the present study was designed (Hacke et al., 2008). Hence, the time for cell therapy initiation seems to be reasonable from a translational perspective. However, the sustained therapeutic benefit of early intravenous BM-MNC application could not be reproduced in our investigation, with the use of healthy animals in aforementioned experiments being the major difference in study design. Indeed, contrary results on a treatment's efficacy depending on the animal strain used in a study with greater infarct size reductions in healthy animals compared to animals with comorbidities were previously reported (Crossley et al., 2008). This observation was even assumed to contribute substantially to the translational failure in experimental stroke research (Crossley et al., 2008). However, despite the use of hypertensive animals in our study and the use of healthy animals in previously published positive studies is the most obvious difference, our study does not provide the final proof that the hypertensive condition of the host or of the donor is the reason for the negative results, since no healthy, non-hypertensive control group was included. Other reasons such as the preparation of the BM-MNCs or the way of cell transplantation might also be relevant for our negative results.

Baker et al. (2007) who also used healthy animals, found reduced infarct volumes and improved functional outcomes after early BM-MNC treatment (Baker et al., 2007). The intraarterial administration of BM-MNCs in this study is a further main difference to our experiments which might explain the contrary results. Although intraarterial procedures after stroke are getting more common and safer, results from a pre-clinical study found that intraarterial cell therapy is associated with an increased mortality (Walczak et al., 2008; Saver et al., 2012). From a translational perspective, intravenous approaches therefore seem to be the first choice in animal models and early-stage clinical trials (Savitz et al., 2011b). So far, one previous study suggested that systemic BM-MNC treatment may not be beneficial when initiated after the onset of ischemia (Kamiya et al., 2008). The significance of this study is, however, limited as the number of animals was rather small ($n = 5$) and only one cell dose was evaluated.

Therapies with cells of different sources were demonstrated to exert anti-inflammatory actions after cerebral ischemia (Lee et al., 2008; Schwarting et al., 2008). With respect to BM-MNC treatment, immunomodulatory mechanisms post-stroke were suggested based on *in vitro* investigations and on studies which found a BM-MNC treatment associated reduction of proinflammatory cytokines (Brenneman et al., 2010; Sharma et al., 2010). Our experiments, which investigated the local microglial immune response, however, did not confirm that BM-MNCs reduce this important element of post-stroke inflammation. A possible explanation for these apparently different observations might be that in the mentioned study cytokine levels of the whole brain were measured (Brenneman et al., 2010). Since healthy animals were used, infarct sizes were smaller after BM-MNC treatment and this reduced volume of injured tissue in turn might be the true reason for the lower cytokine concentrations.

Our study has strengths and limitations. The experiments rigorously adhered to stringent quality criteria in experimental stroke research such as randomization, surgery and evaluations performed in a blinded fashion, and controlled physiological parameters (Dirnagl, 2006; Kilkenny et al., 2011). Additionally, the use of animals with a relevant comorbidity may increase the predictive value of our findings regarding a human stroke patient population. Studies with negative results have a potential general weakness, i.e., the type II error. The type II error means that the null hypothesis is not rejected despite being false. Applied to the present study BM-MNCs in fact would reduce infarct volumes while not doing so in our experiments. However, we performed a thorough *a priori* sample size calculation to detect infarct size reductions of $\geq$20% with a statistical power of 0.8 which corresponds to a type II error of 0.2. Moreover, the use of a range of BM-MNC numbers should have prevented to miss the efficacy of a specific cell dose. Another potential limitation of our study is the generalizability. We cannot rule out that an alternative procedure for cell preparation or the use of healthy animals, animals of another strain or sex might have yield different results. Our negative finding is therefore restricted to the cell preparation, the route of transplantation and the animal model used in this study. Particularly, the negative results cannot attributed to the co-morbid condition of the animals with certainty, since no control groups of healthy animals were included.

Although neutral and negative experimental results are disappointing at a first glance publishing those is certainly of great importance when considering the tremendous costs of clinical studies and the potential risks for patients. The present results add relevant information to the pre-clinical development process of BM-MNCs for stroke therapy. While using a clinically meaningful model for post-stroke neuroprotection, we showed that BM-MNCs do not improve outcome when given in the early phase after the onset of ischemia. This may affect upcoming efficacy studies in patients based on the rational that early induction of BM-MNC therapy may lead to more favorable outcomes.

Indubitably, previous animal experimental stroke studies found improvements after BM-MNC therapy (Giraldi-Guimarães et al., 2009; Brenneman et al., 2010; Nakano-Doi et al., 2010). However, these studies initiated treatment beyond established time windows of acute neuroprotection, thus potentially aiming to restore neurological function by promoting regeneration rather than protecting tissue at risk. Indeed, BM-MNCs can also promote neurological recovery by enhancing neurogenesis and angiogenesis in later phases and were demonstrated to stimulate brain remodeling processes, such as the proliferation of neural progenitor and endothelial cells, and to reduce neurodegeneration (Giraldi-Guimarães et al., 2009; Nakano-Doi

et al., 2010). Since the investigation of these later effects was not in the focus of our investigation, a direct conclusion on the impact of BM-MNC treatment at later stages following experimental stroke may not be valid, but remains for further study. However, the remarkable differences regarding acute neuroprotection in healthy and comorbid animals as revealed by the present study clearly underline the necessity to evaluate relevant treatment effects and the optimal treatment time window in animal models more adequately reflecting the situation of stroke patients. This will help to prepare large scale efficacy studies with maximum rigor regarding our knowledge on therapeutic mechanisms, and thereby efficacy in human patients.

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REFERENCES

Baker, A. H., Sica, V., Work, L. M., Williams-Ignarro, S., de Nigris, F., Lerman, L. O., et al. (2007). Brain protection using autologous bone marrow cell, metalloproteinase inhibitors, and metabolic treatment in cerebral ischemia. *Proc. Natl. Acad. Sci. U S A* 104, 3597–3602. doi: 10.1073/pnas.0611112104

Brenneman, M., Sharma, S., Harting, M., Strong, R., Cox, C. S. Jr., Aronowski, J., et al. (2010). Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J. Cereb. Blood Flow Metab.* 30, 140–149. doi: 10.1038/jcbfm.2009.198

Crossley, N. A., Sena, E., Goehler, J., Horn, J., van der Worp, B., Bath, P. M. W., et al. (2008). Empirical evidence of bias in the design of experimental stroke studies: a metaepidemiologic approach. *Stroke* 39, 929–934. doi: 10.1161/STROKEAHA.107.498725

Dickhout, J. G., and Lee, R. M. (1998). Blood pressure and heart rate development in young spontaneously hypertensive rats. *Am. J. Physiol.* 274, H794–H800.

Dirnagl, U. (2006). Bench to bedside: the quest for quality in experimental stroke research. *J. Cereb. Blood Flow Metab.* 26, 1465–1478. doi: 10.1038/sj.jcbfm.9600298

Dirnagl, U., Iadecola, C., and Moskowitz, M. A. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* 22, 391–397. doi: 10.1016/s0166-2236(99)01401-0

Fisher, M., Feuerstein, G., Howells, D. W., Hurn, P. D., Kent, T. A., Savitz, S. I., et al. (2009). Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke* 40, 2244–2250. doi: 10.1161/STROKEAHA.108.541128

Giraldi-Guimarães, A., Rezende-Lima, M., Bruno, F. P., and Mendez-Otero, R. (2009). Treatment with bone marrow mononuclear cells induces functional recovery and decreases neurodegeneration after sensorimotor cortical ischemia in rats. *Brain Res.* doi: 10.1016/j.brainres.2009.01.062. [Epub ahead of print].

Hacke, W., Kaste, M., Bluhmki, E., Brozman, M., Dávalos, A., Guidetti, D., et al. (2008). Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N. Engl. J. Med.* 359, 1317–1329. doi: 10.1056/NEJMoa0804656

Iihoshi, S., Honmou, O., Houkin, K., Hashi, K., and Kocsis, J. D. (2004). A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. *Brain Res.* 1007, 1–9. doi: 10.1016/j.brainres.2003.09.084

Kamiya, N., Ueda, M., Igarashi, H., Nishiyama, Y., Suda, S., Inaba, T., et al. (2008). Intra-arterial transplantation of bone marrow mononuclear cells immediately after reperfusion decreases brain injury after focal ischemia in rats. *Life Sci.* 83: 433–437. doi: 10.1016/j.lfs.2008.07.018

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., and Altman, D. G. (2011). Animal research: reporting in vivo experiments–the ARRIVE guidelines. *J. Cereb. Blood Flow Metab.* 31, 991–993. doi: 10.1038/jcbfm.2010.220

Lee, S.-T., Chu, K., Jung, K.-H., Kim, S.-J., Kim, D.-H., Kang, K.-M., et al. (2008). Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. *Brain* 131, 616–629. doi: 10.1093/brain/awm306

Longa, E. Z., Weinstein, P. R., Carlson, S., and Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20, 84–91. doi: 10.1161/01.STR.20.1.84

Menzies, S. A., Hoff, J. T., and Betz, A. L. (1992). Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model. *Neurosurgery* 31, 100–106; discussion 106–107. doi: 10.1227/00006123-199207000-00014

Minnerup, J., Kim, J. B., Schmidt, A., Diederich, K., Bauer, H., Schilling, M., et al. (2011). Effects of neural progenitor cells on sensorimotor recovery and endogenous repair mechanisms after photothrombotic stroke. *Stroke* 42, 1757–1763. doi: 10.1161/STROKEAHA.110.599282

Minnerup, J., Seeger, F. H., Kuhnert, K., Diederich, K., Schilling, M., Dimmeler, S., et al. (2010). Intracarotid administration of human bone marrow mononuclear cells in rat photothrombotic ischemia. *Exp. Transl. Stroke Med.* 2:3. doi: 10.1186/2040-7378-2-3

Nakano-Doi, A., Nakagomi, T., Fujikawa, M., Nakagomi, N., Kubo, S., Lu, S., et al. (2010). Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 28, 1292–1302. doi: 10.1002/stem.454

Pösel, C., Möller, K., Fröhlich, W., Schulz, I., Boltze, J., and Wagner, D.-C. (2012). Density gradient centrifugation compromises bone marrow mononuclear cell yield. *PLoS One* 7:e50293. doi: 10.1371/journal.pone.0050293

Saver, J. L., Jahan, R., Levy, E. I., Jovin, T. G., Baxter, B., Nogueira, R. G., et al. (2012). Solitaire flow restoration device versus the Merci Retriever in patients with acute ischaemic stroke (SWIFT): a randomised, parallel-group, non-inferiority trial. *Lancet* 380, 1241–1249. doi: 10.1016/S0140-6736(12)61384-1

Savitz, S. I., Chopp, M., Deans, R., Carmichael, S. T., Phinney, D., and Wechsler, L. (2011a). Stem cell therapy as an emerging paradigm for stroke (STEPS) II. *Stroke* 42, 825–829. doi: 10.1161/strokeaha.110.601914

Savitz, S. I., Misra, V., Kasam, M., Juneja, H., Cox, C. S. Jr., Alderman, S., et al. (2011b). Intravenous autologous bone marrow mononuclear cells for ischemic stroke. *Ann. Neurol.* 70, 59–69. doi: 10.1002/ana.22458

Schäbitz, W.-R., Kollmar, R., Schwaninger, M., Juettler, E., Bardutzky, J., Schölzke, M. N., et al. (2003). Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* 34, 745–751. doi: 10.1161/01.str.0000057814.70180.17

Schneider, A., Krüger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., et al. (2005). The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J. Clin. Invest.* 115, 2083–2098. doi: 10.1172/jci23559

Schwarting, S., Litwak, S., Hao, W., Bähr, M., Weise, J., and Neumann, H. (2008). Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. *Stroke* 39, 2867–2875. doi: 10.1161/strokeaha.108.513978

Sevimli, S., Diederich, K., Strecker, J.-K., Schilling, M., Klocke, R., Nikol, S., et al. (2009). Endogenous brain protection by granulocyte-colony stimulating factor after ischemic stroke. *Exp. Neurol.* 217, 328–335. doi: 10.1016/j.expneurol.2009.03.018

Sharma, S., Yang, B., Strong, R., Xi, X., Brenneman, M., Grotta, J. C., et al. (2010). Bone marrow mononuclear cells protect neurons and modulate microglia in cell culture models of ischemic stroke. *J. Neurosci. Res.* 88, 2869–2876. doi: 10.1002/jnr.22452

Stem Cell Therapies as an Emerging Paradigm in Stroke Participants. (2009). Stem cell therapies as an emerging paradigm in stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. *Stroke* 40, 510–515. doi: 10.1161/STROKEAHA.108.526863

Wagner, D.-C., Bojko, M., Peters, M., Lorenz, M., Voigt, C., Kaminski, A., et al. (2012). Impact of age on the efficacy of bone marrow mononuclear cell transplantation in experimental stroke. *Exp. Transl. Stroke Med.* 4:17. doi: 10.1186/2040-7378-4-17

Walczak, P., Zhang, J., Gilad, A. A., Kedziorek, D. A., Ruiz-Cabello, J., Young, R. G., et al. (2008). Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 39, 1569–1574. doi: 10.1161/strokeaha.107.502047

Zhang, X.-M., Du, F., Yang, D., Wang, R., Yu, C.-J., Huang, X.-N., et al. (2011). Granulocyte colony-stimulating factor increases the therapeutic efficacy of bone marrow mononuclear cell transplantation in cerebral ischemia in mice. *BMC Neurosci.* 12:61. doi: 10.1186/1471-2202-12-61

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Exosomes/miRNAs as mediating cell-based therapy of stroke

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Cell-based therapy, e.g., multipotent mesenchymal stromal cell (MSC) treatment, shows promise for the treatment of various diseases. The strong paracrine capacity of these cells and not their differentiation capacity, is the principal mechanism of therapeutic action. MSCs robustly release exosomes, membrane vesicles (~30–100 nm) originally derived in endosomes as intraluminal vesicles, which contain various molecular constituents including proteins and RNAs from maternal cells. Contained among these constituents, are small non-coding RNA molecules, microRNAs (miRNAs), which play a key role in mediating biological function due to their prominent role in gene regulation. The release as well as the content of the MSC generated exosomes are modified by environmental conditions. Via exosomes, MSCs transfer their therapeutic factors, especially miRNAs, to recipient cells, and therein alter gene expression and thereby promote therapeutic response. The present review focuses on the paracrine mechanism of MSC exosomes, and the regulation and transfer of exosome content, especially the packaging and transfer of miRNAs which enhance tissue repair and functional recovery. Perspectives on the developing role of MSC mediated transfer of exosomes as a therapeutic approach will also be discussed.

Keywords: cell-based therapy, multipotent mesenchymal stromal cell (MSC), Exosome, microRNAs (miRNAs), bio-information transfer, stroke

INTRODUCTION

The therapeutic effects of cell-based therapy, such as for the treatment of stroke, with multipotent mesenchymal stromal cells (MSCs) have demonstrated particular promise. Systemic administration of MSCs as a treatment for stroke (Chen et al., 2001a,b; Li et al., 2001; Chopp and Li, 2002; Hessvik et al., 2013), has demonstrated that MSCs promote central nervous system (CNS) plasticity and neurovascular remodeling which lead to functional benefit (Caplan and Dennis, 2006; Zhang et al., 2006b; Chopp et al., 2008; Dharmasaroja, 2009; Li and Chopp, 2009; Zhang and Chopp, 2009, 2013; Borlongan et al., 2011; Herberts et al., 2011). Instead of the replacement of damaged cells, cell-based therapy provides therapeutic benefit by remodeling of the CNS, i.e., by promoting neuroplasticity, angiogenesis and immunomodulation (Chen et al., 2001b; Chopp and Li, 2002; Chopp et al., 2008; Li and Chopp, 2009; Zhang and Chopp, 2013; Liang et al., 2014). Early studies posited that the therapeutic efficacy of transplanted MSCs was attributed to their subsequent differentiation into parenchymal cells which repairs and replaces damaged tissues. However, studies in animal models and patients demonstrated that only a very small number of transplanted MSCs localize to the damage site and surrounding area, while most of the MSCs were localized in the liver, spleen and lungs (Phinney and Prockop, 2007). In addition, apparent evidence of MSC differentiation likely resulted from the fusion of transplanted MSCs with endogenous cells (Spees et al., 2003; Vassilopoulos et al., 2003; Konig et al., 2005; Ferrand et al., 2011). Supported by robust data, our present understanding of how MSCs promote neurological recovery is through their interaction with brain parenchymal cells. MSCs produce and induce within parenchymal cells biological effectors, e.g., neurotrophic factors, proteases, and morphogens, which subsequently enhance the neurovascular microenvironment surrounding the damaged area, as well as remodel remote tissue (Chen et al., 2002; Lu et al., 2002; Mahmood et al., 2004; Gao et al., 2005, 2008; Xin et al., 2006, 2010, 2011, 2013a; Zhang et al., 2006c, 2009; Qu et al., 2007; Zacharek et al., 2007; Shen et al., 2008, 2010, 2011b; Xu et al., 2010; Hermann and Chopp, 2012; Ding et al., 2013; Zhang and Chopp, 2013). Though the mechanisms which underlie the interaction and communication between the exogenously administered cells, e.g., MSCs, and brain parenchymal cells are not fully understood, the paracrine effect hypothesis has been strengthened by recent evidence that stem cells release extracellular vesicles which elicit similar biological activity to the stem cells themselves (Lai et al., 2011; Camussi et al., 2013; Xin et al., 2013b). These released extracellular lipid vesicles, provide a novel means of intercellular communication (Raposo and Stoorvogel, 2013; Fujita et al., 2014; Record et al., 2014; Turturici et al., 2014; Zhang and Grizzle, 2014). A particularly important class of extracellular vesicles released by stem cells and MSCs, is exosomes, and accumulating data show that MSCs release large amounts of exosomes which mediate the communication of MSCs with other cells (Collino et al., 2010; Hass and Otte, 2012; He et al., 2012; Xin et al., 2012; Lee et al., 2013; Roccaro et al., 2013; Wang et al., 2014). Here, we focus

our discussion on exosomes derived from MSCs, the biogenesis of MSC exosomes, cargo packaging (especially the miRNAs) and intercellular communication, and discuss new opportunities in modifying exosomal cargo to develop exosome-based cell-free therapeutics.

CHARACTERISTIC OF EXOSOMES

Lipid vesicles can be released by various types of cells, and they have been found in the supernatants from a wide variety of cells in culture, as well as in all bodily fluids (Yang et al., 2014; Yellon and Davidson, 2014; Zhang and Grizzle, 2014). The shedding of microvesicles and exosomes is likely a general property of most cells. Initial studies on cell released vesicles were reported in the 1960s (Roth and Luse, 1964; Schrier et al., 1971; Dalton, 1975), and the most common term, exosome, as applied to cell-derived vesicles was first defined by Trams et al. (1981); since they believe that these "exfoliated membrane vesicles may serve a physiologic function" and "it is proposed that they be referred to as exosomes" (Trams et al., 1981), (**Box 1**, nomenclature).

Extracellular released vesicles mainly include exosomes and microvesicles (Momen-Heravi et al., 2013). Exosomes are endocytic origin small-membrane vesicles. Eukaryotic cells periodically engulf small amounts of intracellular fluid in the specific membrane area, forming a small intracellular body called endosome (Thery et al., 2002). The early endosome matures and develops into the late endosome, during the maturation process, the inward budding of the endosomal membrane forms the intraluminal vesicles (ILV) which range in size from approximately 30–100 nm in diameter. The late endosome containing ILVs is also referred to as, a multivesicular body (MVB) and proteins are directly sorted to the MVBs from rough endoplasmic reticulum and Golgi complex (Thery et al., 1999), as are mRNAs, microRNAs, and DNAs (Villarroya-Beltri et al., 2013). The MVBs may either fuse with the lysosome and degrade their contents or fuse with the plasma membrane of the cell, releasing their ILVs to the extracellular environment (**Figure 1**). These vesicles are then referred as exosomes (Van Niel et al., 2006). Microvesicles are small, plasma membrane derived particles that are released into the extracellular environment by the outward budding and fission of the plasma membrane (Amano et al., 2001; Cocucci et al., 2009; Muralidharan-Chari et al., 2010). Unlike the large size of microvesicle (100∼1000 nm in diameter), exosomes have a smaller size, ∼30–100 nm in diameter (Stoorvogel et al., 2002). Exosome density in sucrose is located at 1.13–1.19 g/ml, and exosomes can be collected by ultracentrifugation at 100,000 g (Thery et al., 2006). The exosome membranes are enriched with cholesterol, sphingomyelin, and ceramide which are contained in lipid rafts (Thery et al., 2006). Most exosomes contain conserved proteins such as tetraspanins (CD81, CD63, and CD9), Alix and Tsg101, as well as the unique tissue/cell type specific proteins that reflect their cellular source. A precise and clear distinction between these vesicles (exosomes and microvesicles) is still lacking, and it is technically difficult to definitively separate them from the culture media by currently available methods like ultracentrifugation, density gradient separation, chromatography and immunoaffinity capture methods (Corrado et al., 2013). Exosomes are released by most cell types under physiological conditions. The amount of exosomes released from MSCs is highly related to cellular proliferation rate, and the exosome production is inversely correlated to the developmental maturity of the MSCs (Chen et al., 2013b). The release of extracellular vesicles can be altered by cellular stress and damage (Hugel et al., 2005; Greenwalt, 2006). Increased release of extracellular vesicles is associated with the acute and active phases of several neurological disorders (Hugel et al., 2005; Horstman et al., 2007). The distinctions between exosomes and other extracellular vesicles (such as microvesicles) are beyond the scope of this review and will not be discussed in detail here.

