Chapter 7
Modulation of Post-Stroke Plasticity and Regeneration by Stem Cell Therapy and Exogenic Factors

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Abstract Revascularization therapy in the acute post-stroke phase nowadays is reducing the grade of disability and mortality after cerebral ischemia. Post-acute to chronic therapeutic strategies in the phase of irreversible brain parenchyma damage showed until now controversial results in pre-clinical studies: currently there are no effective treatment strategies apart from neurological rehabilitation aiming at restoration of functional post-ischemic deficits.

Spontaneous functional recovery appears immediately after stroke and was proven to correlate with the endogenous regeneration potential represented by rewiring of

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neuronal circuits through promotion of dendritic and axonal sprouting, improving axonal function, synaptogenesis, neurogenesis, and angiogenesis. These observations have led to numerous preclinical studies investigating a new therapeutic direction after stroke, the neurovascular restoration impacting stroke recovery potential.

This chapter summarizes achievements to date, current challenges and ongoing research in the field of regenerative processes after ischemic stroke, focusing on the formation of functional anatomical pathways responsible for enhanced recovery.

Keywords Stroke • Regeneration • Plasticity • Repair • Stem cells • Neural progenitors • Neurogenesis • Neuroprotection • Endothelial progenitors • Trophic factors

Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AQP	Aquaporin
ATSC	Adipose-tissue stem cell
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMSC	Bone-marrow derived stem cell
BrdU	5-Bromo-2′-deoxyuridine
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CM	Conditioned medium
CNS	Central nervous system
DCX	Doublecortin
EC	Endothelial cell
EPC	Endothelial progenitor cell
Epo	Erythropoietin
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
HIF-1	Hypoxia-inducible factor-1
HuNu	Human nuclear antigen
IL	Interleukin
LTD	Long term depression
LTP	Long term potentiation
LV	Lateral ventricle
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cell

NO	Nitric oxide
NSC	Neural stem cell
PI3K	Phosphoinositide 3-kinase
Robo	Roundabout protein
SEM	Standard error of the mean
SGZ	Subgranular zone
STDP	Spike timing depending plasticity
SVZ	Subventricular zone
TPEN	*N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine
TSP	Thrombospondin
TuJ1	Neuron-specific class III beta-tubulin
UCBC	Umbilical cord blood stem cell
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA-4	Very large antigen-4
Zn	Zinc
Zn^{2+}	Ionic zinc

1 Introduction

The controversial principle of *diaschisis* introduced 100 years ago by *Constantin von Monakow* represents the beginning of understanding the general model of plasticity underlying functional recovery after central nervous system (CNS) damage nowadays. *Diaschisis*, meaning 'shocked throughout' in Greek was defined by an 'interruption of function' in an intact brain region which will lead to a 'struggle for the preservation of the disrupted nervous function, and the CNS is always prepared for a struggle' [1, 2].

Ischemic stroke results from a sudden impairment of blood supply in specific parts of the brain, being the leading cause of long-term disability in adults in industrialized countries [3]. Sensorimotor and cognitive impairment after stroke are often severe with little chance of complete rehabilitation, which is associated with high socio-economic costs. Therefore, there is high demand for the development of new, effective treatment strategies to improve the functional outcome after ischemic brain damage.

The scientific efforts from the last decade showed that not only the brain itself has an intrinsic potential for reorganization and repair after stroke [4], but also that these processes of regeneration can be successfully stimulated by means of extrinsic factors. This plasticity potential is represented at the anatomical level by: 1.) recruitment of pathways that sustain the same function as the destroyed ones but have a different anatomical form, 2.) synaptogenesis, 3.) dendritic arborization, 4.) fortification of functionally silent synaptic connections, 5.) long distance fiber sprouting and branching, and 6.) endogenous neurogenesis [5–8]. These events take place in

the first days up to weeks after the ischemic lesion, and the susceptibility to external therapeutic influence is negatively correlated with time. However, post-stroke plasticity is challenged by unexpected and sudden onset of ischemic damage [9], and by the high complexity of the damaged neural structures [10]. Novel therapeutic approaches like transplantation of stem and progenitor cells or administration of factors influencing endogenous repair capabilities of the post-ischemic brain have been investigated in the last decade. The purpose of this chapter is to provide an overview of the current research on neuroregenerative strategies after stroke focusing on underlying mechanisms of action, therapeutic window and possible implications for targeted neurorehabilitation.