MSCs ROBUSTLY RELEASE EXOSOMES

Human MSC conditioned medium can reduce myocardial infarct size in patients with acute myocardial infarction (Timmers et al., 2007), and Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium, probably by increasing myocardial perfusion (Timmers et al., 2011). These therapeutic effects were then subsequently attributed to MSC derived exosomes (Lai et al., 2010). Thereafter, MSC exosomes were widely observed and tested in several disease models (Lee et al., 2012; Reis et al., 2012; Xin et al., 2012; Li et al., 2013; Tomasoni et al., 2013; Sdrimas and Kourembanas, 2014; Tan et al., 2014; Zhu et al., 2014).

Compared to other cells, MSCs can produce large amounts of exosomes (Yeo et al., 2013). There are no differences in terms of morphological features, isolation and storage conditions between exosomes derived from MSCs and other sources (Yeo et al., 2013). The MSC is the most prolific exosome producer when compared

Box 1 | Nomenclature.

Currently, the use of the term 'exosomes' for MVB-derived extracellular vesicles (EVs) is widely accepted in the field; however, the large variety of EVs secreted by cells and the technical difficult to definitively discriminate small EVs from exosomes in the culture media using currently available methods has led to the less stringent usage of the term, exosomes. Exosomes are presently characterized as either small EVs (of 30–100 nm diameter) measured by transmission electron microscopy (TEM)), or as EVs recovered after 100000g ultracentrifugation. As Gould and Raposo proposed recently, given the absence of perfect identification of EVs' of endosomal origin, researchers are recommended to explicitly state their use of terms, choose their terms based on precedent and logical argument, and apply them consistently throughout a piece of work (Gould and Raposo, 2013). Since the EVs identified and employed in our studies fulfill the above mentioned two characteristics (i.e.,TEM and 100000g untracentrifugation), therefore, exosomes are likely the primary constituents of the EVs. Here, in this manuscript, we use the term 'exosomes' as defined by Trams et al. (Trams et al., 1981), however, we do not exclude the possibility of other non-exosomal microvesicle components within the content of our injected precipitate, and we do not exclude a contribution of non-exosomal microvesicles to mediating stroke recovery.

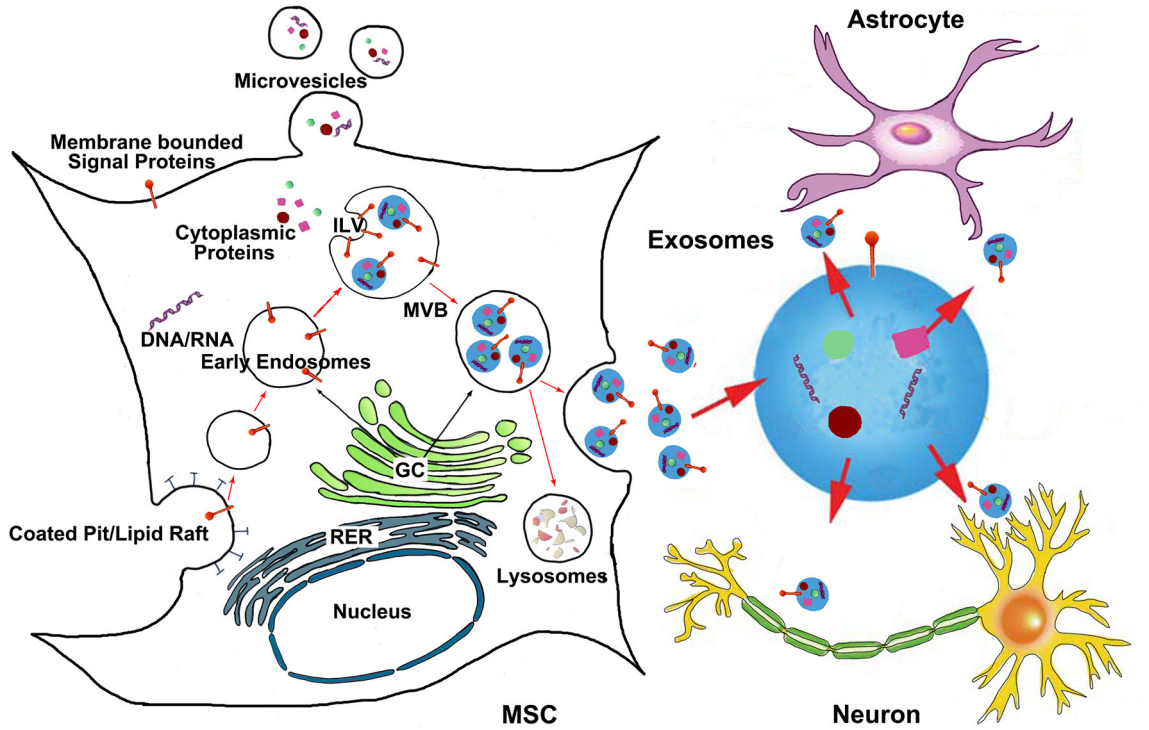

FIGURE 1 | The generation of MSC exosomes and bio-information shuttling between MSCs and brain parenchymal cells via exosomes. Exosomes are generated in the late endosomal compartment by inward budding of the limiting membrane of MVB. The exosome-filled MVBs are either fused with the plasma membrane to release exosomes or sent to lysosomes for degradation. Microvesicles are plasma membrane derived particles that are released into the extracellular environment by the direct outward budding and fission of the plasma membrane. The bio-information carried by MSC exosomes then transfer to brain parenchymal cells like astrocytes and neurons. ILV, intraluminal vesicles; MVB, multivesicular body; GC, Golgi complex; RER, rough endoplasmic reticulum.

to other cell types known to produce exosomes (Yeo et al., 2013). By transfecting human ESC-derived mesenchymal stem cells (hESC-MSCs) with a lentivirus carrying *myc* gene, Chen et al. generated an immortalized hESC-MSCs cell line. Exosomes from MYC-transformed MSCs were able to reduce relative infarct size in a mouse model of myocardial ischemia/reperfusion injury. They found that MYC transformation may be a practical strategy in ensuring an infinite supply of cells for the production of exosomes in the milligram range as either therapeutic agents or delivery vehicles. Additionally, the increased proliferative rate by MYC transformation reduces the time for cell production and thereby reduces production costs. Chen et al. (2011), thus, making MSCs an efficient and effective "factory" for mass production of exosomes.

THE CARGO OF MSC EXOSOMES

Exosomes are complex "living" structures generated by many cell types containing a multitude of cell surface receptors (Shen et al., 2011a; Yang and Gould, 2013), encapsulating proteins, trophic factors, miRNAs, and RNAs (Koh et al., 2010; Lai et al., 2011, 2012, 2013b; Record et al., 2011; Xin et al., 2012; Chen and Lim, 2013; Katakowski et al., 2013; Tomasoni et al., 2013; Yeo et al., 2013). These bioactive molecules can mediate exosomal intercellular communication (Zhang and Grizzle, 2014; Zhang and Wrana, 2014).

The exosome cargo is dependent on the cell type of origin (Raposo and Stoorvogel, 2013). Besides the common surface markers of exosomes, such as CD9 and CD81, MSCs contain specific membrane adhesive molecules, including CD29, CD44, and CD73 that are expressed on the MSC generated exosomes (Lai et al., 2012). Further, the specific conditions of cell preparation affect the exosome cargo (Kim et al., 2005; Park et al., 2010). In the MSC derived exosome, protein components also changed when exosomes were obtained from different MSC cultured media. In their study, Lai et al. found that 379, 432, and 420 unique proteins, detected by means of liquid chromatography-mass spectrometry/mass spectrometry in three independent batches of MSC derived exosomes, and only 154 common proteins are present (Lai et al., 2012). In addition to the protein cargo, RNAs, e.g., messenger RNA (mRNA) and miRNAs are encapsulated in MSC exosomes. MiRNAs encapsulated in MSC-derived microparticles are predominantly in their precursor form (Chen et al., 2010). However, other studies have demonstrated that various miRNAs are present in MSC exosomes, and the miRNA cargo participates in the cell-cell communication to alter the fate of recipient cells (Koh et al., 2010; Xin et al., 2012, 2013c; Katakowski et al., 2013; Lee et al., 2013; Ono et al., 2014).

Environmental challenges, such as activation or stress conditions, influence the composition, biogenesis, and secretion of exosomes. Possibly, exosome secretion is an efficient adaptive

mechanism that cells modulate intracellular stress situations and modify the surrounding environment via the secretion of exosomes. By preconditioning (Yu et al., 2013) or genetic manipulation (Kim et al., 2007b) of dendritic cells, the exosome secretion profile of these cells can be modified. The proteomic profiles of adipocyte-derived exosomes have been characterized (Sano et al., 2014). The authors found that protein content of the exosomes produced from cultured 3T3-L1 adipocytes was changed when they exposed the cells to hypoxic conditions. Quantitative proteomic analysis showed that 231 proteins were identified in the adipocyte-derived exosomes, and the expression levels of some proteins were altered under hypoxic conditions. The total amount of proteins in exosomes increased by 3-4-fold under hypoxic conditions (Sano et al., 2014). Another study found that the miRNA content of dendritic cell exosomes was affected by the maturation of the cells (Montecalvo et al., 2012), and similarly, compared with those from control cells, exosomes from mast cells contain different mRNAs when the cells were exposed to oxidative stress (Eldh et al., 2010). Furthermore, stressed cells that released exosomes conferred resistance against oxidative stress to recipient cells (Eldh et al., 2010), suggesting that cells modulate intracellular stress situations and modify the surrounding environment via the secretion of exosomes. The MSC exosome profile can be modified by pretreatment, as well. When MSCs were *in vitro* exposed to brain tissue extracted from rats subjected to middle cerebral artery occlusion (MCAo), the miR-133b levels in MSCs and their released exosomes were significantly increased compared to MSCs exposed to normal rat brain tissue extracts (Xin et al., 2012), indicating that MSCs used for stroke treatment will modify their gene expression and subsequently affect their exosome cargo. Thus, there is a feedback between the MSC and its environment, and through which ischemic conditions will modify the exosome contents, and consequently, the secreted exosomes affect and modify the tissue environment. Though we only tested one specific miRNA in our study, it is reasonable to propose that other miRNAs or other cargos of MSC exosome were modified by the post ischemic condition. i.e., other groups also demonstrated that miR-22 in MSC exosomes were enriched following ischemic preconditioning (Feng et al., 2014).

MSC DERIVED EXOSOMES TRANSFER BIO-INFORMATION TO RECIPIENT CELLS VIA miRNA

MiRNAs are non-protein coding, short ribonucleic acid (usually 18–25 nucleotides) molecules found in eukaryotic cells. Via binding to complementary sequences on target mRNA transcripts, miRNAs post-transcriptionally control gene expression (Bartel, 2004, 2009). MiRNAs constitute a major regulatory gene family in eukaryotic cells (Bartel, 2004; Zhang et al., 2006a, 2007; Fiore et al., 2008). MiRNAs are master molecular switches, concurrently affecting translation of, possibly, hundreds of mRNAs (Cai et al., 2009; Agnati et al., 2010). Over 1000 miRNAs are encoded by the human genome (Bartel, 2004) and they target about 60% of mammalian genes (Lewis et al., 2005; Friedman et al., 2009), and are abundant in many human cell types (Lim et al., 2003). By affecting gene expression, miRNAs are likely involved in most biological processes (Brennecke et al., 2003; Chen et al., 2004; Cuellar and McManus, 2005; Harfe et al., 2005; Lim et al., 2005). Based on the master gene regulation role of miRNAs, though MSC exosomes have the potential for protein cargo transfer (Zhang et al., 2014), we envisage that compared with the delivery of proteins, transfer of miRNA may have dramatic effects on the network of proteins and RNAs of the recipient cells.

Exosomes are well suited for small functional molecule delivery (Zomer et al., 2010). Increasing evidence indicates that they play a pivotal role in cell-to-cell communication (Mathivanan et al., 2010) and act as biological transporters (Denzer et al., 2000; Fevrier and Raposo, 2004; Lotvall and Valadi, 2007; Smalheiser, 2007; Valadi et al., 2007; Mathivanan et al., 2010; Lee et al., 2011; Record et al., 2011; Von Bartheld and Altick, 2011; Mittelbrunn and Sanchez-Madrid, 2012; Boon and Vickers, 2013; Raposo and Stoorvogel, 2013). Importantly, by being encapsulated and contained within the exosomes, the RNA is protected from the digestion of RNAase or trypsin (Valadi et al., 2007). Multiple studies show that exosomes transfer miRNAs to recipient cells (Valadi et al., 2007; Hergenreider et al., 2012). The transferred miRNAs then modify the recipient cell's characteristics. Shimbo et al. introduced synthetic miR-143 into cells, and the miR-143 was enveloped in released exosomes (Shimbo et al., 2014). The secreted exosome-formed miR-143 is transferred to osteosarcoma cells and subsequently significantly reduced the migration of osteosarcoma cells (Shimbo et al., 2014). Recent studies show that MSC exosomes regulate recipient cell protein expression and modify cell characteristics through the miRNA transfer (Xin et al., 2012; Lee et al., 2013; Wang et al., 2014). Exosomal transfer of miR-23b from the bone marrow may promote breast cancer cell dormancy in a metastatic niche (Ono et al., 2014). The master gene regulation role of miRNAs encapsulated within exosomes, determines their major role in the modification of recipient cells.

EXOSOMES SHUTTLE miRNAs AS REGULATORS FOR STROKE RECOVERY AFTER MSC THERAPY

In the nervous system, exosomes mediate cell-cell communication including the transfer of synaptic proteins, mRNAs and microRNAs (Smalheiser, 2007). The role of miRNAs at various stages of neuronal development and maturation has been recently elucidated (Costa-Mattioli et al., 2009; Saba and Schratt, 2010; Olde Loohuis et al., 2012). Numerous miRNAs are expressed in spatially and temporally controlled manners in the nervous system (Kapsimali et al., 2007; Bak et al., 2008; Dogini et al., 2008; Kocerha et al., 2009; Sethi and Lukiw, 2009; Ziu et al., 2011), suggesting that miRNAs have important functions in the gene regulatory networks involved in adult neural plasticity (Sethi and Lukiw, 2009; Liu and Xu, 2011; Mor et al., 2011; Goldie and Cairns, 2012). Stroke induces changes in the miRNA profile of MSCs and within their released exosomes (Jeyaseelan et al., 2008; Lusardi et al., 2014), and miRNAs actively participate in the recovery process after stroke (Liu et al., 2013).

MiR-133b promotes functional recovery in Parkinson's disease (Kim et al., 2007a) and appears essential for neurite outgrowth and functional recovery after spinal cord injury in adult zebrafish (Yu et al., 2011). Moreover, miR-133b regulates the expression of its targets, connective tissue growth factor (CTGF), a major

inhibitor of axonal growth at injury sites in the CNS in mammals (White and Jakeman, 2008; Duisters et al., 2009) and down-regulates Ras homolog gene family, member A (RhoA) protein expression (Care et al., 2007; Chiba et al., 2009). In our series of studies, we first found that miR-133b is substantially down-regulated in rat brain after MCAo, and MSC administration significantly increased the miR-133b level in the ischemic cerebral tissue. When MSCs were exposed to ischemic brain extracts, the miR-133b level was increased in exosomes released from these MSCs. We then treated primary cultured neurons and astrocytes with these exosomes, and found the miR-133b level in the neurons and astrocytes were increased, suggesting that the exosomes mediate the miR-133b transfer from MSCs to the neurons and astrocytes. Further *in vitro* knockdown of miR-133b in MSCs directly confirmed that the increased miR-133b level in astrocytes is attributed to their transfer from MSCs to neural cells, and exosomal miR-133b from MSCs significantly increased the neurite branch number and total neurite length (Xin et al., 2012). Compared with administration of normal MSCs, *in vivo* administration of MSCs with increased or decreased miR-133b (MSCs modified using lentivirus with miR-133b knocked-in or knocked-down) to rats subjected to MCAo resulted in promotion or inhibition of neurite outgrowth, respectively (Xin et al., 2012). Correspondingly, *in vitro* and *in vivo*, we also observed the transfer of miR-133b from MSCs to astrocytes via exosomes down-regulated CTGF expression, which may thin the glial scar and benefit neurite outgrowth. In contrast, treatment of stroke in rats with MSCs containing increased miR-133b, inhibited RhoA expression in neurons which enhanced the regrowth of the corticospinal tract after injury (Dergham et al., 2002; Holtje et al., 2009). Down-regulation of CTGF and RhoA by miR-133b stimulated neurite outgrowth and thereby improved functional recovery after stroke (Xin et al., 2012). This proof-of-concept study, provides the first demonstration that MSCs communicate with astrocytes and neurons and regulate neurite outgrowth by transfer of miRNAs (miR-133b) via exosomes. The identification of exosomes released from MSCs as a shuttle that carries miR-133b to astrocytes and neurons after cerebral ischemia helps to explain, at least in-part, how the exogenous MSCs contribute to neurological recovery after stroke. Exosome delivery of functional miRNAs, e.g., miR-133b, that promote neurite outgrowth may show benefit in other neurological diseases, in addition to stroke.

EXOSOMES AS AN ALTERNATIVE THERAPEUTIC CANDIDATE OF MSCs ON STROKE

MSC exosomes serve as a vehicle to transfer protein, mRNA, and miRNA to distant recipient cells, altering the gene expression of the recipient cells. Recently, MSC exosomes have been found to be efficacious in an increasing number of animal models for the treatment of diseases such as liver fibrosis (Li et al., 2013), liver injury (Tan et al., 2014), hypoxic pulmonary hypertension (Lee et al., 2012), acute lung injury (Sdrimas and Kourembanas, 2014; Zhu et al., 2014), acute kidney injury (Gatti et al., 2011; Reis et al., 2012; Tomasoni et al., 2013), and cardiovascular diseases (Lai et al., 2011). We demonstrated that systemic treatment of stroke with cell-free exosomes derived from MSCs significantly improve neurological outcome and contribute to neurovascular remodeling (Xin et al., 2013b). This approach is the first to consider treatment of stroke solely with exosomes.

Development of gene therapy vehicles for diffuse delivery to the brain is one of the major challenges for clinical gene therapy. By using miRNA mimics or antagonists, miRNA-based strategies have recently emerged as a promising therapeutic approach for specific diseases. However, despite its exciting potential, the bottleneck of this approach is delivery of miRNA; an optimal delivery system must be found before their clinical application. Researchers developed a number of miRNA delivery systems (Zhang et al., 2013), including liposomes (Lv et al., 2006), and peptide transduction domain–double-stranded RNA-binding domain (Eguchi and Dowdy, 2009). However, synthetic materials which are employed in the above systems, limited their use. Thus, the advantages of exosomes as delivery systems are apparent; they only contain biogenic substances and are readily transferred into target cells, as well as they have potentially wide utility for the delivery of nucleic acids, and possibly for selectively targeting cells. We and others have shown that MSCs can act as "factories" for the generation of exosomes, and that the cargo within these exosomes, including the miRNAs, may be regulated by altering the genetic character of the MSCs, e.g., by transfecting the MSCs with specific genes (Zomer et al., 2010; Bullerdiek and Flor, 2012; Hu et al., 2012; Katakowski et al., 2013; Xin et al., 2013c). We have also successfully modulated the miRNA content of the MSC generated exosomes and thereby modulated neurovascular plasticity and neurological recovery from stroke (Xin et al., 2013c). Given that MSC exosomes promote recovery (Xin et al., 2013b) and MSCs release exosomes *in vivo*, we propose that MSC generated exosomes with enhanced expression of beneficial miRNAs (e.g., miR-133b) may provide improved recovery benefits.

Another development direction for the exosome treatment of disease is the targeting of recipient cells. We demonstrate a significant therapeutic and neuroplasticity effect of systemic exosome administration (Xin et al., 2013b). Considering the nano size of exosomes, they likely enter into the brain (Lakhal and Wood, 2011). Adhesive molecules are expressed on the exosome membrane (Clayton et al., 2004), which may facilitate entry into the brain. Thus, systemic exosome administration may be a means by which to deliver the active components of cell-based therapy to the CNS. To improve exosomal targeting, we may also consider engineering and tailoring cell membrane proteins, e.g., the engineering of dendritic cells to express an exosomal membrane protein, Lamp2b, fused to the neuron-specific RVG peptide3 (Alvarez-Erviti et al., 2011). Alvarez-Erviti et al. demonstrated effective delivery of functional siRNA into mouse brain by systemic injection of exosomes, and targeted the exosomes to neurons (Alvarez-Erviti et al., 2011). These data indicate that specifically targeting neural cells is feasible by modifying exosomal membrane proteins.

CONCLUSION AND PROSPECTS

Exosomes derived from MSCs, carry, and transfer their cargo (e.g., miRNAs) to parenchymal cells, and thereby mediate brain plasticity, and the functional recovery from stroke. For the intricate blend of paracrine factors needed, exosomes may be ideal

carriers for treatment of a complicated disease such as stroke. Specifically modifying the miRNA content of MSC generated exosomes to modulate the therapeutic response for stroke may enhance their therapeutic application.

Cell-based therapies are in clinical trials for stroke and other neurological diseases (Zhou et al., 2013) and there is a robust literature on the efficacy of cell-based therapies for stroke (Hess and Borlongan, 2008). However, there are multiple benefits in transplanting exosomes rather than in transplanting the whole "factory," the cell, into the body. In contrast to exogenously administered cells delivered systemically, exosomes, given their nano dimension may readily enter the brain and easily pass through the blood brain barrier (BBB) (Alvarez-Erviti et al., 2011; Kooijmans et al., 2012; Anthony and Shiels, 2013; Gheldof et al., 2013; Meckes et al., 2013). Exogenously administered MSCs may have many adverse effects, i. e. tumor modulation and malignant transformation. (Herberts et al., 2011; Wong, 2011), and they may lodge and initially obstruct small vessels in organs (Gao et al., 2001; Chen et al., 2013a). Exosomes given their min size, in contrast, have no vascular obstructive effect, and have no apparent adverse effects.

One case has been reported where exosomes were used for treatment for severe acute graft vs. host disease (Kordelas et al., 2014) in which MSC exosomes did not show any side effects. Side effects of exosome therapies were also not observed in any of the tumor vaccination studies which were performed in humans (Mignot et al., 2006; Viaud et al., 2008). Prion diseases are infectious neurodegenerative disorders linked to the accumulation of the abnormally folded prion protein (PrP) scrapie (PrPsc) in the CNS. Once present, PrPsc catalyzes the conversion of naturally occurring cellular PrP (PrPc) to PrPsc. Recent studies show both PrPc and PrPsc were actively released into the extracellular environment by PrP-expressing cells before and after infection with sheep prions, respectively, and the release associated with exosomes. Even though EV administration appears safe and no side effects have been observed so far, it should be noted, that exosomes may contribute to intercellular membrane exchange and the spread of prions (Fevrier et al., 2004; Klohn et al., 2013). Since fetal calf serum is used for *in vitro* culturing MSCs and amplifying the exosomes, it may bring the risk of prion disease spreading by exosomes, but this risk may be carried by *in vitro* cultured MSCs as well. However, the risk associated with exosome therapies is rather low. **Table 1** shows the pros and cons of MSCs based therapy and MSC exosomes based therapy.

Table 1 | Pros and Cons of MSCs based therapy and MSC exosomes based therapy.

MSCs BASED THERAPY	
Pros	Living cell; continuously release exosomes or other soluble factors; potency of differentiation and replacement.
Cons	tumor modulation; malignant transformation; lodge and initially obstruct small vessels in organs.
MSC EXOSOME BASED THERAPY	
Pros	No vascular obstructive effect; no apparent adverse effects; nano size ensure it easily pass through BBB.
Cons	None at present

Technical issues, such as, purity of exosomes must be addressed, since the most common isolation protocol with differential centrifugation and a sucrose gradient yield a heterogeneous product (EL Andaloussi et al., 2013; Lai et al., 2013a). Methods for mass exosome isolation should also be developed to reduce costs. For the modified exosome application, the exosome product needs to be extensively characterized, in order to assess its biological function and to avoid adverse effects.

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REFERENCES

Agnati, L. F., Guidolin, D., Guescini, M., Genedani, S., and Fuxe, K. (2010). Understanding wiring and volume transmission. *Brain Res. Rev.* 64, 137–159. doi: 10.1016/j.brainresrev.2010.03.003

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M. J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* 29, 341–345. doi: 10.1038/nbt.1807

Amano, T., Furuno, T., Hirashima, N., Ohyama, N., and Nakanishi, M. (2001). Dynamics of intracellular granules with CD63-GFP in rat basophilic leukemia cells. *J. Biochem.* 129, 739–744. doi: 10.1093/oxfordjournals.jbchem.a002914

Anthony, D. F., and Shiels, P. G. (2013). Exploiting paracrine mechanisms of tissue regeneration to repair damaged organs. *Transplant. Res.* 2:10. doi: 10.1186/2047-1440-2-10

Bak, M., Silahtaroglu, A., Moller, M., Christensen, M., Rath, M. F., Skryabin, B., et al. (2008). MicroRNA expression in the adult mouse central nervous system. *RNA* 14, 432–444. doi: 10.1261/rna.783108

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. doi: 10.1016/S0092-8674(04)00045-5

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Boon, R. A., and Vickers, K. C. (2013). Intercellular transport of microRNAs. *Arterioscler. Thromb. Vasc. Biol.* 33, 186–192. doi: 10.1161/ATVBAHA.112.300139

Borlongan, C. V., Glover, L. E., Tajiri, N., Kaneko, Y., and Freeman, T. B. (2011). The great migration of bone marrow-derived stem cells toward the ischemic brain: therapeutic implications for stroke and other neurological disorders. *Prog. Neurobiol.* 95, 213–228. doi: 10.1016/j.pneurobio.2011.08.005

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B., and Cohen, S. M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* 113, 25–36. doi: 10.1016/S0092-8674(03)00231-9

Bullerdiek, J., and Flor, I. (2012). Exosome-delivered microRNAs of "chromosome 19 microRNA cluster" as immunomodulators in pregnancy and tumorigenesis. *Mol. Cytogenet.* 5, 27. doi: 10.1186/1755-8166-5-27

Cai, Y., Yu, X., Hu, S., and Yu, J. (2009). A brief review on the mechanisms of miRNA regulation. *Genomics Proteomics Bioinformatics* 7, 147–154. doi: 10.1016/S1672-0229(08)60044-3

Camussi, G., Deregibus, M. C., and Cantaluppi, V. (2013). Role of stem-cell-derived microvesicles in the paracrine action of stem cells. *Biochem. Soc. Trans.* 41, 283–287. doi: 10.1042/BST20120192

Caplan, A. I., and Dennis, J. E. (2006). Mesenchymal stem cells as trophic mediators. *J. Cell. Biochem.* 98, 1076–1084. doi: 10.1002/jcb.20886

Care, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., et al. (2007). MicroRNA-133 controls cardiac hypertrophy. *Nat. Med.* 13, 613–618. doi: 10.1038/nm1582

Chen, C. Z., Li, L., Lodish, H. F., and Bartel, D. P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303, 83–86. doi: 10.1126/science.1091903

Chen, J., Li, Y., Wang, L., Lu, M., Zhang, X., and Chopp, M. (2001a). Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J. Neurol. Sci.* 189, 49–57. doi: 10.1016/S0022-510X(01)00557-3

Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M., et al. (2001b). Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 32, 1005–1011. doi: 10.1161/01.STR.32.4.1005

Chen, K., Page, J. G., Schwartz, A. M., Lee, T. N., Dewall, S. L., Sikkema, D. J., et al. (2013a). False-positive immunogenicity responses are caused by CD20(+) B cell membrane fragments in an anti-ofatumumab antibody bridging assay. *J. Immunol. Methods* 394, 22–31. doi: 10.1016/j.jim.2013.04.011

Chen, T. S., Arslan, F., Yin, Y., Tan, S. S., Lai, R. C., Choo, A. B., et al. (2011). Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *J. Transl. Med.* 9, 47. doi: 10.1186/1479-5876-9-47

Chen, T. S., Lai, R. C., Lee, M. M., Choo, A. B., Lee, C. N., and Lim, S. K. (2010). Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res.* 38, 215–224. doi: 10.1093/nar/gkp857

Chen, T. S., and Lim, S. K. (2013). Measurement of precursor miRNA in exosomes from human ESC-derived mesenchymal stem cells. *Methods Mol. Biol.* 1024, 69–86. doi: 10.1007/978-1-62703-453-1_6

Chen, T. S., Yeo, R. W. Y., Arslan, F., Yin, Y., Tan, S. S., Lai, R. C., et al. (2013b). Efficiency of exosome production correlates inversely with the developmental maturity of MSC donor. *J. Stem Cell Res. Ther.* 3:145. doi: 10.4172/2157-7633.1000145

Chen, X., Li, Y., Wang, L., Katakowski, M., Zhang, L., Chen, J., et al. (2002). Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology* 22, 275–279. doi: 10.1046/j.1440-1789.2002.00450.x

Chiba, Y., Tanabe, M., Goto, K., Sakai, H., and Misawa, M. (2009). Down-regulation of miR-133a contributes to up-regulation of Rhoa in bronchial smooth muscle cells. *Am. J. Respir. Crit. Care Med.* 180, 713–719. doi: 10.1164/rccm.200903-0325OC

Chopp, M., and Li, Y. (2002). Treatment of neural injury with marrow stromal cells. *Lancet Neurol.* 1, 92–100. doi: 10.1016/S1474-4422(02)00040-6

Chopp, M., Li, Y., and Zhang, J. (2008). Plasticity and remodeling of brain. *J. Neurol. Sci.* 265, 97–101. doi: 10.1016/j.jns.2007.06.013

Clayton, A., Turkes, A., Dewitt, S., Steadman, R., Mason, M. D., and Hallett, M. B. (2004). Adhesion and signaling by B cell-derived exosomes: the role of integrins. *FASEB J.* 18, 977–979. doi: 10.1096/fj.03-1094fje

Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 19, 43–51. doi: 10.1016/j.tcb.2008.11.003

Collino, F., Deregibus, M. C., Bruno, S., Sterpone, L., Aghemo, G., Viltono, L., et al. (2010). Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PLoS ONE* 5:e11803. doi: 10.1371/journal.pone.0011803

Corrado, C., Raimondo, S., Chiesi, A., Ciccia, F., De Leo, G., and Alessandro, R. (2013). Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications. *Int. J. Mol. Sci.* 14, 5338–5366. doi: 10.3390/ijms14035338

Costa-Mattioli, M., Sossin, W. S., Klann, E., and Sonenberg, N. (2009). Translational control of long-lasting synaptic plasticity and memory. *Neuron* 61, 10–26. doi: 10.1016/j.neuron.2008.10.055

Cuellar, T. L., and McManus, M. T. (2005). MicroRNAs and endocrine biology. *J. Endocrinol.* 187, 327–332. doi: 10.1677/joe.1.06426

Dalton, A. J. (1975). Microvesicles and vesicles of multivesicular bodies versus "virus-like" particles. *J. Natl. Cancer Inst.* 54, 1137–1148.

Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., and Geuze, H. J. (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* 113(Pt 19), 3365–3374.

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W. D., and McKerracher, L. (2002). Rho signaling pathway targeted to promote spinal cord repair. *J. Neurosci.* 22, 6570–6577.

Dharmasaroja, P. (2009). Bone marrow-derived mesenchymal stem cells for the treatment of ischemic stroke. *J. Clin. Neurosci.* 16, 12–20. doi: 10.1016/j.jocn.2008.05.006

Ding, X., Li, Y., Liu, Z., Zhang, J., Cui, Y., Chen, X., et al. (2013). The sonic hedgehog pathway mediates brain plasticity and subsequent functional recovery after bone marrow stromal cell treatment of stroke in mice. *J. Cereb. Blood Flow Metab.* 33, 1015–1024. doi: 10.1038/jcbfm.2013.50

Dogini, D. B., Ribeiro, P. A., Rocha, C., Pereira, T. C., and Lopes-Cendes, I. (2008). MicroRNA expression profile in murine central nervous system development. *J. Mol. Neurosci.* 35, 331–337. doi: 10.1007/s12031-008-9068-4

Duisters, R. F., Tijsen, A. J., Schroen, B., Leenders, J. J., Lentink, V., Van Der Made, I., et al. (2009). miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. *Circ. Res.* 104, 170–178, 176p following 178. doi: 10.1161/CIRCRESAHA.108.182535

Eguchi, A., and Dowdy, S. F. (2009). siRNA delivery using peptide transduction domains. *Trends Pharmacol. Sci.* 30, 341–345. doi: 10.1016/j.tips.2009.04.009

EL Andaloussi, S., Mager, I., Breakefield, X. O., and Wood, M. J. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* 12, 347–357. doi: 10.1038/nrd3978

Eldh, M., Ekstrom, K., Valadi, H., Sjostrand, M., Olsson, B., Jernas, M., et al. (2010). Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS ONE* 5:e15353. doi: 10.1371/journal.pone.0015353

Feng, Y., Huang, W., Wani, M., Yu, X., and Ashraf, M. (2014). Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22. *PLoS ONE* 9:e88685. doi: 10.1371/journal.pone.0088685

Ferrand, J., Noel, D., Lehours, P., Prochazkova-Carlotti, M., Chambonnier, L., Menard, A., et al. (2011). Human bone marrow-derived stem cells acquire epithelial characteristics through fusion with gastrointestinal epithelial cells. *PLoS ONE* 6:e19569. doi: 10.1371/journal.pone.0019569

Fevrier, B., and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr. Opin. Cell Biol.* 16, 415–421. doi: 10.1016/j.ceb.2004.06.003

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., et al. (2004). Cells release prions in association with exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9683–9688. doi: 10.1073/pnas.0308413101

Fiore, R., Siegel, G., and Schratt, G. (2008). MicroRNA function in neuronal development, plasticity and disease. *Biochim. Biophys. Acta* 1779, 471–478. doi: 10.1016/j.bbagrm.2007.12.006

Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 19, 92–105. doi: 10.1101/gr.082701.108

Fujita, Y., Yoshioka, Y., Ito, S., Araya, J., Kuwano, K., and Ochiya, T. (2014). Intercellular communication by extracellular vesicles and their microRNAs in asthma. *Clin. Ther.* 36, 873–881. doi: 10.1016/j.clinthera.2014.05.006

Gao, J., Dennis, J. E., Muzic, R. F., Lundberg, M., and Caplan, A. I. (2001). The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs* 169, 12–20. doi: 10.1159/000047856

Gao, Q., Katakowski, M., Chen, X., Li, Y., and Chopp, M. (2005). Human marrow stromal cells enhance connexin43 gap junction intercellular communication in cultured astrocytes. *Cell Transplant.* 14, 109–117. doi: 10.3727/000000005783983205

Gao, Q., Li, Y., Shen, L., Zhang, J., Zheng, X., Qu, R., et al. (2008). Bone marrow stromal cells reduce ischemia-induced astrocytic activation *in vitro*. *Neuroscience* 152, 646–655. doi: 10.1016/j.neuroscience.2007.10.069

Gatti, S., Bruno, S., Deregibus, M. C., Sordi, A., Cantaluppi, V., Tetta, C., et al. (2011). Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol. Dial. Transplant* 26, 1474–1483. doi: 10.1093/ndt/gfr015

Gheldof, D., Mullier, F., Chatelain, B., Dogne, J. M., and Chatelain, C. (2013). Inhibition of tissue factor pathway inhibitor increases the sensitivity of thrombin generation assay to procoagulant microvesicles. *Blood Coagul. Fibrinolysis* 24, 567–572. doi: 10.1097/MBC.0b013e328360a56e

Goldie, B. J., and Cairns, M. J. (2012). Post-transcriptional trafficking and regulation of neuronal gene expression. *Mol. Neurobiol.* 45, 99–108. doi: 10.1007/s12035-011-8222-0

Gould, S. J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J. Extracell. Vesicles* 2:20389. doi: 10.3402/jev.v2i0.20389

Greenwalt, T. J. (2006). The how and why of exocytic vesicles. *Transfusion* 46, 143–152. doi: 10.1111/j.1537-2995.2006.00692.x

Harfe, B. D., McManus, M. T., Mansfield, J. H., Hornstein, E., and Tabin, C. J. (2005). The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10898–10903. doi: 10.1073/pnas.0504834102

Hass, R., and Otte, A. (2012). Mesenchymal stem cells as all-round supporters in a normal and neoplastic microenvironment. *Cell Commun. Signal.* 10, 26. doi: 10.1186/1478-811X-10-26

He, J., Wang, Y., Sun, S., Yu, M., Wang, C., Pei, X., et al. (2012). Bone marrow stem cells-derived microvesicles protect against renal injury in the mouse remnant kidney model. *Nephrology (Carlton)* 17, 493–500. doi: 10.1111/j.1440-1797.2012.01589.x

Herberts, C. A., Kwa, M. S., and Hermsen, H. P. (2011). Risk factors in the development of stem cell therapy. *J. Transl. Med.* 9:29. doi: 10.1186/1479-5876-9-29

Hergenreider, E., Heydt, S., Treguer, K., Boettger, T., Horrevoets, A. J., Zeiher, A. M., et al. (2012). Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat. Cell Biol.* 14, 249–256. doi: 10.1038/ncb2441

Hermann, D. M., and Chopp, M. (2012). Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurol.* 11, 369–380. doi: 10.1016/S1474-4422(12)70039-X

Hess, D. C., and Borlongan, C. V. (2008). Cell-based therapy in ischemic stroke. *Expert Rev. Neurother.* 8, 1193–1201. doi: 10.1586/14737175.8.8.1193

Hessvik, N. P., Sandvig, K., and Llorente, A. (2013). Exosomal miRNAs as Biomarkers for Prostate Cancer. *Front. Genet.* 4:36. doi: 10.3389/fgene.2013.00036

Holtje, M., Djalali, S., Hofmann, F., Munster-Wandowski, A., Hendrix, S., Boato, F., et al. (2009). A 29-amino acid fragment of Clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation. *FASEB J.* 23, 1115–1126. doi: 10.1096/fj.08-116855

Horstman, L. L., Jy, W., Minagar, A., Bidot, C. J., Jimenez, J. J., Alexander, J. S., et al. (2007). Cell-derived microparticles and exosomes in neuroinflammatory disorders. *Int. Rev. Neurobiol.* 79, 227–268. doi: 10.1016/S0074-7742(07)79010-4

Hu, G., Drescher, K. M., and Chen, X. M. (2012). Exosomal miRNAs: biological properties and therapeutic potential. *Front. Genet.* 3:56. doi: 10.3389/fgene.2012.00056

Hugel, B., Martinez, M. C., Kunzelmann, C., and Freyssinet, J. M. (2005). Membrane microparticles: two sides of the coin. *Physiology (Bethesda)* 20, 22–27. doi: 10.1152/physiol.00029.2004

Jeyaseelan, K., Lim, K. Y., and Armugam, A. (2008). MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 39, 959–966. doi: 10.1161/STROKEAHA.107.500736

Kapsimali, M., Kloosterman, W. P., De Bruijn, E., Rosa, F., Plasterk, R. H., and Wilson, S. W. (2007). MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. *Genome Biol.* 8, R173. doi: 10.1186/gb-2007-8-8-r173

Katakowski, M., Buller, B., Zheng, X., Lu, Y., Rogers, T., Osobamiro, O., et al. (2013). Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett.* 335, 201–204. doi: 10.1016/j.canlet.2013.02.019

Kim, J., Inoue, K., Ishii, J., Vanti, W. B., Voronov, S. V., Murchison, E., et al. (2007a). A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* 317, 1220–1224. doi: 10.1126/science.1140481

Kim, S. H., Bianco, N. R., Shufesky, W. J., Morelli, A. E., and Robbins, P. D. (2007b). Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4. *J. Immunol.* 179, 2242–2249. doi: 10.4049/jimmunol.179.4.2242

Kim, S. H., Lechman, E. R., Bianco, N., Menon, R., Keravala, A., Nash, J., et al. (2005). Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J. Immunol.* 174, 6440–6448. doi: 10.4049/jimmunol.174.10.6440

Klohn, P. C., Castro-Seoane, R., and Collinge, J. (2013). Exosome release from infected dendritic cells: a clue for a fast spread of prions in the periphery? *J. Infect.* 67, 359–368. doi: 10.1016/j.jinf.2013.07.024

Kocerha, J., Kauppinen, S., and Wahlestedt, C. (2009). microRNAs in CNS disorders. *Neuromolecular Med.* 11, 162–172. doi: 10.1007/s12017-009-8066-1

Koh, W., Sheng, C. T., Tan, B., Lee, Q. Y., Kuznetsov, V., Kiang, L. S., et al. (2010). Analysis of deep sequencing microRNA expression profile from human embryonic stem cells derived mesenchymal stem cells reveals possible role of let-7 microRNA family in downstream targeting of hepatic nuclear factor 4 alpha. *BMC Genomics* 11(Suppl. 1), S6. doi: 10.1186/1471-2164-11-S1-S6

Konig, H. G., Kogel, D., Rami, A., and Prehn, J. H. (2005). TGF-{beta}1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa}B signaling. *J. Cell Biol.* 168, 1077–1086. doi: 10.1083/jcb.200407027

Kooijmans, S. A., Vader, P., Van Dommelen, S. M., Van Solinge, W. W., and Schiffelers, R. M. (2012). Exosome mimetics: a novel class of drug delivery systems. *Int. J. Nanomedicine* 7, 1525–1541. doi: 10.2147/IJN.S29661

Kordelas, L., Rebmann, V., Ludwig, A. K., Radtke, S., Ruesing, J., Doeppner, T. R., et al. (2014). MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28, 970–973. doi: 10.1038/leu.2014.41