2 Clinical Aspects of Stroke Treatment Research

The human brain detains its own rescue mechanisms in case of acute ischemic injury such as: 1.) recruitment of existing collateral blood vessels and induction of angiogenesis, preparing them for takeover in case of sudden obstruction [11], 2.) glial scar formation in the close vicinity of the ischemic core with neuroprotective potential, 3.) self-regeneration by reactivation of ontogenetic repair mechanisms [12]. These three pathways observed after stroke were further investigated in preclinical and clinical studies, giving rise to three therapeutic directions: re-establishing cerebral blood flow (revascularization), neuroprotection and neuroregeneration.

2.1 Revascularization

Mechanical thrombectomy with stent retrievers after large artery occlusion has been proven in recently published randomized studies [13] to re-establish blood flow and to reduce the functional disabilities, being nowadays the gold standard of acute stroke therapy (for review see *Balami et al.* [14]). The reperfusion of the ischemic tissue in the therapeutic window is meant to save the penumbra, limiting the ischemic damage, as well as to prevent vasogenic edema. It also sets the basic conditions for regenerative processes in the peri-infarct zone after stroke.

2.2 Neuroprotection

Neuroprotection is a broad term for mechanisms and strategies aiming at preventing neuronal cell death, therefore reducing deleterious effects of ischemic injury. This terminology is being used in preclinical research with regard to treatments that

prevent or interrupt the molecular injury cascade in the penumbra and preventing secondary neuronal death [15, 16].

Neuroprotective strategies were developed on all progression pathways of ischemic injury described earlier: molecular injury, brain edema, inflammation, excitotoxicity, apoptosis, and spreading depression [17, 18].

Glutamate antagonists were studied with regard to their inhibitory effect upon peri-infarct depolarization and proved to reduce the size of ischemic lesion [19, 20]. Trying to reverse or stop the cascade of molecular injury after stroke lead to the development of different strategies like: stopping neuronal death by excitotoxicity by glutamate antagonists, using antioxidant substances to stop the formation of reactive species of oxygen or of peroxynitrite, antiapoptotic substances meant to stop the delayed neuronal death [17].

Formation of cytotoxic edema was considered as a target for aquaporin (AQP) channels, which are located in the plasma membrane and facilitate water transport. Inhibition of AQP water conductance was demonstrated to reduce the severity of ischemic brain edema [21]. Later studies proved that an intrinsic mechanism of early induction of AQPs may decrease cytotoxic edema formation after stroke but has no influence upon blood-brain barrier (BBB) disruption and therefore has a limited time effect after stroke [22].

The cellular inflammatory response after ischemia was proven to have both detrimental effects contributing to lesion expansion but also to play an important role in the orchestration of lesion repair, the outcome after stroke being seen as a result of the interaction between the injured brain and the immune system [23].

The most active inflammatory pathway after stroke is lead by cytokines and their answer after stroke. Especially the cytokine interleukin (IL)1-beta was for a long time considered a strong neuroprotective target, since administration of IL1-beta receptor antagonists reduces infarct size [24].

The translation of these therapies failed repeatedly, despite the convincing preclinical and phase IIb available data. The SAINT-II (Stroke Acute Ischemic NXY Treatment) study investigating the antioxidative agent NXY-059 as neuroprotective therapy after stroke in patients had to be stopped due to lack of efficiency in the beginning of the phase III trial [25, 26].

2.3 Neurovascular Restoration

Since the main clinical impact of stroke is due to its long time disability effect and because neuroprotective studies did not succeed in the clinical trials, the focus of stroke research changed in the last years on neuroregenerative approaches. The observation of endogenous regeneration potential after stroke, by means of neurogenesis [8, 27, 28] angiogenesis [29], axonal and dendritic sprouting potential [30] and synaptogenesis [30] started a new therapeutic direction after stroke, called neurovascular restoration.