Lai, R. C., Arslan, F., Lee, M. M., Sze, N. S., Choo, A., Chen, T. S., et al. (2010). Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 4, 214–222. doi: 10.1016/j.scr.2009.12.003

Lai, R. C., Chen, T. S., and Lim, S. K. (2011). Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen. Med.* 6, 481–492. doi: 10.2217/rme.11.35

Lai, R. C., Tan, S. S., Teh, B. J., Sze, S. K., Arslan, F., De Kleijn, D. P., et al. (2012). Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int. J. Proteomics* 2012, 971907. doi: 10.1155/2012/971907

Lai, R. C., Yeo, R. W., Tan, K. H., and Lim, S. K. (2013a). Exosomes for drug delivery–a novel application for the mesenchymal stem cell. *Biotechnol. Adv.* 31, 543–551. doi: 10.1016/j.biotechadv.2012.08.008

Lai, R. C., Yeo, R. W., Tan, K. H., and Lim, S. K. (2013b). Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen. Med.* 8, 197–209. doi: 10.2217/rme.13.4

Lakhal, S., and Wood, M. J. (2011). Exosome nanotechnology: an emerging paradigm shift in drug delivery: exploitation of exosome nanovesicles for systemic *in vivo* delivery of RNAi heralds new horizons for drug delivery across biological barriers. *Bioessays* 33, 737–741. doi: 10.1002/bies.201100076

Lee, C., Mitsialis, S. A., Aslam, M., Vitali, S. H., Vergadi, E., Konstantinou, G., et al. (2012). Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 126, 2601–2611. doi: 10.1161/CIRCULATIONAHA.112.114173

Lee, J. K., Park, S. R., Jung, B. K., Jeon, Y. K., Lee, Y. S., Kim, M. K., et al. (2013). Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* 8:e84256. doi: 10.1371/journal.pone.0084256

Lee, T. H., D'asti, E., Magnus, N., Al-Nedawi, K., Meehan, B., and Rak, J. (2011). Microvesicles as mediators of intercellular communication in cancer–the emerging science of cellular 'debris'. *Semin. Immunopathol.* 33, 455–467. doi: 10.1007/s00281-011-0250-3

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20. doi: 10.1016/j.cell.2004.12.035

Li, T., Yan, Y., Wang, B., Qian, H., Zhang, X., Shen, L., et al. (2013). Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev.* 22, 845–854. doi: 10.1089/scd.2012.0395

Li, Y., Chen, J., Wang, L., Lu, M., and Chopp, M. (2001). Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* 56, 1666–1672. doi: 10.1212/WNL.56.12.1666

Li, Y., and Chopp, M. (2009). Marrow stromal cell transplantation in stroke and traumatic brain injury. *Neurosci. Lett.* 456, 120–123. doi: 10.1016/j.neulet.2008.03.096

Liang, X., Ding, Y., Zhang, Y., Tse, H. F., and Lian, Q. (2014). Paracrine mechanisms of mesenchymal Stem cell-based therapy: current status and perspectives. *Cell Transplant*. 23, 1045–1059. doi: 10.3727/096368913X667709

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., et al. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773. doi: 10.1038/nature03315

Lim, L. P., Lau, N. C., Weinstein, E. G., Abdelhakim, A., Yekta, S., Rhoades, M. W., et al. (2003). The microRNAs of *Caenorhabditis elegans*. *Genes Dev.* 17, 991–1008. doi: 10.1101/gad.1074403

Liu, F. J., Lim, K. Y., Kaur, P., Sepramaniam, S., Armugam, A., Wong, P. T., et al. (2013). microRNAs involved in regulating spontaneous recovery in embolic stroke model. *PLoS ONE* 8:e66393. doi: 10.1371/journal.pone.0066393

Liu, N. K., and Xu, X. M. (2011). MicroRNA in central nervous system trauma and degenerative disorders. *Physiol. Genomics* 43, 571–580. doi: 10.1152/physiolgenomics.00168.2010

Lotvall, J., and Valadi, H. (2007). Cell to cell signalling via exosomes through esRNA. *Cell Adh. Migr.* 1, 156–158. doi: 10.4161/cam.1.3.5114

Lu, D., Li, Y., Mahmood, A., Wang, L., Rafiq, T., and Chopp, M. (2002). Neural and marrow-derived stromal cell sphere transplantation in a rat model of traumatic brain injury. *J. Neurosurg.* 97, 935–940. doi: 10.3171/jns.2002.97.4.0935

Lusardi, T. A., Murphy, S. J., Phillips, J. I., Chen, Y., Davis, C. M., Young, J. M., et al. (2014). MicroRNA responses to focal cerebral ischemia in male and female mouse brain. *Front. Mol. Neurosci.* 7:11. doi: 10.3389/fnmol.2014.00011

Lv, H., Zhang, S., Wang, B., Cui, S., and Yan, J. (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* 114, 100–109. doi: 10.1016/j.jconrel.2006.04.014

Mahmood, A., Lu, D., and Chopp, M. (2004). Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. *J. Neurotrauma* 21, 33–39. doi: 10.1089/089771504772695922

Mathivanan, S., Ji, H., and Simpson, R. J. (2010). Exosomes: extracellular organelles important in intercellular communication. *J. Proteomics* 73, 1907–1920. doi: 10.1016/j.jprot.2010.06.006

Meckes, D. G. Jr., Gunawardena, H. P., Dekroon, R. M., Heaton, P. R., Edwards, R. H., Ozgur, S., et al. (2013). Modulation of B-cell exosome proteins by gamma herpesvirus infection. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2925–E2933. doi: 10.1073/pnas.1303906110

Mignot, G., Roux, S., Thery, C., Segura, E., and Zitvogel, L. (2006). Prospects for exosomes in immunotherapy of cancer. *J. Cell. Mol. Med.* 10, 376–388. doi: 10.1111/j.1582-4934.2006.tb00406.x

Mittelbrunn, M., and Sanchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nat. Rev. Mol. Cell Biol.* 13, 328–335. doi: 10.1038/nrm3335

Momen-Heravi, F., Balaj, L., Alian, S., Mantel, P. Y., Halleck, A. E., Trachtenberg, A. J., et al. (2013). Current methods for the isolation of extracellular vesicles. *Biol. Chem.* 394, 1253–1262. doi: 10.1515/hsz-2013-0141

Montecalvo, A., Larregina, A. T., Shufesky, W. J., Stolz, D. B., Sullivan, M. L., Karlsson, J. M., et al. (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 119, 756–766. doi: 10.1182/blood-2011-02-338004

Mor, E., Cabilly, Y., Goldshmit, Y., Zalts, H., Modai, S., Edry, L., et al. (2011). Species-specific microRNA roles elucidated following astrocyte activation. *Nucleic Acids Res.* 39, 3710–3723. doi: 10.1093/nar/gkq1325

Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., and D'souza-Schorey, C. (2010). Microvesicles: mediators of extracellular communication during cancer progression. *J. Cell Sci.* 123, 1603–1611. doi: 10.1242/jcs.064386

Olde Loohuis, N. F., Kos, A., Martens, G. J., Van Bokhoven, H., Nadif Kasri, N., and Aschrafi, A. (2012). MicroRNA networks direct neuronal development and plasticity. *Cell. Mol. Life Sci.* 69, 89–102. doi: 10.1007/s00018-011-0788-1

Ono, M., Kosaka, N., Tominaga, N., Yoshioka, Y., Takeshita, F., Takahashi, R. U., et al. (2014). Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci. Signal.* 7, ra63. doi: 10.1126/scisignal.2005231

Park, J. E., Tan, H. S., Datta, A., Lai, R. C., Zhang, H., Meng, W., et al. (2010). Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. *Mol. Cell. Proteomics* 9, 1085–1099. doi: 10.1074/mcp.M900381-MCP200

Phinney, D. G., and Prockop, D. J. (2007). Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair–current views. *Stem Cells* 25, 2896–2902. doi: 10.1634/stemcells.2007-0637

Qu, R., Li, Y., Gao, Q., Shen, L., Zhang, J., Liu, Z., et al. (2007). Neurotrophic and growth factor gene expression profiling of mouse bone marrow stromal cells induced by ischemic brain extracts. *Neuropathology* 27, 355–363. doi: 10.1111/j.1440-1789.2007.00792.x

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. doi: 10.1083/jcb.201211138

Record, M., Carayon, K., Poirot, M., and Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. *Biochim. Biophys. Acta* 1841, 108–120. doi: 10.1016/j.bbalip.2013.10.004

Record, M., Subra, C., Silvente-Poirot, S., and Poirot, M. (2011). Exosomes as intercellular signalosomes and pharmacological effectors. *Biochem. Pharmacol.* 81, 1171–1182. doi: 10.1016/j.bcp.2011.02.011

Reis, L. A., Borges, F. T., Simoes, M. J., Borges, A. A., Sinigaglia-Coimbra, R., and Schor, N. (2012). Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats. *PLoS ONE* 7:e44092. doi: 10.1371/journal.pone.0044092

Roccaro, A. M., Sacco, A., Maiso, P., Azab, A. K., Tai, Y. T., Reagan, M., et al. (2013). BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J. Clin. Invest.* 123, 1542–1555. doi: 10.1172/JCI66517

Roth, L. M., and Luse, S. A. (1964). Fine structure of the neurohypophysis of the opossum (*Didelphis Virginiana*). *J. Cell Biol.* 20, 459–472. doi: 10.1083/jcb.20.3.459

Saba, R., and Schratt, G. M. (2010). MicroRNAs in neuronal development, function and dysfunction. *Brain Res.* 1338, 3–13. doi: 10.1016/j.brainres.2010.03.107

Sano, S., Izumi, Y., Yamaguchi, T., Yamazaki, T., Tanaka, M., Shiota, M., et al. (2014). Lipid synthesis is promoted by hypoxic adipocyte-derived exosomes in 3T3-L1 cells. *Biochem. Biophys. Res. Commun.* 445, 327–333. doi: 10.1016/j.bbrc.2014.01.183

Schrier, S. L., Godin, D., Gould, R. G., Swyryd, B., Junga, I., and Seeger, M. (1971). Characterization of microvesicles produced by shearing of human erythrocyte membranes. *Biochim. Biophys. Acta* 233, 26–36. doi: 10.1016/0005-2736(71)90354-3

Sdrimas, K., and Kourembanas, S. (2014). MSC microvesicles for the treatment of lung disease: a new paradigm for cell-free therapy. *Antioxid. Redox Signal.* 21, 1905–1915. doi: 10.1089/ars.2013.5784

Sethi, P., and Lukiw, W. J. (2009). Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex. *Neurosci. Lett.* 459, 100–104. doi: 10.1016/j.neulet.2009.04.052

Shen, B., Wu, N., Yang, J. M., and Gould, S. J. (2011a). Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J. Biol. Chem.* 286, 14383–14395. doi: 10.1074/jbc.M110.208660

Shen, L. H., Li, Y., and Chopp, M. (2010). Astrocytic endogenous glial cell derived neurotrophic factor production is enhanced by bone marrow stromal cell transplantation in the ischemic boundary zone after stroke in adult rats. *Glia* 58, 1074–1081. doi: 10.1002/glia.20988

Shen, L. H., Li, Y., Gao, Q., Savant-Bhonsale, S., and Chopp, M. (2008). Down-regulation of neurocan expression in reactive astrocytes promotes axonal regeneration and facilitates the neurorestorative effects of bone marrow stromal cells in the ischemic rat brain. *Glia* 56, 1747–1754. doi: 10.1002/glia.20722

Shen, L. H., Xin, H., Li, Y., Zhang, R. L., Cui, Y., Zhang, L., et al. (2011b). Endogenous tissue plasminogen activator mediates bone marrow stromal cell-induced neurite remodeling after stroke in mice. *Stroke* 42, 459–464. doi: 10.1161/STROKEAHA.110.593863

Shimbo, K., Miyaki, S., Ishitobi, H., Kato, Y., Kubo, T., Shimose, S., et al. (2014). Exosome-formed synthetic microRNA-143 is transferred to osteosarcoma cells and inhibits their migration. *Biochem. Biophys. Res. Commun.* 445, 381–387. doi: 10.1016/j.bbrc.2014.02.007

Smalheiser, N. R. (2007). Exosomal transfer of proteins and RNAs at synapses in the nervous system. *Biol. Direct* 2, 35. doi: 10.1186/1745-6150-2-35

Spees, J. L., Olson, S. D., Ylostalo, J., Lynch, P. J., Smith, J., Perry, A., et al. (2003). Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2397–2402. doi: 10.1073/pnas.0437997100

Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002). The biogenesis and functions of exosomes. *Traffic* 3, 321–330. doi: 10.1034/j.1600-0854.2002.30502.x

Tan, C. Y., Lai, R. C., Wong, W., Dan, Y. Y., Lim, S. K., and Ho, H. K. (2014). Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res. Ther.* 5, 76. doi: 10.1186/scrt465

Thery, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol.* Chapter 3, Unit 3.22. doi: 10.1002/0471143030.cb0322s30

Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., et al. (1999). Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* 147, 599–610. doi: 10.1083/jcb.147.3.599

Thery, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2, 569–579. doi: 10.1038/nri855

Timmers, L., Lim, S. K., Arslan, F., Armstrong, J. S., Hoefer, I. E., Doevendans, P. A., et al. (2007). Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res.* 1, 129–137. doi: 10.1016/j.scr.2008.02.002

Timmers, L., Lim, S. K., Hoefer, I. E., Arslan, F., Lai, R. C., Van Oorschot, A. A., et al. (2011). Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res.* 6, 206–214. doi: 10.1016/j.scr.2011.01.001

Tomasoni, S., Longaretti, L., Rota, C., Morigi, M., Conti, S., Gotti, E., et al. (2013). Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. *Stem Cells Dev.* 22, 772–780. doi: 10.1089/scd.2012.0266

Trams, E. G., Lauter, C. J., Salem, N. Jr., and Heine, U. (1981). Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim. Biophys. Acta* 645, 63–70. doi: 10.1016/0005-2736(81)90512-5

Turturici, G., Tinnirello, R., Sconzo, G., and Geraci, F. (2014). Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. *Am. J. Physiol. Cell Physiol.* 306, C621–C633. doi: 10.1152/ajpcell.00228.2013

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., and Lotvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Van Niel, G., Porto-Carreiro, I., Simoes, S., and Raposo, G. (2006). Exosomes: a common pathway for a specialized function. *J. Biochem.* 140, 13–21. doi: 10.1093/jb/mvj128

Vassilopoulos, G., Wang, P. R., and Russell, D. W. (2003). Transplanted bone marrow regenerates liver by cell fusion. *Nature* 422, 901–904. doi: 10.1038/nature01539

Viaud, S., Ullrich, E., Zitvogel, L., and Chaput, N. (2008). Exosomes for the treatment of human malignancies. *Horm. Metab. Res.* 40, 82–88. doi: 10.1055/s-2007-1022548

Villarroya-Beltri, C., Gutierrez-Vazquez, C., Sanchez-Cabo, F., Perez-Hernandez, D., Vazquez, J., Martin-Cofreces, N., et al. (2013). Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* 4, 2980. doi: 10.1038/ncomms3980

Von Bartheld, C. S., and Altick, A. L. (2011). Multivesicular bodies in neurons: distribution, protein content, and trafficking functions. *Prog. Neurobiol.* 93, 313–340. doi: 10.1016/j.pneurobio.2011.01.003

Wang, J., Hendrix, A., Hernot, S., Lemaire, M., De Bruyne, E., Van Valckenborgh, E., et al. (2014). Bone marrow stromal cell-derived exosomes as communicators in drug resistance in multiple myeloma cells. *Blood* 124, 555–566. doi: 10.1182/blood-2014-03-562439

White, R. E., and Jakeman, L. B. (2008). Don't fence me in: harnessing the beneficial roles of astrocytes for spinal cord repair. *Restor. Neurol. Neurosci.* 26, 197–214.

Wong, R. S. (2011). Mesenchymal stem cells: angels or demons? *J. Biomed. Biotechnol.* 2011, 459510. doi: 10.1155/2011/459510

Xin, H., Chopp, M., Shen, L. H., Zhang, R. L., Zhang, L., Zhang, Z. G., et al. (2013a). Multipotent mesenchymal stromal cells decrease transforming growth factor beta1 expression in microglia/macrophages and down-regulate plasminogen activator inhibitor 1 expression in astrocytes after stroke. *Neurosci. Lett.* 542, 81–86. doi: 10.1016/j.neulet.2013.02.046

Xin, H., Li, Y., Buller, B., Katakowski, M., Zhang, Y., Wang, X., et al. (2012). Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 30, 1556–1564. doi: 10.1002/stem.1129

Xin, H., Li, Y., Chen, X., and Chopp, M. (2006). Bone marrow stromal cells induce BMP2/4 production in oxygen-glucose-deprived astrocytes, which promotes an astrocytic phenotype in adult subventricular progenitor cells. *J. Neurosci. Res.* 83, 1485–1493. doi: 10.1002/jnr.20834

Xin, H., Li, Y., Cui, Y., Yang, J. J., Zhang, Z. G., and Chopp, M. (2013b). Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J. Cereb. Blood Flow Metab.* 33, 1711–1715. doi: 10.1038/jcbfm.2013.152

Xin, H., Li, Y., Liu, Z., Wang, X., Shang, X., Cui, Y., et al. (2013c). MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 31, 2737–2746. doi: 10.1002/stem.1409

Xin, H., Li, Y., Shen, L. H., Liu, X., Hozeska-Solgot, A., Zhang, R. L., et al. (2011). Multipotent mesenchymal stromal cells increase tPA expression and concomitantly decrease PAI-1 expression in astrocytes through the sonic hedgehog signaling pathway after stroke (*in vitro* study). *J. Cereb. Blood Flow Metab.* 31, 2181–2188. doi: 10.1038/jcbfm.2011.116

Xin, H., Li, Y., Shen, L. H., Liu, X., Wang, X., Zhang, J., et al. (2010). Increasing tPA activity in astrocytes induced by multipotent mesenchymal stromal cells facilitate neurite outgrowth after stroke in the mouse. *PLoS ONE* 5:e9027. doi: 10.1371/journal.pone.0009027

Xu, J., Liu, X., Chen, J., Zacharek, A., Cui, X., Savant-Bhonsale, S., et al. (2010). Cell-cell interaction promotes rat marrow stromal cell differentiation into endothelial cell via activation of TACE/TNF-alpha signaling. *Cell Transplant.* 19, 43–53. doi: 10.3727/096368909X474339

Yang, J. M., and Gould, S. J. (2013). The cis-acting signals that target proteins to exosomes and microvesicles. *Biochem. Soc. Trans.* 41, 277–282. doi: 10.1042/BST20120275

Yang, X., Weng, Z., Mendrick, D. L., and Shi, Q. (2014). Circulating extracellular vesicles as a potential source of new biomarkers of drug-induced liver injury. *Toxicol. Lett.* 225, 401–406. doi: 10.1016/j.toxlet.2014.01.013

Yellon, D. M., and Davidson, S. M. (2014). Exosomes: nanoparticles involved in cardioprotection? *Circ. Res.* 114, 325–332. doi: 10.1161/CIRCRESAHA.113.300636

Yeo, R. W., Lai, R. C., Zhang, B., Tan, S. S., Yin, Y., Teh, B. J., et al. (2013). Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv. Drug Deliv. Rev.* 65, 336–341. doi: 10.1016/j.addr.2012.07.001

Yu, L., Yang, F., Jiang, L., Chen, Y., Wang, K., Xu, F., et al. (2013). Exosomes with membrane-associated TGF-beta1 from gene-modified dendritic cells inhibit murine EAE independently of MHC restriction. *Eur. J. Immunol.* 43, 2461–2472. doi: 10.1002/eji.201243295

Yu, Y. M., Gibbs, K. M., Davila, J., Campbell, N., Sung, S., Todorova, T. I., et al. (2011). MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. *Eur. J. Neurosci.* 33, 1587–1597. doi: 10.1111/j.1460-9568.2011.07643.x

Zacharek, A., Chen, J., Cui, X., Li, A., Li, Y., Roberts, C., et al. (2007). Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC treatment amplifies angiogenesis and vascular stabilization after stroke. *J. Cereb. Blood Flow Metab.* 27, 1684–1691. doi: 10.1038/sj.jcbfm.9600475

Zhang, B., Pan, X., Cobb, G. P., and Anderson, T. A. (2006a). Plant microRNA: a small regulatory molecule with big impact. *Dev. Biol.* 289, 3–16. doi: 10.1016/j.ydbio.2005.10.036

Zhang, B., Wang, M., Gong, A., Zhang, X., Wu, X., Zhu, Y., et al. (2014). HucMSC-exosome mediated -Wnt4 signaling is required for cutaneous wound healing. *Stem Cells*. doi: 10.1002/stem.1771. [Epub ahead of print].

Zhang, B., Wang, Q., and Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. *J. Cell. Physiol.* 210, 279–289. doi: 10.1002/jcp.20869

Zhang, H. G., and Grizzle, W. E. (2014). Exosomes: a novel pathway of local and distant intercellular communication that facilitates the growth and metastasis of neoplastic lesions. *Am. J. Pathol.* 184, 28–41. doi: 10.1016/j.ajpath.2013.09.027

Zhang, H., Huang, Z., Xu, Y., and Zhang, S. (2006b). Differentiation and neurological benefit of the mesenchymal stem cells transplanted into the rat brain following intracerebral hemorrhage. *Neurol. Res.* 28, 104–112. doi: 10.1179/016164106X91960

Zhang, J., Brodie, C., Li, Y., Zheng, X., Roberts, C., Lu, M., et al. (2009). Bone marrow stromal cell therapy reduces proNGF and p75 expression in mice with experimental autoimmune encephalomyelitis. *J. Neurol. Sci.* 279, 30–38. doi: 10.1016/j.jns.2008.12.033

Zhang, J., and Chopp, M. (2013). Cell-based therapy for ischemic stroke. *Expert Opin. Biol. Ther.* doi: 10.1517/14712598.2013.804507

Zhang, J., Li, Y., Lu, M., Cui, Y., Chen, J., Noffsinger, L., et al. (2006c). Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice. *J. Neurosci. Res.* 84, 587–595. doi: 10.1002/jnr.20962

Zhang, L., and Wrana, J. L. (2014). The emerging role of exosomes in Wnt secretion and transport. *Curr. Opin. Genet. Dev.* 27C, 14–19. doi: 10.1016/j.gde.2014.03.006

Zhang, Y., Wang, Z., and Gemeinhart, R. A. (2013). Progress in microRNA delivery. *J. Control. Release* 172, 962–974. doi: 10.1016/j.jconrel.2013.09.015

Zhang, Z. G., and Chopp, M. (2009). Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurol.* 8, 491–500. doi: 10.1016/S1474-4422(09)70061-4

Zhou, Y., Xu, H., Xu, W., Wang, B., Wu, H., Tao, Y., et al. (2013). Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis *in vivo* and *in vitro*. *Stem Cell Res. Ther.* 4, 34. doi: 10.1186/scrt194

Zhu, Y. G., Feng, X. M., Abbott, J., Fang, X. H., Hao, Q., Monsel, A., et al. (2014). Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 32, 116–125. doi: 10.1002/stem.1504

Ziu, M., Fletcher, L., Rana, S., Jimenez, D. F., and Digicaylioglu, M. (2011). Temporal differences in microRNA expression patterns in astrocytes and neurons after ischemic injury. *PLoS ONE* 6:e14724. doi: 10.1371/journal.pone.0014724

Zomer, A., Vendrig, T., Hopmans, E. S., Van Eijndhoven, M., Middeldorp, J. M., and Pegtel, D. M. (2010). Exosomes: fit to deliver small RNA. *Commun. Integr. Biol.* 3, 447–450. doi: 10.4161/cib.3.5.12339

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Therapeutic outcomes of transplantation of amniotic fluid-derived stem cells in experimental ischemic stroke

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Accumulating preclinical evidence suggests the use of amnion as a source of stem cells for investigations of basic science concepts related to developmental cell biology, but also for stem cells' therapeutic applications in treating human disorders. We previously reported isolation of viable rat amniotic fluid-derived stem (AFS) cells. Subsequently, we recently reported the therapeutic benefits of intravenous transplantation of AFS cells in a rodent model of ischemic stroke. Parallel lines of investigations have provided safety and efficacy of stem cell therapy for treating stroke and other neurological disorders. This review article highlights the need for investigations of mechanisms underlying AFS cells' therapeutic benefits and discusses lab-to-clinic translational gating items in an effort to optimize the clinical application of the cell transplantation for stroke.

Keywords: cerebral ischemia, neural stem/progenitor cells, regenerative medicine, neurogenesis, neurotrophic factors

WHY IS THERE A NEED FOR NOVEL TREATMENTS IN STROKE?

Stroke, the fourthleading cause of death and the leading cause of disability in the United States (Roger et al., 2012), has only one FDA-approved drug, namely tissue plasminogen activator (tPA). Due to tPA limitations and complications, which include a limited therapeutic window (4.5 h from disease onset to tPA administration) and adverse effects associated with delayed treatment (i.e., hemorrhagic transformation), only a mere 3 percent of ischemic stroke patients actually benefit from the tPA treatment (Graham, 2003; Yip and Demaerschalk, 2007). This significant unmet clinical need for stroke has prompted investigations to increase the therapeutic window with innovative treatment strategies specifically targeting the restorative phase, which begins days to weeks post-stroke (Matsukawa et al., 2009; Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011).

DO STEM CELLS EXIST IN THE AMNION?

Stem cells have emerged as a prospective restorative agent for stroke due to their ability to abrogate sub-acute and chronic secondary cell death associated with the disease (Borlongan et al., 1997; Nishino and Borlongan, 2000). We previously reported the isolation of viable rat amniotic fluid-derived stem (AFS) cells (Tajiri et al., 2012). Together with other research teams, the grafted stem cells' production of trophic factors and cytokines, as well as the increase in levels of neurotrophic factors and reduced inflammatory response in the ischemic stroke region, have been directly attributed to the positive effects by transplantation of AFS cells (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Furthermore, the inhibition of apoptosis and oxidative stress, in tandem with stimulation of angiogenesis, neurogenesis, and synaptogenesis, may be linked as a benefit of AFS cells against stroke deficits (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Even though stem cells can be harvested from various sources (Borlongan et al., 2004, 2005; Hematti et al., 2004; Clavel and Verfaillie, 2008; Burns et al., 2009; Ou et al., 2010), including bone marrow, fetal and embryonic tissues, amnion-derived stem cells are an appealing choice because of many logistical and ethical advantages including the ease of isolation of the stem cells from amnion tissue and the fluid (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Similar to amniotic-tissue derived cells, the harvest of AFS cells poses negligible risk of injury to the fetus. These cells are isolated from amniotic fluid collected from amniocentesis, a pre-natal exam performed at around 15–20 weeks of gestation. Accordingly, since AFS cells can be isolated much earlier, compared to amniotic tissue-derived

cells, AFS cells possess properties that closely resemble embryonic and mesenchymal cell markers, which could be more beneficial at treating diseases. The low immunogenicity, low tumorgenicity, high proliferative capacity and anti-inflammatory characteristics, are phenotypic features of transplantable cells (Newman et al., 2005) that support AFS cells to be a safe and effective donor cell for stroke (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Another distinguishing feature of AFS cells when compared to amniotic tissue-derived cells is the sterility involved. AFS cells are harvested via amniocentesis under aseptic condition, but the sterility may be compromised when stem cells are extracted from amnion tissue during child delivery.

AFS cells can phenotypically commit to various lineages (Prusa and Hengstschlager, 2002; In't Anker et al., 2003; Prusa et al., 2003; Fauza, 2004; Tsai et al., 2004, 2006; McLaughlin et al., 2006). A misleading concept is the term "fluid" associated with AFS cells because cells isolated during amniocentesis are comprised of multiple stem cells originating from extra-embryonic and embryonic tissues (Prusa and Hengstschlager, 2002) and their properties differ with gestational age (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Accordingly, phenotypic characterization of AFS cells reveal a plethora of stem cell subtypes, from pluripotent embryonic stem cells to multipotent adult stem cells owing likely to the age-dependent tissue plasticity potential (De Coppi et al., 2007; Mauro et al., 2010). Indeed, AFS cells from second trimester amniotic fluid display the ability to differentiate into all three germ layers and express Oct-4, Nanog, and SSEA-4 (Roubelakis et al., 2007), which are pluripotent embryonic stem cell markers (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). Although considered as "adult stem cells", the doubling time for the AFS cells population is approximately 30–36 h with a high regeneration capacity that can be extended for over 250 doublings without any measurable loss of chromosomal telomere length (De Coppi et al., 2007). Altogether, these studies support the amnion as a potent source of stem cells for investigations of basic science concepts related to developmental cell biology, but also for therapeutic purposes such as AFS cell transplantation for treating human disorders, especially stroke.

ARE AFS CELLS TRANSPLANTABLE AND DO THEY EXERT FUNCTIONAL BENEFITS?

The following protocol allows the isolation of AFS cells from isolated amniotic fluid samples obtained from timed pregnant Sprague-Dawley rats at gestation age 16–18 weeks (Tajiri et al., 2012). For each sample, 2–3 ml of amniotic fluid, corresponding to a cell number ranging from 2×10^3 to 2×10^6 are centrifuged for 10 min at 1800 rpm. The high variance in the amount of cells which can be isolated from amniotic fluid is influenced by factors such as child birth, genetics, and fluid cell isolation. It is expected, however, that these discrepancies can be overcome when technology is able to be standardized with appropriate quality control and assurance. This will yield a more accurate and efficient cell isolation in the near future. Pellets are then resuspended in Iscove's modified Dulbecco's medium supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), 2 mM L-glutamine, 5 ng/ml basic fibroblast growth factor (FGF2) and incubated at 37°C with 5% humidified CO_2. After 7 days, non-adherent cells are removed and the adherent cells are allowed to grow in the same medium, which is changed every 4 days. When the cell culture reaches confluency (about 20 days after the primary culture), cells are treated with 0.05% trypsin and 0.02% EDTA, then counted and replaced in 25 cm^2 culture flasks. For routine AFS cell procedure, an approximate yield of about 22 million stem cells per amnion fluid aspirate is anticipated. The viability yield of AFS cells is about 70%, which would be an estimated 15 million stem cells per amnion fluid aspirate. An estimated range of several million AFS cells per milliliter of amnion fluid are therefore expected to be obtained.

This protocol allows ample AFS cells for transplantation studies. Indeed, accumulating preclinical data have demonstrated the potential of transplantation of AFS cells for treating experimental models of brain diseases (Yu et al., 2009; Antonucci et al., 2011; Kaneko et al., 2011; Manuelpillai et al., 2011). For example, AFS cells are a potentially valuable source of stem cells to treat Parkinson's disease because under standard neuronal induction protocols for stem cells, AFS cells preferentially differentiate into a dopaminergic phenotype (Pisani et al., 2005). In parallel, transplantation of AFS cells has been examined in stroke, with AFS transplanted ischemic stroke mice exhibiting reduced short-term memory impairment and improved sensorimotor ability, somatosensory functions, and motor coordination (Rehni et al., 2007). Although this study shows the beneficial effects of AFS cell transplantation in experimental stroke, the mechanism underlying the observed therapeutic benefits remained underexplored. Perhaps AFS cells are mediating specific neurotransmitter release to restore cellular function in an injured brain (Broughton et al., 2013). This may explain a reversal of disease-induced impairment of memory following AFS cell transplantation in an ischemic model of stroke when transfer latency time (TLT) is used as a parameter of memory. If administration of AFS cells following middle cerebral artery occlusion and reperfusion significantly attenuated ischemia-reperfusion induced increase in day 7 TLT, it is likely that the AFS cells are secreting the neurotransmitters necessary to ameliorate memory and subsequently, cognition in general (Rehni et al., 2007).

HOW DO AFS CELLS AFFORD REPAIR OF THE STROKE BRAIN?

Our group transplanted AFS cells in experimental stroke animals (via occlusion of the middle cerebral artery; Borlongan et al., 1998) using motor and cognitive tests, and subsequent histological analysis of the brain to assess the mechanism of action associated with AFS cell therapy for stroke. Although still impaired compared to sham-operated animals (Acosta et al., 2010), intravenous transplantation of AFS cells (1 million viable cells) in adult Sprague-Dawley rats at 30 days after experimental stroke revealed that motor and cognitive deficits were reduced at 60 days post-MCAo compared to vehicle-infused stroke animals (Tajiri et al., 2012). Moreover, histological analyses revealed that the AFS cell-transplanted stroke animals significantly decreased infarct volumes by 92% compared to the vehicle-infused stroke animals, which likely facilitated the recovery of motor and cognitive functions. In addition, cell proliferation was significantly

upregulated in the neurogenic subventrical zone of the AFS cell-transplanted stroke animals compared to the vehicle-infused stroke animals. In tandem, immature neuronal cells also increased in the subventrical zone of AFS cell-transplanted stroke animals compared to the vehicle-infused stroke animals. The increased cell proliferation and reduced neuronal loss in the subventrical zone was similarly observed in the dentate gyrus, another major neurogenic niche. That AFS cell transplantation attenuated stroke-induced behavioral and histological deficits coincided with increased cell proliferation and neuronal differentiation in the two neurogenic sites, namely subventricular zone and dentate gyrus, implicates a major role of graft-induced host tissue repair in the brain remodeling process following stroke (**Table 1**).

CAN WE TRANSLATE AFS CELL GRAFT-MEDIATED FUNCTIONAL RECOVERY IN EXPERIMENTAL STROKE TO THE CLINIC?

Many stroke victims present with symptoms characterized by sensorimotor and cognitive functions (Grefkes et al., 2008; Rush et al., 2010; Lin et al., 2011). Up to now, most stroke animal models have focused on motor impairments, disregarding the cognitive declines that proceed after the brain offense (Borlongan, 2009; Chopp et al., 2009; STEPS, 2009). Findings on the pathology of ischemic stroke support that secondary cell death may extend beyond the routine cortical damage towards the hippocampus, which is the key brain structure for learning and memory consolidation (Scoville and Milner, 1957; Shors et al., 2002; Saxe et al., 2006; Dupret et al., 2008). A decline in adult neurogenesis is magnified during aging and correlates with worsening of cognitive functions (McDonald and Wojtowicz, 2005; Drapeau and Nora Abrous, 2008; Encinas et al., 2011). Interestingly, the impaired neurogenesis and the cognitive decline in the aging hippocampus (Freret et al., 2009; Dhawan et al., 2010; Zvejniece et al., 2012) also accompany stroke (Dhawan et al., 2010; Wattanathorn et al., 2011; Zvejniece et al., 2012).

Whereas our cognitive test results did not show any difference in learning performance between the transplanted and vehicle-infused stroke animals, the AFS cell-transplanted stroke animals demonstrated a significantly improved reference memory compared to the vehicle-infused stroke animals. This improvement in the memory task directly correlates with increased neurogenesis in the hippocampus of AFS cell-transplanted stroke animals relative to vehicle-infused stroke animals. In order to increase the clinical relevance of cell therapy for stroke, especially when evaluating cognitive deficits, is to employ a hippocampal dependent task for spatial memory and reference memory (Gallagher et al., 1993; Duva et al., 1997; Clarke et al., 1999; Anisman and McIntyre, 2002; Broadbent et al., 2004; Clark et al., 2007; Gillani et al., 2010; Bergado et al., 2011; Jurgens et al., 2012), and long-term potentiation (Bliss and Collingridge, 1993; Norris and Foster, 1999; Gusev and Alkon, 2001; Richardson et al., 2002; Vorhees and Williams, 2006; Pisu et al., 2011; Xu et al., 2012). Our data advanced the notion that transplanted AFS cells selectively aid in the recovery of the reference memory, but not task acquisition. Cognitive improvements in stem/progenitor cell transplanted stroke animals have been linked previously with the decline in cerebral infarct volumes (Nishino et al., 1993; Fukunaga et al., 1999, 2003; Mimura et al., 2005), as well as reduced secondary cell death loss in brain areas known to modulate motor and cognitive functions (Ebrahimi et al., 1992; Pisani et al., 2005; Takahashi et al., 2008; Tabuse et al., 2010; Miyoshi et al., 2012). That the AFS cell transplanted stroke animals displayed improved motor and cognitive performance, reduced the brain infarcts and the secondary cell death, and enhanced the level of neurogenesis provide insights on possible mechanisms of action underlying

Table 1 | Effects of AFS cells and amniotic tissue-derived cell in stroke-related disease.

Protective effects of AFS and amniotic tissue-derived stem cells in stroke-related disease	Reference
Stroke rats manifest a robust reduction of infarct volumes by 92% and reduced local inflammation.	Liu et al. (2008), Tao et al. (2012) and Broughton et al. (2013).
Cell proliferation, neuronal differentiation, and immature neuronal cells significantly upregulated in the subventrical zone and dentate gyrus of stroke rats.	Ekdahl et al. (2009), Jezierski et al. (2010), Zhang et al. (2010) and Prasongchean et al. (2012).
Reduced short term memory impairment and improved sensorimotor ability, somatosensory functions, and motor coordination in stroke rats.	Rehni et al. (2007) and Broughton et al. (2013).
Ischemic rats present an increase of neurogenesis in the hippocampus, leading to improved reference memory.	Tajiri et al. (2012).
Reversal of hemi-parkisonian syndrome and behavioral improvement thanks to the formation of new dopaminergic fibers in the denervated striatum.	Sheng et al. (1993) and Bankiewicz et al. (1994).
Rats subjected to middle cerebral artery occlusion model express MAP2, Nestin, and glial fibrillary acidic protein that improve behavioral recovery.	Liu et al. (2008) and Tao et al. (2012).
The administration of melatonin significantly increases the proliferation and survival of human amniotic epithelial cells and boosts neuronal differentiation.	Kaneko et al. (2011).
Human amniotic membrane-derived mesenchymal stem cells lack major histocompatibility complex class I molecule greatly reducing transplant rejection.	Tao et al. (2012) and Broughton et al. (2013).
Amniotic membrane mesenchymal stem cells have innate capacity to express factors for endothelialization and angiogenesis. Crucial for wound recovery in ischemic diseases.	Warrier et al. (2012) and Broughton et al. (2013).
Amniotic membrane cell grafts enhance the recovery of cardiac function.	Cargnoni et al. (2009).

A synopsis of the beneficial behavioral and cognitive effects observed in experimental animals models of stroke.

the functional benefits afforded by AFS cell transplantation. Our observed transplant-mediated recovery of motor and cognitive functions confirms a recent report of AFS therapeutic effects in stroke (Rehni et al., 2007), but our study expanded the short timeline of 7 days from the previous study to 63 days.

The transplantation of AFS cells shows extensive therapeutic benefits such as reduction in infarct volume, enhanced cell proliferation, increased neuronal differentiation, and improved memory and motor skills, seen with other stem cells (Yasuhara et al., 2006a,b, 2008; Hara et al., 2007). The exact underlying mechanism of these benefits is still unknown and understanding therapeutic pathways could potentially yield promising insights into optimizing AFS cell transplantation towards the clinical trial stage. Clinical trials that closely reflect the preclinical data on safety and efficacy of AFS cells in animal models of stroke will enhance the functional outcome of this cell therapy in stroke patients. Based on preclinical data demonstrating AFS cell safety and efficacy, patients who suffer a stroke and show significant inflammation of the brain or who display short-term memory loss due to the accompanying injury to the hippocampus seem to be the best candidates for future clinical trials of AFS cells. Moreover, AFS cell therapy trials could be extended to patients who have suffered from cardiac stroke, as animal models show promising improvement in cardiac functions following AFS cell transplantation (Bollini et al., 2011). In addition, a better understanding of the mechanism(s) involved in the functional improvement seen in transplanted stroke animals will likely further optimize AFS cell therapy.

When contemplating with clinical applications of AFS cells, the route of transplantation is key to successful outcomes of targeted stroke patient population. Intracerebral (Rehni et al., 2007; Liu et al., 2008), intraperitoneal (Ghionzoli et al., 2010), and intravenous (Tajiri et al., 2012) have been explored in AFS transplantation in experimental models of cerebral ischemia. The intravenous route of transplantation is a minimally invasive procedure and poses less risk to the patient compared to intracerebral transplantation. A peripheral injection method may be favored over the direct transplantation route so that the stem cells can be administered quickly after the onset of a stroke. A limited FDA-approved clinical trial for intravenous transplantation of placenta/amnion-derived stem cells in sub-acute stroke patients was terminated with no available safety and efficacy results (ClinicalTrials.gov., 2014).

The recommendations of Stem cell Therapeutics as an Emerging Paradigm for Stroke (STEPS; Borlongan, 2009; Chopp et al., 2009; STEPS, 2009) are likely to facilitate the translational research on AFS cell grafts for stroke. Such translational blueprint for cell therapy in stroke provides important guidance in experimental stroke testing and stem cell therapy, including the need for multiple strains, both genders, and age groups to evaluate safety and efficacy of AFS cells (Borlongan, 2009; Chopp et al., 2009; STEPS, 2009). Equally important is the phenotypic characterization of AFS cells in order to allow replications of the results, but also to maintain quality control and quality assurance of the cells for transplantation therapy in the clinic. With this in mind, another key component of translating laboratory studies into clinical applications is to carefully and critically analyze the type of transplantation procedure to be employed in the clinic. The efficient isolation of AFS cells (i.e., during amniocentesis) (Kalogiannidis et al., 2011) may prove applicable to allogenic grafts in the stroke patients. The animal model shows the efficacy of AFS cells 30 days after stroke, which suggests its beneficial application to chronic stroke patients.

The collection of the AFS cells will likely require banking of cells associated with storage costs that may not be covered by health insurance. This will in turn require the donor to shoulder the costs ranging to thousands of dollars. The studies conducted so far exploring the immunogenic properties of human amniotic cells have revealed that these cells inhibit the function of different immune cells of the innate and adaptive immune response (Borlongan et al., 1996; Insausti et al., 2014); however, it is also documented that even autologous grafts have the potential to elicit an immune response. Therefore, further investigations to determine whether AFS cells are truly immune-free need to be conducted. In addition, the heterogenic population of AFS cells contains many cell phenotypes. While such variety of cell lineages suggests differentiation capability of AFS cells to multiple cell phenotypes and thus allowing many disease indications for AFS cell therapy, the stability of phenotypic expression and functional effects of AFS cells remain to be fully investigated. Altogether, these challenges may seem to hinder translational potential of AFS cells, but they pose as open avenues of research waiting to be discovered towards realizing the full potential of AFS cells for transplant therapy.