2.3.1 Endogenous Neural Stem Cells as a Possible Pool for Regeneration After Stroke

Formation of neural stem cells (NSCs) starts in the gastrulation phase and continues throughout the embryonic brain by a continuous proliferation of NSCs and subsequent differentiation and migration of neural progenitor cells (NPCs) [31, 32]. After birth there are still neurogenic niches situated in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus [33]. Accordingly, proliferation of residential NSCs is observed in the adult brain in the SVZ, SGZ and the posterior periventricular area [34–37]. This represents an endogenous pool of NSCs which was proven to be activated by focal ischemia [38] both in the ipsilesional and in the contralesional hemisphere [39, 40], presenting a well determined timing following transient focal ischemia by reaching the peak point 1–2 weeks after stroke and returning to sham levels by 3–4 weeks [41, 42].

The process of neurogenesis includes three major anatomical steps: proliferation, migration and differentiation [43], which have to be followed by functional integration of the newborn neurons, including integration in the extracellular matrix environment and electrophysiological integration in neuronal circuits.

Proliferation of neurons after stroke was intensively studied until now with regards to different growth factors, some of the most promising being erythropoietin (Epo) and vascular endothelial growth factor (VEGF). Ischemia was shown to stimulate the hypoxia-inducible factor-1 (HIF-1) pathway as a main player in the signal cascade after stroke. The smallest reduction in oxygen partial pressure in the brain leads to a strong activation of HIF-1. Both VEGF and Epo are responsible for downstream effects of the transcription factor HIF-1 cascade. Epo knock-out mice have deficiencies in post-ischemic neurogenesis [44, 45] and VEGF was proven to promote neurogenesis both *in vitro* and *in vivo* [46]. Further neuroregeneration-specific aspects of these two growth factors are going to be discussed later in detail.

Migration and differentiation of endogenous NPCs in the normal brain was demonstrated to follow the route of the rostral migratory stream towards the olfactory bulb, whereas in the ischemic preconditioned brain, the NPCs migrate towards the injured areas in the brain [47, 48]. Important mediators in this process of migration and maturation are represented by matrix metalloproteinases (MMPs), especially the MMP9 molecule, which is upregulated in the infarcted cortex at 7–14 days in rats and was shown to colocalize with the NPC marker doublecortin (DCX) and proliferating 5-bromo-2′-deoxyuridine (BrdU) positive cells migrating from the SVZ [49]. *Wang et al.* proved in their studies that conditioned medium from Epo-treated epithelial cell cultures significantly promoted NPC migration, which was blocked by specific MMP inhibitors [50].

Even if there is ample evidence for migration and maturation of NPCs to the ischemic lesion, an aspect that still causes controversies involves the functionality of these neurons, their long-time survival and their integration in the peri-neural and angiogenetic milieu in order to sustain the beneficial recovery after stroke. Among other molecules, VEGF and Epo are thought to be promising candidates to facilitate this functional integration.

2.3.2 Angiogenesis and Neurovascular Remodeling After Stroke

A strong intercellular orchestration is needed to create the permissive conditions for functionally relevant axonal and dendritic sprouting after ischemic injury of the brain. The vascular and the nervous system share multiple similarities in their development, both of them using long-distance projections to reach their targets, being guided by gradients of chemokines and growth factors. Especially in the peripheral nervous tissue, the parallel tracking of blood vessels and nerves is obvious. In the CNS, neurogenesis takes place in the embryological vascular niches where endothelial cells (ECs) proliferate. This is why the two systems have to be taken into consideration as a homeostatic unit, especially in neuropathological conditions such as stroke.