It is also important to discuss the practical issues associated with stroke therapy in adults. The unpredictable occurrence of a stroke event can be addressed by the ready availability of AFS cells. However, the route of stem cell administration may be dictated by the stroke disease phase. For example, acute stroke will require rapid delivery of cells via minimally invasive route, while chronic stroke may be amenable to intracerebral delivery of the cells. In addition, the ready availability of the amnion cells and timing of transplantation for both acute and chronic phases of stroke will require cryopreserved cells, thereby requiring high viability of cells following freezing and thawing storage. In this case, a panel of cell release criteria monitoring high viability of cells, normal karyotyping, and phenotypic stability (among other cell quality assurance issues) need to be in place prior to transplantation of AFS cells as these are common cell release criteria in place for other donor stem cell types. Ultimately, the maintenance of therapeutic efficacy due to scarce data to date providing "efficacy" readout for cells prior to transplantation is a challenge. A simple assay of neurotransmitter release or growth factor secretion after thawing may need to be developed as an efficacy release criterion which should enhance the therapeutic outcome of AFS cell transplantation.

Transplanted AFS cells-induced cell proliferation, in tandem with a decrease in neuronal loss, produced robust reduction in cerebral infarcts (Tajiri et al., 2012). The subventricular zone and dentate gyrus, two neurogenic niches in the brain, are critical to the repair of damaged brain tissue (Ekdahl et al., 2009; Jezierski et al., 2010; Zhang et al., 2010; Prasongchean et al., 2012). The results imply that AFS cell transplantation may have enhanced endogenous repair mechanisms by maximizing the potential of

these two neurogenic sites to confer a host brain remodeling process. Intravenous AFS cell transplantation in the chronic stage of experimental stroke decreased motor and cognitive impairments, maintained cell proliferation, and increased cell differentiation in the host brain (Tajiri et al., 2012). The mechanism of action appears to involve AFS grafted cells' capacity to solicit endogenous stem cells for brain repair. These observations offer insights into therapeutic pathways of brain repair, and guide the design of clinical trials of cell therapy in stroke.

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REFERENCES

Acosta, S., Jernberg, J., Sanberg, C. D., Sanberg, P. R., Small, B. J., Gemma, C., et al. (2010). NT-020, a natural therapeutic approach to optimize spatial memory performance and increase neural progenitor cell proliferation and decrease inflammation in the aged rat. *Rejuvenation Res.* 13, 581–588. doi: 10.1089/rej.2009.1011

Anisman, H., and McIntyre, D. C. (2002). Conceptual, spatial and cue learning in the Morris water maze in fast or slow kindling rats: attention deficit comorbidity. *J. Neurosci.* 22, 7809–7817.

Antonucci, I., Stuppia, L., Kaneko, Y., Yu, S., Tajiri, N., Bae, E., et al. (2011). Amniotic fluid as a rich source of mesenchymal stromal cells for transplantation therapy. *Cell Transplant.* 20, 789–795. doi: 10.3727/096368910x539074

Bankiewicz, K. S., Palmatier, M., Plunkett, R. J., Cummins, A., and Oldfield, E. H. (1994). Reversal of hemiparkinsonian syndrome in nonhumans primates by amnion implantation into caudate nucleus. *J. Neurosurg.* 81, 869–876. doi: 10.3171/jns.1994.81.6.0869

Bergado, J. A., Almaguer, W., Rojas, Y., Capdevila, V., and Frey, J. U. (2011). Spatial and emotional memory in aged rats: a behavioral-statistical analysis. *Neuroscience* 172, 256–269. doi: 10.1016/j.neuroscience.2010.10.064

Bliss, T. V., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39. doi: 10.1038/361031a0

Bollini, S., Cheung, K. K., Riegler, J., Dong, X., Smart, N., Ghionzoli, M., et al. (2011). Amniotic fluid stem cells are cardioprotective following acute myocardial infarction. *Stem Cells Dev.* 20, 1985–1994. doi: 10.1089/scd.2010.0424

Borlongan, C. V. (2009). Cell therapy for stroke: remaining issues to address before embarking on clinical trials. *Stroke* 40, S146–S148. doi: 10.1161/STROKEAHA.108.533091

Borlongan, C. V., Evans, A., Yu, G., and Hess, D. C. (2005). Limitations of intravenous human bone marrow CD133+ cell grafts in stroke rats. *Brain Res.* 1048, 116–122. doi: 10.1016/j.brainres.2005.04.087

Borlongan, C. V., Hadman, M., Sanberg, C. D., and Sanberg, P. R. (2004). Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke* 35, 2385–2389. doi: 10.1161/01.str.0000141680.49960.d7

Borlongan, C. V., Hida, H., and Nishino, H. (1998). Early assessment of motor dysfunctions aids in successful occlusion of the middle cerebral artery. *Neuroreport* 9, 3615–3621. doi: 10.1097/00001756-199811160-00012

Borlongan, C. V., Koutouzis, T. K., Jorden, J. R., Martinez, R., Rodriguez, A. I., Poulos, S. G., et al. (1997). Neural transplantation as an experimental treatment modality for cerebral ischemia. *Neurosci. Biobehav. Rev.* 21, 79–90. doi: 10.1016/0149-7634(95)00063-1

Borlongan, C. V., Stahl, C. E., Cameron, D. F., Saporta, S., Freeman, T. B., Cahill, D. W., et al. (1996). CNS immunological modulation of neural graft rejection and survival. *Neurol. Res.* 18, 297–304.

Broadbent, N. J., Squire, L. R., and Clark, R. E. (2004). Spatial memory, recognition memory and the hippocampus. *Proc. Natl. Acad. Sci. U S A* 101, 14515–14520. doi: 10.1073/pnas.0406344101

Broughton, B. R., Lim, R., Arumugam, T. V., Drummond, G. R., Wallace, E. M., and Sobey, C. G. (2013). Post-stroke inflammation and the potential efficacy of novel stem cell therapies: focus on amnion epithelial cells. *Front. Cell. Neurosci.* 6:66. doi: 10.3389/fncel.2012.00066

Burns, T. C., Verfaillie, C. M., and Low, W. C. (2009). Stem cells for ischemic brain injury: a critical review. *J. Comp. Neurol.* 515, 125–144. doi: 10.1002/cne.22038

Cargnoni, A., Di Marcello, M., Campagnol, M., Nassuato, C., Albertini, A., and Parolini, O. (2009). Amniotic membrane patching promotes ischemic rat heart repair. *Cell Transplant.* 18, 1147–1159. doi: 10.3727/096368909x12483162196764

Chopp, M., Steinberg, G. K., Kondziolka, D., Lu, M., Bliss, T. M., Li, Y., et al. (2009). Who's in favor of translational cell therapy for stroke: STEPS forward please? *Cell Transplant.* 18, 691–693. doi: 10.3727/096368909X470883

Clark, R. E., Broadbent, N. J., and Squire, L. R. (2007). The hippocampus and spatial memory: findings with a novel modification of the water maze. *J. Neurosci.* 27, 6647–6654. doi: 10.1523/jneurosci.0913-07.2007

Clarke, M. S., Prendergast, M. A., and Terry, A. V. Jr. (1999). Plasma membrane ordering agent pluronic F-68 (PF-68) reduces neurotransmitter uptake and release and produces learning and memory deficits in rats. *Learn. Mem.* 6, 634–649. doi: 10.1101/lm.6.6.634

Clavel, C., and Verfaillie, C. M. (2008). Bone-marrow-derived cells and heart repair. *Curr. Opin. Organ Transplan.* 13, 36–43. doi: 10.1097/MOT.0b013e3282f428d1

ClinicalTrials.gov. (2014). *A Phase 2A, Prospective, Multi-Center, Randomized, Double-Blind, Placebo-Controlled, Dose-Escalation Study to Evaluate the Safety of Intravenous Infusion of Human Placenta-Derived Cells (PDA001) for the Treatment of Adults Following Ischemic Stroke*. Available online at: http://clinicaltrials.gov/ct2/show/NCT01310114

De Coppi, P., Bartsch, G. Jr., Siddiqui, M. M., Xu, T., Santos, C. C., Perin, L., et al. (2007). Isolation of amniotic stem cell lines with potential for therapy. *Nat. Biotechnol.* 25, 100–106. doi: 10.1038/nbt1274

Dhawan, J., Benveniste, H., Nawrocky, M., Smith, S. D., and Biegon, A. (2010). Transient focal ischemia results in persistent and widespread neuroinflammation and loss of glutamate NMDA receptors. *Neuroimage* 51, 599–605. doi: 10.1016/j.neuroimage.2010.02.073

Drapeau, E., and Nora Abrous, D. (2008). Stem cell review series: role of neurogenesis in age-related memory disorders. *Aging Cell.* 7, 569–589. doi: 10.1111/j.1474-9726.2008.00369.x

Dupret, D., Revest, J. M., Koehl, M., Ichas, F., De Giorgi, F., Costet, P., et al. (2008). Spatial relational memory requires hippocampal adult neurogenesis. *PLoS One* 3:e1959. doi: 10.1371/journal.pone.0001959

Duva, C. A., Floresco, S. B., Wunderlich, G. R., Lao, T. L., Pinel, J. P., and Phillips, A. G. (1997). Disruption of spatial but not object-recognition memory by neurotoxic lesions of the dorsal hippocampus in rats. *Behav. Neurosci.* 111, 1184–1196. doi: 10.1037/0735-7044.111.6.1184

Ebrahimi, A., Pochet, R., and Roger, M. (1992). Topographical organization of the projections from physiologically identified areas of the motor cortex to the striatum in the rat. *Neurosci. Res.* 14, 39–60. doi: 10.1016/s0168-0102(05)80005-7

Ekdahl, C. T., Kokaia, Z., and Lindvall, O. (2009). Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* 158, 1021–1029. doi: 10.1016/j.neuroscience.2008.06.052

Encinas, J. M., Michurina, T. V., Peunova, N., Park, J. H., Tordo, J., Peterson, D. A., et al. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* 8, 566–579. doi: 10.1016/j.stem.2011.03.010

Fauza, D. (2004). Amniotic fluid and placental stem cells. *Best Pract. Res. Clin. Obstet. Gynaecol.* 18, 877–891. doi: 10.1016/j.bpobgyn.2004.07.001

Freret, T., Bouet, V., Leconte, C., Roussel, S., Chazalviel, L., Divoux, D., et al. (2009). Behavioral deficits after distal focal cerebral ischemia in mice: usefulness of adhesive removal test. *Behav. Neurosci.* 123, 224–230. doi: 10.1037/a0014157

Fukunaga, A., Kawase, T., and Uchida, K. (2003). Functional recovery after simultaneous transplantation with neuro-epithelial stem cells and adjacent mesenchymal tissues into infarcted rat brain. *Acta Neurochir. (Wien)* 145, 473–480; discussion 480–471.

Fukunaga, A., Uchida, K., Hara, K., Kuroshima, Y., and Kawase, T. (1999). Differentiation and angiogenesis of central nervous system stem cells implanted with mesenchyme into ischemic rat brain. *Cell Transplant.* 8, 435–441.

Gallagher, M., Burwell, R., and Burchinal, M. (1993). Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behav. Neurosci.* 107, 618–626. doi: 10.1037/0735-7044.107.4.618

Ghionzoli, M., Cananzi, M., Zani, A., Rossi, C. A., Leon, F. F., Pierro, A., et al. (2010). Amniotic fluid stem cell migration after intraperitoneal injection in pup rats: implication for therapy. *Pediatr. Surg. Int.* 26, 79–84. doi: 10.1007/s00383-009-2504-x

Gillani, R. L., Tsai, S. Y., Wallace, D. G., O'Brien, T. E., Arhebamen, E., Tole, M., et al. (2010). Cognitive recovery in the aged rat after stroke and anti-Nogo-A immunotherapy. *Behav. Brain Res.* 208, 415–424. doi: 10.1016/j.bbr.2009.12.015

Graham, G. D. (2003). Tissue plasminogen activator for acute ischemic stroke in clinical practice: a meta-analysis of safety data. *Stroke* 34, 2847–2850. doi: 10.1161/01.str.0000101752.23813.c3

Grefkes, C., Nowak, D. A., Eickhoff, S. B., Dafotakis, M., Kust, J., Karbe, H., et al. (2008). Cortical connectivity after subcortical stroke assessed with functional magnetic resonance imaging. *Ann. Neurol.* 63, 236–246. doi: 10.1002/ana.21228

Gusev, P. A., and Alkon, D. L. (2001). Intracellular correlates of spatial memory acquisition in hippocampal slices: long-term disinhibition of CA1 pyramidal cells. *J. Neurophysiol.* 86, 881–899.

Hara, K., Matsukawa, N., Yasuhara, T., Xu, L., Yu, G., Maki, M., et al. (2007). Transplantation of post-mitotic human neuroteratocarcinoma-overexpressing Nurr1 cells provides therapeutic benefits in experimental stroke: in vitro evidence of expedited neuronal differentiation and GDNF secretion. *J. Neurosci. Res.* 85, 1240–1251. doi: 10.1002/jnr.21234

Hematti, P., Hong, B. K., Ferguson, C., Adler, R., Hanawa, H., Sellers, S., et al. (2004). Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol.* 2:e423. doi: 10.1371/journal.pbio.0020423

Insausti, C. L., Blanquer, M., Garcia-Hernandez, A. M., Castellanos, G., and Moraleda, J. M. (2014). Amniotic membrane-derived stem cells: immunomodulatory properties and potential clinical application. *Stem Cells Cloning* 7, 53–63. doi: 10.2147/SCCAA.S58696

In't Anker, P. S., Scherjon, S. A., Kleijburg-van der Keur, C., Noort, W. A., Claas, F. H., Willemze, R., et al. (2003). Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 102, 1548–1549. doi: 10.1182/blood-2003-04-1291

Jezierski, A., Gruslin, A., Tremblay, R., Ly, D., Smith, C., Turksen, K., et al. (2010). Probing stemness and neural commitment in human amniotic fluid cells. *Stem Cell Rev.* 6, 199–214. doi: 10.1007/s12015-010-9116-7

Jurgens, H. A., Amancherla, K., and Johnson, R. W. (2012). Influenza infection induces neuroinflammation, alters hippocampal neuron morphology and impairs cognition in adult mice. *J. Neurosci.* 32, 3958–3968. doi: 10.1523/JNEUROSCI.6389-11.2012

Kalogiannidis, I., Prapa, S., Dagklis, T., Karkanaki, A., Petousis, S., Prapas, Y., et al. (2011). Amniocentesis-related adverse outcomes according to placental location and risk factors for fetal loss after midtrimester amniocentesis. *Clin. Exp. Obstet. Gynecol.* 38, 239–242.

Kaneko, Y., Hayashi, T., Yu, S., Tajiri, N., Bae, E. C., Solomita, M. A., et al. (2011). Human amniotic epithelial cells express melatonin receptor MT1, but not melatonin receptor MT2: a new perspective to neuroprotection. *J. Pineal Res.* 50, 272–280. doi: 10.1111/j.1600-079x.2010.00837.x

Lin, M. S., Chiu, M. J., Wu, Y. W., Huang, C. C., Chao, C. C., Chen, Y. H., et al. (2011). Neurocognitive improvement after carotid artery stenting in patients with chronic internal carotid artery occlusion and cerebral ischemia. *Stroke* 42, 2850–2854. doi: 10.1161/STROKEAHA.111.613133

Liu, T., Wu, J., Huang, Q., Hou, Y., Jiang, Z., Zang, S., et al. (2008). Human amniotic epithelial cells ameliorate behavioral dysfunction and reduce infarct size in the rat middle cerebral artery occlusion model. *Shock* 29, 603–611. doi: 10.1097/SHK.0b013e318157e845

Manuelpillai, U., Moodley, Y., Borlongan, C. V., and Parolini, O. (2011). Amniotic membrane and amniotic cells: potential therapeutic tools to combat tissue inflammation and fibrosis? *Placenta* 32(Suppl. 4), S320–S325. doi: 10.1016/j.placenta.2011.04.010

Matsukawa, N., Yasuhara, T., Hara, K., Xu, L., Maki, M., Yu, G., et al. (2009). Therapeutic targets and limits of minocycline neuroprotection in experimental ischemic stroke. *BMC Neurosci.* 10:126. doi: 10.1186/1471-2202-10-126

Mauro, A., Turriani, M., Loannoni, A., Russo, V., Martelli, A., Di Giacinto, O., et al. (2010). Isolation, characterization and in vitro differentiation of ovine amniotic stem cells. *Vet. Res. Commun.* 34(Suppl. 1), S25–S28. doi: 10.1007/s11259-010-9393-2

McDonald, H. Y., and Wojtowicz, J. M. (2005). Dynamics of neurogenesis in the dentate gyrus of adult rats. *Neurosci. Lett.* 385, 70–75. doi: 10.1016/j.neulet.2005.05.022

McLaughlin, D., Tsirimonaki, E., Vallianatos, G., Sakellaridis, N., Chatzistamatiou, T., Stavropoulos-Gioka, C., et al. (2006). Stable expression of a neuronal dopaminergic progenitor phenotype in cell lines derived from human amniotic fluid cells. *J. Neurosci. Res.* 83, 1190–1200. doi: 10.1002/jnr.20828

Mimura, T., Dezawa, M., Kanno, H., and Yamamoto, I. (2005). Behavioral and histological evaluation of a focal cerebral infarction rat model transplanted with neurons induced from bone marrow stromal cells. *J. Neuropathol. Exp. Neurol.* 64, 1108–1117. doi: 10.1097/01.jnen.0000190068.03009.b5

Miyoshi, E., Wietzikoski, E. C., Bortolanza, M., Boschen, S. L., Canteras, N. S., Izquierdo, I., et al. (2012). Both the dorsal hippocampus and the dorsolateral striatum are needed for rat navigation in the Morris water maze. *Behav. Brain Res.* 226, 171–178. doi: 10.1016/j.bbr.2011.09.011

Newman, M. B., Misiuta, I., Willing, A. E., Zigova, T., Karl, R. C., Borlongan, C. V., et al. (2005). Tumorigenicity issues of embryonic carcinoma-derived stem cells: relevance to surgical trials using NT2 and hNT neural cells. *Stem Cells Dev.* 14, 29–43. doi: 10.1089/scd.2005.14.29

Nishino, H., and Borlongan, C. V. (2000). Restoration of function by neural transplantation in the ischemic brain. *Prog. Brain Res.* 127, 461–476. doi: 10.1016/s0079-6123(00)27022-2

Nishino, H., Koide, K., Aihara, N., Kumazaki, M., Sakurai, T., and Nagai, H. (1993). Striatal grafts in the ischemic striatum improve pallidal GABA release and passive avoidance. *Brain Res. Bull.* 32, 517–520. doi: 10.1016/0361-9230(93)90300-z

Norris, C. M., and Foster, T. C. (1999). MK-801 improves retention in aged rats: implications for altered neural plasticity in age-related memory deficits. *Neurobiol. Learn. Mem.* 71, 194–206. doi: 10.1006/nlme.1998.3864

Ou, Y., Yu, S., Kaneko, Y., Tajiri, N., Bae, E. C., Chheda, S. H., et al. (2010). Intravenous infusion of GDNF gene-modified human umbilical cord blood CD34+ cells protects against cerebral ischemic injury in spontaneously hypertensive rats. *Brain Res.* 1366, 217–225. doi: 10.1016/j.brainres.2010.09.098

Pisani, A., Centonze, D., Bernardi, G., and Calabresi, P. (2005). Striatal synaptic plasticity: implications for motor learning and Parkinson's disease. *Mov. Disord.* 20, 395–402. doi: 10.1002/mds.20394

Pisu, M. G., Dore, R., Mostallino, M. C., Loi, M., Pibiri, F., Mameli, R., et al. (2011). Down-regulation of hippocampal BDNF and Arc associated with improvement in aversive spatial memory performance in socially isolated rats. *Behav. Brain Res.* 222, 73–80. doi: 10.1016/j.bbr.2011.03.021

Prasongchean, W., Bagni, M., Calzarossa, C., De Coppi, P., and Ferretti, P. (2012). Amniotic fluid stem cells increase embryo survival following injury. *Stem Cells Dev.* 21, 675–688. doi: 10.1089/scd.2011.0281

Prusa, A. R., and Hengstschlager, M. (2002). Amniotic fluid cells and human stem cell research: a new connection. *Med. Sci. Monit.* 8, RA253–RA257.

Prusa, A. R., Marton, E., Rosner, M., Bernaschek, G., and Hengstschlager, M. (2003). Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? *Hum. Reprod.* 18, 1489–1493. doi: 10.1093/humrep/deg279

Rehni, A. K., Singh, N., Jaggi, A. S., and Singh, M. (2007). Amniotic fluid derived stem cells ameliorate focal cerebral ischaemia-reperfusion injury induced behavioural deficits in mice. *Behav. Brain Res.* 183, 95–100. doi: 10.1016/j.bbr.2007.05.028

Richardson, D. P., Byrnes, M. L., Brien, J. F., Reynolds, J. N., and Dringenberg, H. C. (2002). Impaired acquisition in the water maze and hippocampal long-term potentiation after chronic prenatal ethanol exposure in the guinea-pig. *Eur. J. Neurosci.* 16, 1593–1598. doi: 10.1046/j.1460-9568.2002.02214.x

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., et al. (2012). Executive summary: heart disease and stroke statistics—2012 update: a report from the american heart association. *Circulation* 125, 188–197. doi: 10.1161/CIR.0b013e3182456d46

Roubelakis, M. G., Pappa, K. I., Bitsika, V., Zagoura, D., Vlahou, A., Papadaki, H. A., et al. (2007). Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. *Stem Cells Dev.* 16, 931–952. doi: 10.1089/scd.2007.0036

Rush, B. K., McNeil, R. B., Gamble, D. M., Luke, S. H., Richie, A. N., Albers, C. S., et al. (2010). Behavioral symptoms in long-term survivors of ischemic stroke. *J. Stroke Cerebrovasc. Dis.* 19, 326–332. doi: 10.1016/j.jstrokecerebrovasdis.2009.09.009

Saxe, M. D., Battaglia, F., Wang, J. W., Malleret, G., David, D. J., Monckton, J. E., et al. (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc. Natl. Acad. Sci. U S A* 103, 17501–17506. doi: 10.1073/pnas.0607207103

Scoville, W. B., and Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry* 20, 11–21. doi: 10.1136/jnnp.20.1.11

Sheng, J. G., McShane, L. M., Plunkett, R. J., Cummins, A. C., Oldfield, E. H., Kopin, I. J., et al. (1993). Dopaminergic neuronal sprouting and behavioral recovery in hemi-parkinsonian rats after implantation of amnion cells. *Exp. Neurol.* 123, 192–203. doi: 10.1006/exnr.1993.1152

Shors, T. J., Townsend, D. A., Zhao, M., Kozorovitskiy, Y., and Gould, E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus* 12, 578–584. doi: 10.1002/hipo.10103

STEPS Participants. (2009). Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. *Stroke* 40, 510–515. doi: 10.1161/STROKEAHA.108.526863

Tabuse, M., Yaguchi, M., Ohta, S., Kawase, T., and Toda, M. (2010). A simple behavioral test for locomotor function after brain injury in mice. *J. Clin. Neurosci.* 17, 1412–1416. doi: 10.1016/j.jocn.2010.01.056

Tajiri, N., Acosta, S., Glover, L. E., Bickford, P. C., Jacotte Simancas, A., Yasuhara, T., et al. (2012). Intravenous grafts of amniotic fluid-derived stem cells induce endogenous cell proliferation and attenuate behavioral deficits in ischemic stroke rats. *PLoS One* 7:e43779. doi: 10.1371/journal.pone.0043779

Takahashi, K., Yasuhara, T., Shingo, T., Muraoka, K., Kameda, M., Takeuchi, A., et al. (2008). Embryonic neural stem cells transplanted in middle cerebral artery occlusion model of rats demonstrated potent therapeutic effects, compared to adult neural stem cells. *Brain Res.* 1234, 172–182. doi: 10.1016/j.brainres.2008.07.086

Tao, J., Ji, F., Liu, B., Wang, F., Dong, F., and Zhu, Y. (2012). Improvement of deficits by transplantation of lentiviral vector-modified human amniotic mesenchymal cells after cerebral ischemia in rats. *Brain Res.* 1448, 1–10. doi: 10.1016/j.brainres.2012.01.069

Tsai, M. S., Hwang, S. M., Tsai, Y. L., Cheng, F. C., Lee, J. L., Chang, Y. G., et al. (2006). Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells. *Biol. Reprod.* 74, 545–551. doi: 10.1095/biolreprod.105.046029

Tsai, M. S., Lee, J. L., Chang, Y. J., and Hwang, S. M. (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum. Reprod.* 19, 1450–1456. doi: 10.1093/humrep/deh279

Vorhees, C. V., and Williams, M. T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat. Protoc.* 1, 848–858. doi: 10.1038/nprot.2006.116

Warrier, S., Haridas, N., and Bhonde, R. (2012). Inherent propensity of amnion-derived mesenchymal stem cells towards endothelial lineage: vascularization from an avascular tissue. *Placenta* 33, 850–858. doi: 10.1016/j.placenta.2012.07.001

Wattanathorn, J., Jittiwat, J., Tongun, T., Muchimapura, S., and Ingkaninan, K. (2011). Zingiber officinale mitigates brain damage and improves memory impairment in focal cerebral ischemic rat. *Evid. Based Complement. Alternat. Med.* 2011:429505. doi: 10.1155/2011/429505

Xu, J., Wang, Y., Li, N., Xu, L., Yang, H., and Yang, Z. (2012). L-3-n-butylphthalide improves cognitive deficits in rats with chronic cerebral ischemia. *Neuropharmacology* 62, 2424–2429. doi: 10.1016/j.neuropharm.2012.02.014

Yasuhara, T., Borlongan, C. V., and Date, I. (2006a). Ex vivo gene therapy: transplantation of neurotrophic factor-secreting cells for cerebral ischemia. *Front. Biosci.* 11, 760–775. doi: 10.2741/1834

Yasuhara, T., Hara, K., Maki, M., Mays, R. W., Deans, R. J., Hess, D. C., et al. (2008). Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells in neonatal hypoxic-ischemic rats. *J. Cereb. Blood Flow Metab.* 28, 1804–1810. doi: 10.1038/jcbfm.2008.68

Yasuhara, T., Matsukawa, N., Yu, G., Xu, L., Mays, R. W., Kovach, J., et al. (2006b). Behavioral and histological characterization of intrahippocampal grafts of human bone marrow-derived multipotent progenitor cells in neonatal rats with hypoxic-ischemic injury. *Cell Transplant.* 15, 231–238. doi: 10.3727/000000006783982034

Yip, T. R., and Demaerschalk, B. M. (2007). Estimated cost savings of increased use of intravenous tissue plasminogen activator for acute ischemic stroke in Canada. *Stroke* 38, 1952–1955. doi: 10.1161/strokeaha.106.479477

Yu, S. J., Soncini, M., Kaneko, Y., Hess, D. C., Parolini, O., Borlongan, C. V., et al. (2009). Amnion: a potent graft source for cell therapy in stroke. *Cell Transplant.* 18, 111–118. doi: 10.3727/096368909788341243

Zhang, C., Chopp, M., Cui, Y., Wang, L., Zhang, R., Zhang, L., et al. (2010). Cerebrolysin enhances neurogenesis in the ischemic brain and improves functional outcome after stroke. *J. Neurosci. Res.* 88, 3275–3281. doi: 10.1002/jnr.22495

Zvejniece, L., Svalbe, B., Liepinsh, E., Pulks, E., and Dambrova, M. (2012). The sensorimotor and cognitive deficits in rats following 90- and 120-min transient occlusion of the middle cerebral artery. *J. Neurosci. Methods* 208, 197–204. doi: 10.1016/j.jneumeth.2012.05.018

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Stem cell-based treatments against stroke: observations from human proof-of-concept studies and considerations regarding clinical applicability

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Ischemic stroke remains a heavy burden for industrialized countries. The only causal therapy is the recanalization of occluded vessels via thrombolysis, which due to a narrow time window still can be offered only to a minority of patients. Since the majority of patients continues to exhibit neurological deficits even following successful thrombolysis, restorative therapies are urgently needed that promote brain remodeling and repair once stroke injury has occurred. Due to their unique properties of action, stem cell-based strategies gained increasing interest during recent years. Using various stroke models in both rodents and primates, the transplantation of stem cells, namely of bone marrow derived mesenchymal stem cells (MSCs) or neural progenitor cells (NPCs), has been shown to promote neurological recovery most likely via indirect bystander actions. In view of promising observations, clinical proof-of-concept studies are currently under way, in which effects of stem and precursor cells are evaluated in human stroke patients. In this review we summarize already published studies, which due to the broad experience in other medical contexts mostly employed bone marrow-derived MSCs by means of intravenous transplantation. With the overall number of clinical trials limited in number, only a fraction of these studies used non-treated control groups, and only single studies were adequately blinded. Despite these limitations, first promising results justify the need for more elaborate clinical trials in order to make stem cell transplantation a success for stroke treatment in the future.

Keywords: stem cells, stroke, mesenchymal stem cells, neural progenitor cells, trials

STEM AND PROGENITOR CELLS IN EXPERIMENTAL STROKE MODELS

In defined areas of the adult brain such as the subgranular zone of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles, endogenous neurogenesis persists during adulthood (Alvarez-Buylla and Garcia-Verdugo, 2002; Taupin and Gage, 2002; Silva-Vargas et al., 2013; Braun and Jessberger, 2014; Jessberger and Gage, 2014; Sawada et al., 2014). These neurogenic niches comprise astrocyte like neural stem and progenitor cells (NPC) that give rise to neurons under physiologic conditions (Doetsch et al., 1997, 1999). In rodent and primate stroke models, cerebral ischemia stimulates endogenous neurogenesis, and NPCs migrate from the SVZ towards the site of injury where they proliferate and differentiate into neurons (Liu et al., 1998; Arvidsson et al., 2002; Tonchev et al., 2003; Yamashita et al., 2006). Unfortunately, both differentiation and survival rates of endogenous NPCs are low (Parent, 2003; Haas et al., 2005; Doeppner et al., 2009). For this reason, the functional contribution of endogenous neurogenesis to post-ischemic neurological recovery remains a matter of debate.

In view of their restorative properties, efforts have been made to promote neurological recovery by transplantation of stem or progenitor cells in ischemic stroke. Although embryonic stem cells have the capacity to give rise to all cell lineages, their therapeutic potential is limited due to teratoma formation and ethical concerns (Blum and Benvenisty, 2008, 2009). Consequently, a wide variety of adult stem and progenitor cells from different species and various tissue sources have been used for therapeutic purposes, which were transplanted either locally or systemically in experimental models of focal cerebral ischemia (Bacigaluppi et al., 2008, 2009; Schwarting et al., 2008; Bliss et al., 2010; Zheng et al., 2010; Banerjee et al., 2012; Doeppner et al., 2012; Leong et al., 2012). Although grafted cells are not thought to be integrated into residing neural networks, they do promote neurological recovery via paracrine (indirect) mechanisms that involve the stimulation of endogenous angioneurogenesis and neural plasticity, stabilization of the blood brain barrier as well as modulation of peripheral and central immune responses (Doeppner et al., 2012; Hermann and Chopp, 2012; Mora-Lee et al., 2012; Zhang and Chopp, 2013). Despite the fact that questions related to the optimal cell type, the most adequate cell delivery

Table 1 | Clinical trials using stem cells or progenitor cells against stroke.

Authors	Year	Cell type	Key findings
Bang et al.	2005	MSCs	Cells were intravenously grafted twice within 9 weeks post-stroke. Better outcome in Barthel index after 1 year, but no effect on NIHSS and MRI scan.
Lee et al.	2010	MSCs	Intravenous cell grafting twice post-stroke with observation period of 5 years. Better outcome in mRS.
Bhasin et al.	2011	MSCs	Autologous intravenous MSC transplantation. Within 24 weeks, no significant side effects observed plus putative increased neural plasticity.
Bhasin et al.	2013	MSCs	Intravenous MSC transplantation followed by observation period of 24 weeks. Statistically improved modified Barthel Index and increased neural plasticity after stem cell treatment. No side effects.
Honmou et al.	2011	MSCs	Intravenous cell transplantation showed no side effects during 1 year of follow-up. Reduction of lesion volume by >20% after 1 week.
Savitz et al.	2011	MSCs	Intravenous transplantation of MSCs within 72 h post-stroke plus observation period of 6 months. No study-related side effects. Median NIHSS 13 before cell grafting and 3 after 6 months.
Barbosa da Fonseca et al.	2010	MSCs	Intraarterial delivery of 99mTc-labeled MSCs. Significantly reduced intracerebral numbers of grafted cells after 24 h. No significant side effects for as long as 120 days.
Moniche et al.	2012	MSCs	Intraarterial infusion of MSCs between 5-9 days post-stroke. After 6 months, no side effects but also no improved functional outcome.
Suárez-Monteagudo et al.	2009	MSCs	Stereotactic transplantation of cells into 5 patients. Authors claim discrete functional improvement after 1 year.
Kondziolka et al.	2000	Cultured neuronal cells	Stereotactic delivery of cells with observation period of 18 months. Some functional improvement. No relevant safety issues.
Kondziolka et al.	2005	Cultured neuronal cells	Stereotactic cell delivery with maximal observation period of 24 months. Some functional improvement, but primary outcome was not met. No significant adverse events.
Savitz et al.	2005	Fetal lateral eminescence (=neural) cells	Cells were pre-treated with anti-MHC I antibody and intracerebrally delivered. Study was stopped after 5 patients. Significant side effects.
Rabinovich et al.	2005	Cell suspension from immature nervous and hemopoietic tissue	Intrathecal cell delivery in 10 patients. No significant side effects during 6 months of observation.

The table describes the studies quoted in the main text with special regard to key findings, the cell type used and the year of publication. MRI: magnetic resonance imaging, mRS: modified Rankin Scale, MSCs: mesenchymal stem cells, NIHSS: National Institutes of Health Stroke Scale.

time point and the route of cell delivery are still vividly discussed, clinical trials have been on the way with some of them holding promising results. Due to the broad experience with these cells in other clinical contexts, the most widely used cell source in clinical stroke studies are mesenchymal stem cells (MSCs), which are mostly derived from bone marrow. Besides, NPCs are also used in stroke patients. The following paragraphs provide an overview on major clinical trials (**Table 1**), which due to the existence of multiple investigator-driven smaller trials by no means claims to be exhaustive.

CLINICAL STROKE TRIALS USING STEM OR PROGENITOR CELLS

MESENCHYMAL STEM CELL SOURCES

MSCs have been successfully used under various experimental paradigms (Chen et al., 2001, 2003; Li et al., 2002; Kurozumi et al., 2005; Ukai et al., 2007; Onda et al., 2008; Yoo et al., 2008; Kranz et al., 2010; Sheikh et al., 2011). Due to the long-lasting experience with MSC transplantation in other clinical contexts, namely in malignancies of the blood, MSCs have evolved as the preferred candidate for clinical transplantation studies. Characteristics of MSCs include adherence on plastic surfaces, expression of CD markers such as CD105, CD73 and CD90 as well as differentiation into fat, bone and cartilage tissue (Dominici et al., 2006). MSCs can be easily obtained from various tissue sources including bone marrow and adipose tissue (Bliss et al., 2007; Doeppner and Hermann, 2010). They can also be easily expanded *in vitro* and are regarded to be immunologically inert, which reduces the risk of rejection of grafted cells in allogeneic transplantation settings (Aggarwal and Pittenger, 2005; Beyth et al., 2005). Although MSCs can be induced to differentiate into neural tissue *in vitro* (Pittenger et al., 1999), their potential for neural differentiation is low. Nevertheless, transplantation of MSCs improves neurological outcome

in experimental stroke models, which is attributed to paracrine effects of grafted cells (Caplan, 2009). As such, promising preclinical data lead to clinical studies using MSCs or bone marrow-derived mononuclear progenitor cells (subsequently subsumed together as MSCs), the most relevant of which are presented in the next paragraph.

CLINICAL PROOF-OF-CONCEPT STUDIES USING MESENCHYMAL STEM CELL TRANSPLANTATION

In one of the very first randomized controlled phase I/II clinical trials, Bang et al. (2005) intravenously transplanted autologous MSCs twice within 9 weeks after stroke onset. During the observation period of 1 year, patients receiving MSCs showed better improvement of the Barthel Index than control patients, whereas National Institutes of Health Stroke Scale (NIHSS) score and brain injury assessed by magnetic resonance imaging (MRI) did not differ between groups. Patient numbers in this trial were low (5 MSC transplanted and 25 control patients), thus possibly explaining the lack of significant results in the latter readouts. Taking this lack of statistical power and concerns regarding the safety of fetal calf serum (Spees et al., 2004) that was used for *ex vivo* MSC expansion into account, the same study group performed an additional open-label and observer-blinded clinical trial on patients suffering from severe strokes using an observation period of 5 years (Lee et al., 2010). As in their earlier study, MSCs were intravenously transplanted twice in a total of 16 patients, whereas controls received no injection. Although 4 patients from the MSC group died during the observation period, no significant side effects or comorbidities attributed to the transplantation itself were observed. Importantly, the modified Rankin Scale (mRS) score was significantly improved in patients receiving MSCs within the observation period. This improvement was associated with increased levels of stromal cell-derived factor-1 that is upregulated upon stroke in rodents and thought to be involved in MSC homing (Shen et al., 2007). Although Lee et al. (2010) significantly extended the observation period to 5 years, more definite conclusions about the efficacy of MSCs cannot be drawn from this study, which lacked randomization and true blinding. Interestingly however, beneficial effects related to MSC treatment seemed to correlate with the involvement of the SVZ into the stroke. Thus, MSC transplantation was more effective when the SVZ was not part of the evolving stroke lesion, suggesting an indirect action of MSCs that promote endogenous neuroregeneration. According to a recent MRI study, the SVZ is located in close proximity to stroke lesions in a large percentage of stroke patients. In a prospective cohort of 108 included patients with first-ever stroke, the distance from the nearest margin of the infarct to the SVZ was $\leq$2 mm in half of all patients exhibiting visible diffusion weighted image (DWI) lesions (Delavaran et al., 2013). Thus, the relationship between stroke lesion and SVZ may represent a hitherto under-recognized factor influencing responses to neurorestorative therapies (Lee et al., 2010).

In the meantime, further clinical trials provided evidence that intravenous MSC transplantation is safe and feasible in humans (Bhasin et al., 2011, 2013; Honmou et al., 2011; Savitz et al., 2011). However, scientific conclusions from these trials are hampered due to the heterogeneous study design, the different timing of cell delivery and considerable differences in size of study groups. As a matter of fact, only two of the aforementioned studies included non-treated control groups (Bhasin et al., 2011; Savitz et al., 2011). Nevertheless, these studies were not randomized or blinded, and beneficial effects due to MSC transplantation as described by Bhasin et al. therefore need further evaluation.

Since the homing of MSCs and other stem cells into the brain is limited after intravenous transplantation, a Brazilian group performed intraarterial infusions of ^{99m}Tc-labeled MSCs in six stroke patients with the aim of increasing the amount of grafted cells within the brain (Barbosa da Fonseca et al., 2010). Although significant cell homing was observed as early as 2 h post-stroke within the ischemic hemisphere, the latter was greatly diminished 24 h post-stroke. Within an observation period of 120 days, no significant side effects related to cell grafting were observed. Similar findings were reported by Battistella et al. (2011) for an observation period of 180 days after intraarterial MSC infusion during the chronic phase of the stroke. Noteworthy, conclusions from the aforementioned studies are limited by low patient numbers and a lack of appropriate blinding. Since MSCs are known to home into peripheral organs such as the lungs, questions remain about the safety of required interventional procedures, particularly in more severely affected patients suffering from serious comorbidities. Patient safety will have to be taken into account carefully in future treatment studies after a recent study did not observe a beneficial effect of intraarterial MSC transplantation in patients suffering from stroke (Moniche et al., 2012). In animal studies, intraarterial MSC delivery was not superior to intravenous delivery (Yang et al., 2013).

In an even smaller clinical study, Suarez-Monteagudo et al. successfully transplanted autologous MSCs stereotactically into the brain of five stroke patients, followed by an observation period of 1 year (Suárez-Monteagudo et al., 2009). Although the authors claimed a discrete functional improvement over time, a scientific evaluation of this observation is certainly misplaced due to the low patient number and the study trial itself with no adequate control group. A sample of five patients is even far too low to draw conclusions about therapeutic safety. Reports of neurological improvement in small patient cohorts are hampered by the fact that complication rates may not be adequately determined. The MSC delivery into the brain parenchyma should, if at all, be considered with great caution.

At present, further studies that analyze both safety and feasibility of MSCs in stroke patients are on the way. The U.S. National Institutes of Health[1] currently list 10 clinical trials ranging from phases I to III upon the keywords "mesenchymal stem cells" and "stroke". The majority of these studies use intravenous cell delivery with primary outcome measurements of either safety or functional neurological improvement. However, the recruitment status of these studies remains heterogeneous with some trials having not yet recruited at all.

[1] www.clinicaltrials.gov

CRITICAL CONSIDERATIONS REGARDING MESENCHYMAL STEM CELL TRANSPLANTATION IN HUMANS

Although clinical follow-up studies with observation periods of one or 5 years did not show significant side effects (Bang et al., 2005; Lee et al., 2010), safety issues carefully need to be taken into account when considering MSCs for the treatment of ischemic stroke. MSC treatment was associated with improved outcome of stroke patients in at least some of the aforementioned studies, suggesting that MSC transplantation is safe. Likewise, a large meta-analysis on clinical trials under various pathological conditions not exclusively related to stroke did not show any evidence for severe side effects due to MSC transplantation (Lalu et al., 2012). This work analyzed safety issues in 36 studies covering a total of 1012 participants that had suffered from stroke, Crohn's disease, cardiomyopathy, myocardial infarction or graft vs. host disease. Some studies also included MSC transplantation in healthy non-affected volunteers. The meta-analysis did not detect significant side effects related to MSC transplantation, such as acute infusion-related toxicity, complications in peripheral organ systems, infection, death, or tumor formation. There was, however, a consistent observation of MSC transplantation-related transient fever.