Angiogenesis in the adult brain is the hypoxia-driven sprouting of new capillaries from postcapillary venules [51]. Tissue hypoxia in the adult brain stimulates the activation of HIF-1α expression, which then stimulates the transcription of VEGF, VEGF receptors flt-1 and neuropilin-1, and angiopoietin [52]. Besides the molecular aspect of angiogenesis activation, there are two further systems that are implicated immediately after stroke: loss of vascular integrity and cell matrix degradation [53]. These two processes activate growth factors, their receptors and the guidance molecules which were until then in a dormant phase by being incorporated in the cellular matrix. One of the most important activated growth factors is VEGF, which induces endothelial cell proliferation and their migration [54, 55]. The VEGF family comprises 5 related genes: VEGF-A, -B, -C, -D and PIGF (placenta induced growth factor). VEGF-A is the vascular permeability factor and is known in several isoforms (VEGF-A_{204}, -A_{189}, -A_{165}, -A_{145}, -A_{121}), with different amino acid length and has the capacity of binding to heparin sulfates. VEGF-A_{165} has some degree of heparan sulfate binding which reduces its diffusibility, but at the same time increases its ability to stimulate VEGF receptors [56].

As discussed for neurorestoration, an important aspect of angiogenesis is its functionality translated either by a significant increase in overall blood flow to the tissue that suffered an ischemic damage or by creating the foundation for late restoration processes in the ischemic tissue together with the neural network. The formation of new blood vessels after stroke seems to develop parallel to neurogenesis, being initiated rather late at 48 h after stroke [57, 58]. Because of this delay, there are no reasons to assume that angiogenesis influences brain hemodynamics during an acute ischemic stroke in a relevant way [53]. However, the observed timing hints towards a coupling of neurovascular remodeling after stroke in order to prepare the necessary background for long term restorative processes, e.g. axonal sprouting.

Another important aspect in the process of new blood vessel formation is the VEGF induced disruption of the BBB immediately after stroke, leading to edema formation. Early post-ischemic administration of VEGF in rats increased BBB leakage and infarction volume, whereas its late administration (48 h) enhanced angiogenesis and decreased BBB leakage, resulting in improved recovery volume [59].

2.3.3 Axonal Sprouting and Plasticity

An interesting general observation in the maturation of the corticospinal tract was that the early widespread distribution reaches the specific mature distribution by means of collateral selection. Neuronal cell death is not known to take place in the developing brain, so the hypothesis of postnatal reorganization could be explained just by means of collateral elimination. Using retrograde fluorescent tracer injections into the pyramidal decussation at the spinomedullary junction in adult versus postnatal rats, *Stanfield et al.* could prove that beside the frontal and parietal cortex, the occipital cortex was involved in building the corticospinal tract in postnatal rats [60]. These studies lead to the conclusion that transient pyramidal tract axons are eliminated during development, e.g. being found as projections to the superior colliculus and/or the pons [61, 62]. The understanding of developmental sculpturing of cortical efferent systems is important in further perception of remodeling processes in the adult brain after stroke or other types of injury.

It is now well accepted that the CNS has an intrinsic recovery capacity after stroke, by means of reactivating the ontogenetic machinery stimulating gene expression, protein synthesis, and cellular genesis, reconstructing the needed environment for recovery [63]. Preclinical and clinical studies on unilateral ischemic brain damage demonstrate an increased amount of corticospinal projections and shift of cortical sensorimotor functions to the intact hemisphere. Whether the intact hemisphere increases functionality after contralateral stroke just by means of increasing pyramidal corticospinal projections is not clear. A series of recent studies could identify stimulation of interhemispheric, cortico-reticular or cortico-thalamic pathways [5, 6, 64]. The involvement of the intact pyramidal tract in taking over functions of the damaged contralateral pyramidal system requires large–scale reorganization and a competition between the two cerebral hemispheres for spinal synaptic space. This implies that the degree of abnormality of these corticospinal projections following unilateral lesions might not reflect simply the extent of the initial lesion but also the consecutive competitive disadvantage of the surviving corticospinal projections. This competitive disadvantage would lead to cortico-spinal projections from the intact hemisphere progressively replacing a part of the surviving cortico-spinal projections from the damaged hemisphere and thus to a progressive degeneration.