Nevertheless, setbacks and unfavorable reports in experimental stroke models deserve special attention. Using intraarterial transplantation paradigms in a rat stroke model, Mitkari et al. could not show improved functional outcome after MSC transplantation, although grafted MSCs were attracted towards the lesion site and post-ischemic angiogenesis was significantly increased (Mitkari et al., 2014). In line with this, Steiner and colleagues did not observe post-stroke neuroprotection after systemic MSC transplantation in rodents, which was attributed to homing of grafted MSCs into peripheral organs and not into the brain (Steiner et al., 2012). Most importantly, transplantation risks might increase when comorbidities such as diabetes are taken into account. As such, Chen et al. did not show a beneficial effect of MSCs in diabetic stroke rats, but even reported increased mortality in treated animals that was associated with enhanced brain hemorrhage as a consequence of maladaptive angiogenesis (Chen et al., 2011).

Although MSCs themselves are not tumorigenic, they might migrate to existing primary tumors and modify or even stimulate tumor growth due to their immunomodulatory properties (Lazennec and Jorgensen, 2008). Accordingly, MSC-induced bystander effects might change the biological behavior of tumor cells with unpredictable consequences for the patient. Hence, further clinical trials using larger study cohorts with extended observation periods are urgently needed before more definite conclusions about the safety of MSC transplantation may be drawn. In view of risks related to invasive procedures, such studies should preferably use intravenous instead of delivery strategies from the authors' point of view.

NEURAL STEM AND PROGENITOR CELLS AND OTHER STEM CELL SOURCES

In comparison with MSCs, other cell types have been less frequently evaluated in clinical studies. The hesitation of delivering these cells to human stroke patients is a consequence of the fact that unlike MSCs, which are used for bone marrow transplantation, these cells had not been used before as therapeutics in other medical contexts. In case of NPCs, concerns remain about malignant transformation, which cannot be ruled out completely even when fetal or adult cell sources are used. NPCs derived from the SVZ of the lateral ventricles induce potent neuroprotection and brain remodeling, both when systemically and locally (i.e., intracerebrally) delivered. This aspect deserves special attention, since cells are not integrated into neural networks but act mainly via paracrine bystander mechanisms (Bacigaluppi et al., 2008, 2009; Doeppner et al., 2010, 2012). Two clinical trials have demonstrated the feasibility of stereotactic delivery of cultured neuronal cells derived from a teratocarcinoma cell line (Kondziolka et al., 2000, 2005). Although cell transplantation was followed by some functional improvement, the primary outcome measure of the study, the change in the European Stroke Scale (ESS) motor score, was not met. Again, the sample size (4–7 patients per group) was too small to infer conclusions regarding the therapeutic efficacy of NPC transplantation.

Another study using fetal porcine NPCs pre-treated with an anti-MHC class I antibody for prevention of graft rejection could not confirm its high expectations regarding safety and feasibility (Savitz et al., 2005). Noteworthy, this trial was stopped after intracerebral transplantation into five patients resulted in significant side effects in two patients. Thus, temporary worsening of motor deficits was noted in one patient 3 weeks after transplantation, while another patient developed epileptic seizures 1 week after transplantation (Savitz et al., 2005). MRI in both patients demonstrated areas of contrast enhancement remote from the grafting site, which resolved on subsequent imaging. In contrast to this study, another study investigating the intrathecal transplantation of cell suspensions derived from immature nervous and hematopoietic tissues did not detect any side effects in 10 patients over an observation period of 6 months (Rabinovich et al., 2005). Although the number of published clinical studies investigating cell sources other than MSCs is low, additional studies are on the way in stroke patients using cells from different tissue sources. Among these ones, studies using genetically modified NPCs are noteworthy, which are delivered by stereotactic intracerebral transplantation (NCT02117635 and NCT01151124). In other studies, human placenta-derived cells (NCT01310114) or olfactory ensheathing cells (NCT01327768) are applied. While the former study is already completed but not yet published, the status of the latter is currently unknown.[2] Unfortunately, the aforementioned studies make use of intraparenchymal transplantation strategies, which reduces their clinical relevance, even in case they prove to be successful.

CONCLUSION AND OUTLOOK

Whereas application of stem cells has become a clinical routine for treatment of hematological diseases, neurorestaurative treatment paradigms using stem cells against stroke have not found their way into the clinic, yet. However, first proof-of-concept studies evaluating MSC transplantation in human stroke patients achieved promising data, which justify more systematic studies. These

[2] www.clinicaltrials.gov

observations will have to be confirmed in larger cohorts in the near future, before more definite conclusions regarding the safety of stem cell treatment can be made. Unfortunately, recruitment in some of the ongoing studies was rather slow in recent years, which delays progress in the field of stem cell therapies. A major problem of several previous studies is the lack of appropriate control groups. On the other hand, clinical trials using cell sources other than MSCs are still scarce and need further evaluation. In the absence of clinical experience in larger patient cohorts, questions of long-term safety remain a concern for most of the latter cell sources, even when fetal or adult cells are used.

AUTHOR AND CONTRIBUTIONS

Thorsten R. Doeppner and Dirk M. Hermann wrote the manuscript.

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REFERENCES

Aggarwal, S., and Pittenger, M. F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105, 1815–1822. doi: 10.1182/blood-2004-04-1559

Alvarez-Buylla, A., and Garcia-Verdugo, J. M. (2002). Neurogenesis in adult subventricular zone. *J. Neurosci.* 22, 629–634.

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat. Med.* 8, 963–970. doi: 10.1038/nm747

Bacigaluppi, M., Pluchino, S., Martino, G., Kilic, E., and Hermann, D. M. (2008). Neural stem/precursor cells for the treatment of ischemic stroke. *J. Neurol. Sci.* 265, 73–77. doi: 10.1016/j.jns.2007.06.012

Bacigaluppi, M., Pluchino, S., Peruzzotti Jametti, L., Kilic, E., Kilic, U., Salani, G., et al. (2009). Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 132, 2239–2251. doi: 10.1093/brain/awp174

Banerjee, S., Williamson, D. A., Habib, N., and Chataway, J. (2012). The potential benefit of stem cell therapy after stroke: an update. *Vasc. Health Risk Manag.* 8, 569–580. doi: 10.2147/VHRM.s25745

Bang, O. Y., Lee, J. S., Lee, P. H., and Lee, G. (2005). Autologous mesenchymal stem cell transplantation in stroke patients. *Ann. Neurol.* 57, 874–882. doi: 10.1002/ana.20501

Barbosa da Fonseca, L. M., Gutfilen, B., Rosado de Castro, P. H., Battistella, V., Goldenberg, R. C., Kasai-Brunswick, T., et al. (2010). Migration and homing of bone-marrow mononuclear cells in chronic ischemic stroke after intra-arterial injection. *Exp. Neurol.* 221, 122–128. doi: 10.1016/j.expneurol.2009.10.010

Battistella, V., de Freitas, G. R., da Fonseca, L. M., Mercante, D., Gutfilen, B., Goldenberg, R. C., et al. (2011). Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke. *Regen. Med.* 6, 45–52. doi: 10.2217/rme.10.97

Beyth, S., Borovsky, Z., Mevorach, D., Liebergall, M., Gazit, Z., Aslan, H., et al. (2005). Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 105, 2214–2219. doi: 10.1182/blood-2004-07-2921

Bhasin, A., Srivastava, M. V., Kumaran, S. S., Mohanty, S., Bhatia, R., Bose, S., et al. (2011). Autologous mesenchymal stem cells in chronic stroke. *Cerebrovasc. Dis. Extra* 1, 93–104. doi: 10.1159/000333381

Bhasin, A., Srivastava, M. V., Mohanty, S., Bhatia, R., Kumaran, S. S., and Bose, S. (2013). Stem cell therapy: a clinical trial of stroke. *Clin. Neurol. Neurosurg.* 115, 1003–1008. doi: 10.1016/j.clineuro.2012.10.015

Bliss, T. M., Andres, R. H., and Steinberg, G. K. (2010). Optimizing the success of cell transplantation therapy for stroke. *Neurobiol. Dis.* 37, 275–283. doi: 10.1016/j.nbd.2009.10.003

Bliss, T., Guzman, R., Daadi, M., and Steinberg, G. K. (2007). Cell transplantation therapy for stroke. *Stroke* 38, 817–826. doi: 10.1161/01.str.0000247888.25985.62

Blum, B., and Benvenisty, N. (2008). The tumorigenicity of human embryonic stem cells. *Adv. Cancer Res.* 100, 133–158. doi: 10.1016/S0065-230X(08)00005-5

Blum, B., and Benvenisty, N. (2009). The tumorigenicity of diploid and aneuploid human pluripotent stem cells. *Cell Cycle* 8, 3822–3830. doi: 10.4161/cc.8.23.10067

Braun, S. M., and Jessberger, S. (2014). Adult neurogenesis: mechanisms and functional significance. *Development* 141, 1983–1986. doi: 10.1242/dev.104596

Caplan, A. I. (2009). Why are MSCs therapeutic? New data: new insight. *J. Pathol.* 217, 318–324. doi: 10.1002/path.2469

Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M., et al. (2001). Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 32, 1005–1011. doi: 10.1161/01.str.32.4.1005

Chen, J., Ye, X., Yan, T., Zhang, C., Yang, X. P., Cui, X., et al. (2011). Adverse effects of bone marrow stromal cell treatment of stroke in diabetic rats. *Stroke* 42, 3551–3558. doi: 10.1161/STROKEAHA.111.627174

Chen, J., Zhang, Z. G., Li, Y., Wang, L., Xu, Y. X., Gautam, S. C., et al. (2003). Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ. Res.* 92, 692–699. doi: 10.1161/01.res.0000063425.51108.8d

Delavaran, H., Sjunnesson, H., Arvidsson, A., Lindvall, O., Norrving, B., van Westen, D., et al. (2013). Proximity of brain infarcts to regions of endogenous neurogenesis and involvement of striatum in ischaemic stroke. *Eur. J. Neurol.* 20, 473–479. doi: 10.1111/j.1468-1331.2012.03877.x

Doeppner, T. R., Dietz, G. P., El Aanbouri, M., Gerber, J., Witte, O. W., Bähr, M., et al. (2009). TAT-Bcl-x(L) improves survival of neuronal precursor cells in the lesioned striatum after focal cerebral ischemia. *Neurobiol. Dis.* 34, 87–94. doi: 10.1016/j.nbd.2008.12.013

Doeppner, T. R., El Aanbouri, M., Dietz, G. P., Weise, J., Schwarting, S., and Bähr, M. (2010). Transplantation of TAT-Bcl-xL-transduced neural precursor cells: long-term neuroprotection after stroke. *Neurobiol. Dis.* 40, 265–276. doi: 10.1016/j.nbd.2010.05.033

Doeppner, T. R., Ewert, T. A., Tönges, L., Herz, J., Zechariah, A., Elali, A., et al. (2012). Transduction of neural precursor cells with TAT-heat shock protein 70 chaperone: therapeutic potential against ischemic stroke after intrastriatal and systemic transplantation. *Stem Cells* 30, 1297–1310. doi: 10.1002/stem.1098

Doeppner, T. R., and Hermann, D. M. (2010). Mesenchymal stem cells in the treatment of ischemic stroke: progress and possibilities. *Stem Cells Cloning* 3, 157–163. doi: 10.2147/SCCAA.s7820

Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716. doi: 10.1016/s0092-8674(00)80783-7

Doetsch, F., García-Verdugo, J. M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.* 17, 5046–5061.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8, 315–317. doi: 10.1080/14653240600855905

Haas, S., Weidner, N., and Winkler, J. (2005). Adult stem cell therapy in stroke. *Curr. Opin. Neurol.* 18, 59–64. doi: 10.1097/00019052-200502000-00012

Hermann, D. M., and Chopp, M. (2012). Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurol.* 11, 369–380. doi: 10.1016/S1474-4422(12)70039-X

Honmou, O., Houkin, K., Matsunaga, T., Niitsu, Y., Ishiai, S., Onodera, R., et al. (2011). Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 134, 1790–1807. doi: 10.1093/brain/awr063

Jessberger, S., and Gage, F. H. (2014). Adult neurogenesis: bridging the gap between mice and humans. *Trends Cell Biol.* 24, 558–563. doi: 10.1016/j.tcb.2014.07.003

Kondziolka, D., Steinberg, G. K., Wechsler, L., Meltzer, C. C., Elder, E., Gebel, J., et al. (2005). Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J. Neurosurg.* 103, 38–45. doi: 10.3171/jns.2005.103.1.0038

Kondziolka, D., Wechsler, L., Goldstein, S., Meltzer, C., Thulborn, K. R., Gebel, J., et al. (2000). Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* 55, 565–569. doi: 10.1212/wnl.55.4.565

Kranz, A., Wagner, D. C., Kamprad, M., Scholz, M., Schmidt, U. R., Nitzsche, F., et al. (2010). Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats. *Brain Res.* 1315, 128–136. doi: 10.1016/j.brainres.2009.12.001

Kurozumi, K., Nakamura, K., Tamiya, T., Kawano, Y., Ishii, K., Kobune, M., et al. (2005). Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Mol. Ther.* 11, 96–104. doi: 10.1016/j.ymthe.2004.09.020

Lalu, M. M., McIntyre, L., Pugliese, C., Fergusson, D., Winston, B. W., Marshall, J. C., et al. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One* 7:e47559. doi: 10.1371/journal.pone.0047559

Lazennec, G., and Jorgensen, C. (2008). Concise review: adult multipotent stromal cells and cancer: risk or benefit? *Stem cells* 26, 1387–1394. doi: 10.1634/stemcells.2007-1006

Lee, J. S., Hong, J. M., Moon, G. J., Lee, P. H., Ahn, Y. H., Bang, O. Y., et al. (2010). A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 28, 1099–1106. doi: 10.1002/stem.430

Leong, W. K., Henshall, T. L., Arthur, A., Kremer, K. L., Lewis, M. D., Helps, S. C., et al. (2012). Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. *Stem Cells Transl. Med.* 1, 177–187. doi: 10.5966/sctm.2011-0039

Li, Y., Chen, J., Chen, X. G., Wang, L., Gautam, S. C., Xu, Y. X., et al. (2002). Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* 59, 514–523. doi: 10.1212/wnl.59.4.514

Liu, J., Solway, K., Messing, R. O., and Sharp, F. R. (1998). Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci.* 18, 7768–7778.

Mitkari, B., Nitzsche, F., Kerkelä, E., Kuptsova, K., Huttunen, J., Nystedt, J., et al. (2014). Human bone marrow mesenchymal stem/stromal cells produce efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with cerebral ischemia, but this is not translated to behavioral recovery. *Behav. Brain Res.* 259, 50–59. doi: 10.1016/j.bbr.2013.10.030

Moniche, F., Gonzalez, A., Gonzalez-Marcos, J. R., Carmona, M., Piñero, P., Espigado, I., et al. (2012). Intra-arterial bone marrow mononuclear cells in ischemic stroke: a pilot clinical trial. *Stroke* 43, 2242–2244. doi: 10.1161/STROKEAHA.112.659409

Mora-Lee, S., Sirerol-Piquer, M. S., Gutiérrez-Pérez, M., Gomez-Pinedo, U., Roobrouck, V. D., López, T., et al. (2012). Therapeutic effects of hMAPC and hMSC transplantation after stroke in mice. *PLoS One* 7:e43683. doi: 10.1371/journal.pone.0043683

Onda, T., Honmou, O., Harada, K., Houkin, K., Hamada, H., and Kocsis, J. D. (2008). Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J. Cereb. Blood Flow Metab.* 28, 329–340. doi: 10.1038/sj.jcbfm.9600527

Parent, J. M. (2003). Injury-induced neurogenesis in the adult mammalian brain. *Neuroscientist* 9, 261–272. doi: 10.1177/1073858403252680

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147. doi: 10.1126/science.284.5411.143

Rabinovich, S. S., Seledtsov, V. I., Banul, N. V., Poveshchenko, O. V., Senyukov, V. V., Astrakov, S. V., et al. (2005). Cell therapy of brain stroke. *Bull. Exp. Biol. Med.* 139, 126–128.

Savitz, S. I., Dinsmore, J., Wu, J., Henderson, G. V., Stieg, P., and Caplan, L. R. (2005). Neurotransplantation of fetal porcine cells in patients with basal ganglia infarcts: a preliminary safety and feasibility study. *Cerebrovasc. Dis.* 20, 101–107. doi: 10.1159/000086518

Savitz, S. I., Misra, V., Kasam, M., Juneja, H., Cox, C. S. Jr., Alderman, S., et al. (2011). Intravenous autologous bone marrow mononuclear cells for ischemic stroke. *Ann. Neurol.* 70, 59–69. doi: 10.1002/ana.22458

Sawada, M., Matsumoto, M., and Sawamoto, K. (2014). Vascular regulation of adult neurogenesis under physiological and pathological conditions. *Front. Neurosci.* 8:53. doi: 10.3389/fnins.2014.00053

Schwarting, S., Litwak, S., Hao, W., Bähr, M., Weise, J., and Neumann, H. (2008). Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. *Stroke* 39, 2867–2875. doi: 10.1161/strokeaha.108.513978

Sheikh, A. M., Nagai, A., Wakabayashi, K., Narantuya, D., Kobayashi, S., Yamaguchi, S., et al. (2011). Mesenchymal stem cell transplantation modulates neuroinflammation in focal cerebral ischemia: contribution of fractalkine and IL-5. *Neurobiol. Dis.* 41, 717–724. doi: 10.1016/j.nbd.2010.12.009

Shen, L. H., Li, Y., Chen, J., Zacharek, A., Gao, Q., Kapke, A., et al. (2007). Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J. Cereb. Blood Flow Metab.* 27, 6–13. doi: 10.1038/sj.jcbfm.9600311

Silva-Vargas, V., Crouch, E. E., and Doetsch, F. (2013). Adult neural stem cells and their niche: a dynamic duo during homeostasis, regeneration and aging. *Curr. Opin. Neurobiol.* 23, 935–942. doi: 10.1016/j.conb.2013.09.004

Spees, J. L., Gregory, C. A., Singh, H., Tucker, H. A., Peister, A., Lynch, P. J., et al. (2004). Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol. Ther.* 9, 747–756. doi: 10.1016/j.ymthe.2004.02.012

Steiner, B., Roch, M., Holtkamp, N., and Kurtz, A. (2012). Systemically administered human bone marrow-derived mesenchymal stem home into peripheral organs but do not induce neuroprotective effects in the MCAo-mouse model for cerebral ischemia. *Neurosci. Lett.* 513, 25–30. doi: 10.1016/j.neulet.2012.01.078

Suárez-Monteagudo, C., Hernández-Ramírez, P., Alvarez-González, L., García-Maeso, I., de la Cuétara-Bernal, K., Castillo-Díaz, L., et al. (2009). Autologous bone marrow stem cell neurotransplantation in stroke patients. *Restor. Neurol. Neurosci.* 27, 151–161. doi: 10.3233/RNN-2009-0483

Taupin, P., and Gage, F. H. (2002). Adult neurogenesis and neural stem cells of the central nervous system in mammals. *J. Neurosci. Res.* 69, 745–749. doi: 10.1002/jnr.10378

Tonchev, A. B., Yamashima, T., Zhao, L., and Okano, H. (2003). Differential proliferative response in the postischemic hippocampus, temporal cortex and olfactory bulb of young adult macaque monkeys. *Glia* 42, 209–224. doi: 10.1002/glia.10209

Ukai, R., Honmou, O., Harada, K., Houkin, K., Hamada, H., and Kocsis, J. D. (2007). Mesenchymal stem cells derived from peripheral blood protects against ischemia. *J. Neurotrauma* 24, 508–520. doi: 10.1089/neu.2006.0161

Yamashita, T., Ninomiya, M., Hernández Acosta, P., García-Verdugo, J. M., Sunabori, T., Sakaguchi, M., et al. (2006). Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. *J. Neurosci.* 26, 6627–6636. doi: 10.1523/jneurosci.0149-06.2006

Yang, B., Migliati, E., Parsha, K., Schaar, K., Xi, X., Aronowski, J., et al. (2013). Intra-arterial delivery is not superior to intravenous delivery of autologous bone marrow mononuclear cells in acute ischemic stroke. *Stroke* 44, 3463–3472. doi: 10.1161/strokeaha.111.000821

Yoo, S. W., Kim, S. S., Lee, S. Y., Lee, H. S., Kim, H. S., Lee, Y. D., et al. (2008). Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. *Exp. Mol. Med.* 40, 387–397. doi: 10.3858/emm.2008.40.4.387

Zhang, J., and Chopp, M. (2013). Cell-based therapy for ischemic stroke. *Expert Opin. Biol. Ther.* 13, 1229–1240. doi: 10.1517/14712598.2013.804507

Zheng, W., Honmou, O., Miyata, K., Harada, K., Suzuki, J., Liu, H., et al. (2010). Therapeutic benefits of human mesenchymal stem cells derived from bone marrow after global cerebral ischemia. *Brain Res.* 1310, 8–16. doi: 10.1016/j.brainres.2009.11.012

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