Post-ischemic endogenous responses of the CNS go in line with an enhanced sensitivity to rehabilitative [65] and plasticity-promoting [66, 67] treatments, opening a time window in which ontogenetic brain repair mechanisms might successfully be reactivated [12, 68]. Stroke recovery is associated with reorganization of neuronal circuits both at the cortical and subcortical level. A series of events set the stage for brain reorganization in the intact hemisphere, such as increased angiogenesis [69] and axonal sprouting [66, 70].

Recruitment of contralesional brain areas correlated with a better recovery from stroke in animal studies [66, 70]. By administering anterograde tract tracers into the contralesional motor cortex, these authors suggested that contralateral projections may be recruited by plasticity-promoting therapies, underlining the relevance of contralesional reorganization for neurological recovery. However, models of permanent

focal cerebral ischemia were used in the latter studies, in which motor cortex tissue was irreversibly destroyed. Brain plasticity ipsilateral to the stroke was not systematically assessed in these studies.

The vascular system is strongly linked to the neural system due to common ontogenic developing pathways. When investigating the circulatory system in cases of neurovascular pathologies of the brain, a series of dynamic processes were identified, which modulate development, survival and differentiation of neurons, rewriting the embryologic developmental phase in a restricted time and space manner [71]. This interconnected developmental network also depends on an important common regulator: the VEGF protein family and its receptor system.

2.3.4 Dendritic Elaboration and Dendritic Spine Proliferation

Whereas in the uninjured adult brain dendritic branching and spines are considered to be stable entities [72], important changes in density of dendritic spines were observed in ischemic situations [73].

By means of two-photon imaging techniques, *Brown et al.* demonstrated an increase in dendritic spine formation with a peak around 1–2 weeks and lasting around 6 weeks in the peri-infarct region after cortical ischemic injury [74]. Until now, no direct link between the rate of dendritic spine formation and functional recovery after stroke has been shown. Due to the spatial and temporal coincidence between dendritic branching and spine reorganization and changes in functional representation of the peri-infarct region [75] and functional recovery [76], restorative therapies focused also on stimulation of dendritic branching and spine density in the early phase after stroke.

After an ischemic injury that affects the axons of pyramidal neurons in the cortex without having a direct effect upon dendrites, significant dendritic spine loss was observed, which was proven to be a result of profound deafferentation. With time, this is followed by an increase in dendritic spine densities and neuritic outgrowth. There are two main sprouting directions involved: the horizontal cortico-cortical connections [6, 77] and the vertical connections from the contralesional hemisphere that travel through the corpus callosum [78].

Large-scale dendritic plasticity was proven to depend on the balanced elongation-retraction of pyramidal dendrites in the peri-infarct cortex after a small photo-thrombotic stroke [79]. Layer V of pyramidal neurons in the cortex is considered the main excitatory zone, therefore differences in dendritic branching and connectivity in this region will be expected to have a major impact upon cortical circuits. Still the evidence that this branching takes place is controversial, and some studies done with the MCAO stroke model found no differences in dendritic branching and spine densities between lesioned and control animals [80, 81]. By repetitive imaging *in vivo* over 3 months after ischemia, another study failed to prove evidence of *de novo* branching formation in the surviving L5 pyramidal neurons in the peri-infarct cortex [82].

The failure of spontaneous regeneration to induce functional recovery after stroke was proved not to be a failure of forming new connections [83], but mainly to be dependent on the non-permissive environment. The main players in the inhibition of the sprouting are the growth-inhibitory proteins, parts of the CNS myelin [84].

2.3.5 Synaptic Plasticity

Synaptic plasticity during the developing phase experiences a burst early in the postnatal period especially in the occipital cortex, reaching a density that is approximately twice the density in the adult brain [85]. Waves of synaptic plasticity appear also in parieto-temporal and then frontal regions during development, reaching their peaks around early adolescence [86, 87].

In physiological situations in the brain, synapses are modulated (strengthened or weakened) and shaped by activity-induced mechanisms called also Hebbian plasticity. Whereas regulation of individual synapses by means of long term potentiation (LTP) or long term depression (LTD) is the main mechanism responsible for learning and memory processes. In addition, mechanisms regulating levels of activity are considered important in synaptic plasticity for network function [88]. The Hebbian plasticity describes positive-feedback mechanisms responsible either for strengthening of effective synapses or for weakening of passive synapses. Regulation of neuronal activity during synaptic modulation was theoretically and experimentally devised in three possible underlying processes: synaptic scaling, spike timing depending plasticity (STDP) and synaptic redistribution.

Synaptic scaling refers to the competitive interaction between synapses coupling on the same neuron. The biological substrates that lead to synaptic scaling are dependent of glutamate receptors number. This leads to specialization of neuronal pathways depending on their synaptic stimulus. Whereas synaptic scaling is non-Hebbian plasticity forming active neuronal pathways by means of mainly post-synaptic firing rate, STDP is thought to respect Hebbian plasticity by considering both pre- and postsynaptic activity. Synaptic redistribution was observed in some forms of cortical neurons in which short-term plasticity of synapses induced by LTD can be modified by LTP, which increases the presynaptic elimination of the neurotransmitter. The mechanism of synaptic redistribution is not completely understood. For further information please see the review of *Abbott and Nelson* [88].

In the ischemic cortex, synaptogenesis is progressively increased in the peri-infarct region together with markers of axonal sprouting such as GAP-43, underlining the importance of the penumbral region in synaptic reorganization after stroke [30].

2.3.6 Modulation of the Immune Response and Inflammation

MMPs represent a family of zinc endopeptidases with a major role during development of the CNS and distinct functions in pathological states. Recent data showed specific roles of MMPs in different time periods after stroke. Whereas acute

post-ischemic activation of MMPs leads to increased ischemic injury by enhancing the neuroinflammatory response [89], the postacute activation was shown to contribute to neurovascular remodeling and promote stroke recovery [90].

3 Cell Therapy and Brain Plasticity After Stroke

In the last decade, extensive research efforts have been carried out to establish cell-based therapies, e.g. transplantation of NSCs and/or NPCs, bone-marrow derived stem cells (BMSCs), umbilical cord blood stem cells (UCBCs), adipose-tissue derived stem cells (ATSCs), and embryonic stem cells (ESCs) as a possible experimental therapeutic avenue for ischemic stroke and other disorders of the CNS (for review see *Lindvall et al.* [91]). A series of clinical studies has already demonstrated the feasibility and safety of this approach in clinical practice (for review see *Bliss et al.* [92]).

Transplanted NPCs have been shown to survive, migrate and integrate in the post-ischemic host brain, thereby acquiring adequate neuronal and glial phenotypes and display functional electrophysiological integration into neuronal circuitry (for review see *Hermann et al.* [93]).

Investigations on the possible underlying mechanisms of cell transplantation have revealed that processes other than direct replacement of neurons and glial cells by the grafted NSCs or NPCs are involved in promoting neurological recovery after stroke [94]. The grafted cells orchestrate tissue plasticity of the host brain [95, 96]. These effects include secretion of neurotrophic factors, immunomodulation, and angiogenesis [97–102].

We have previously shown that transplantation of human NPCs improved functional outcome after experimental stroke in rats with temporal coincidence of increased dendritic arborization of layer V pyramidal neurons and promotion of cortico-cortical, cortico-striatal and cortico-spinal axonal projections [103].

Extensive migration of grafted cells towards the perilesional area was observed (Fig. 7.1). Only a very small percentage (about 6%) of the grafted NPCs showed differentiation into the neuronal lineage, most of them stayed in an undifferentiated state. At the same time, grafted animals showed robust functional recovery demonstrated with a battery of neurological tests, as compared to sham-operated controls (Fig. 7.1). Impaired axonal transport processes were partially restored in grafted animals, as demonstrated by reduced amyloid precursor protein accumulation. Using *in vitro* assays with indirect co-culture of human NPCs and cortical neurons, we demonstrated that increased dendritic and axonal plasticity depends on molecules secreted by NPCs. In a further step, some of these mediating factors were identified as VEGF, thrombospondins 1 and 2, and slit using immunodepletion assays (Fig. 7.2). Endogenous remapping of the ipsi- and contralesional hemispheres is a well-known phenomenon in recovery after ischemic damage [104–109]. It is reasonable that the above-mentioned soluble factors secreted by the grafted NPCs and their progeny, among many others, influence the host cells during this process by promoting dendritic and axonal regeneration. Other types of stem cells,

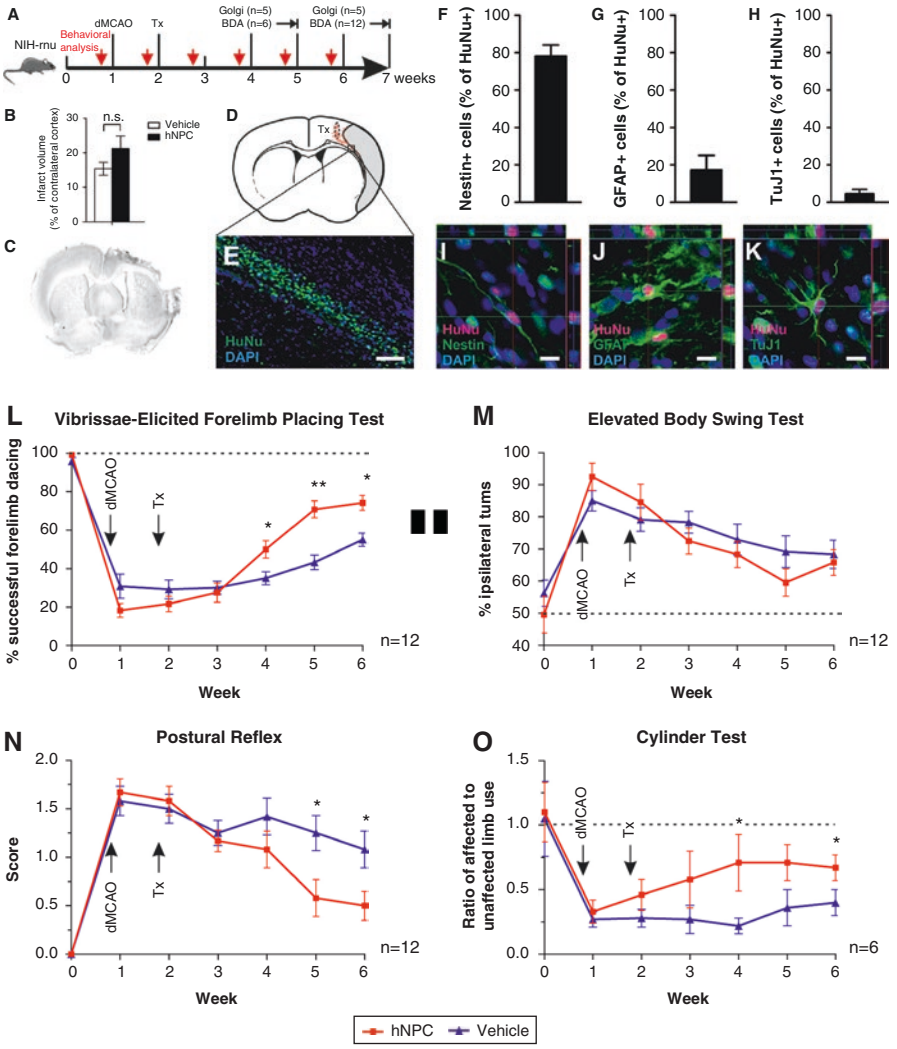

Fig. 7.1 Fate of transplanted human NPCs (hNPCs) and behavioral recovery after experimental stroke in rats. Experimental setup (**a**). Transplanted animals (hNPC) showed a tendency to smaller infarct volumes than controls (Vehicle) (**b**). Distal middle cerebral artery occlusion (dMCAO) resulted in consistent cortical infarction (**c**). 4 weeks after transplantation, the majority of human HuNu+ cells are found in the ischemic boundary zone (**d, e**). Most of the cells remain in a undifferentiated state (Nestin+; **f**), while smaller portions show astrocytic (GFAP; **g**) or neuronal (TuJ1; **h**) differentiation. Confocal immunofluorescence photomicrographs of Nestin+ (**i**), GFAP+ (**j**) and TuJ1+ (**k**) cells co-localizing with HuNu in the peri-infarct area. hNPC-grafted animals (hNPC) demonstrate improved functional recovery as compared to sham-operated controls (Vehicle) using the vibrissae-elicited forelimb placing test (**l**), the elevated body swing test (**m**), the postural reflex test (**n**), and the cylinder test (**o**). Scale bars: **e**: 50 μm, **i–k**: 10 μm. Mean ± SEM; *p < 0.05, **p < 0.01. *Data partially published in Andres et al. Brain 2011 Jun;134(Pt 6):1777-89. doi: 10.1093/brain/awr094, with kind permission from Oxford University Press*

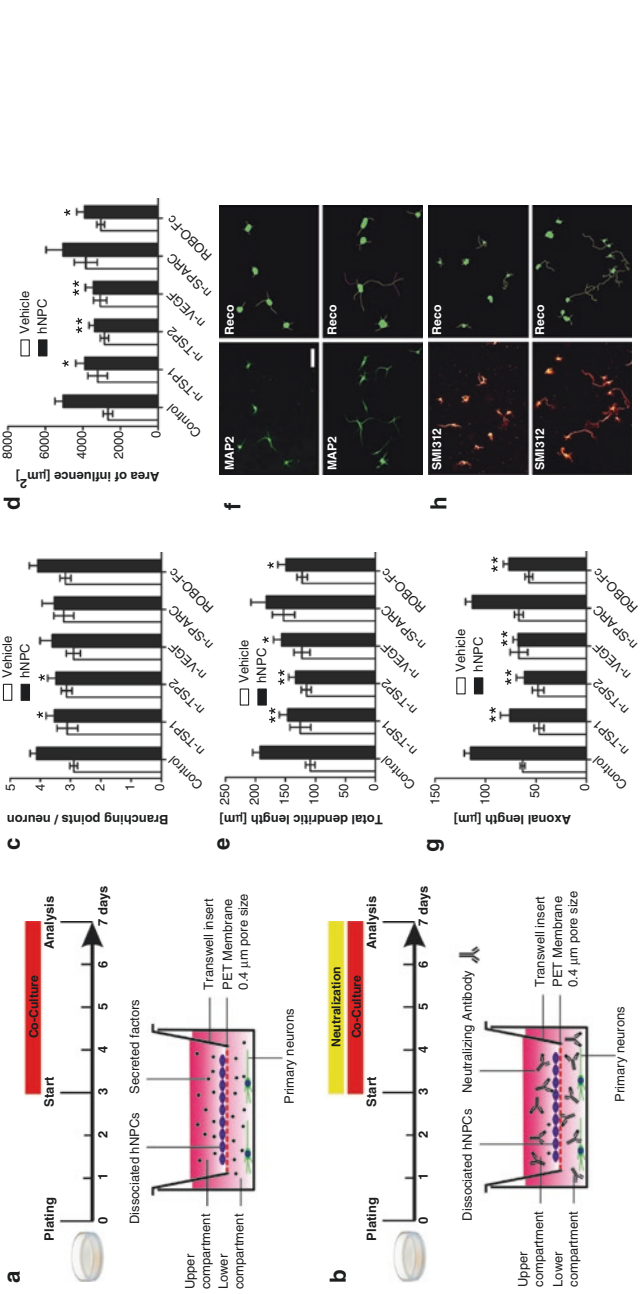

Fig. 7.2 Identification of human NPC (hNPC)-secreted factors mediating the effects on dendritic plasticity and axonal outgrowth *in vitro*. Experimental setup of co-culture experiments (**a**) and immunodepletion studies for identification of specific molecules (**b**). hNPCs were indirectly co-cultured with rat E14 primary cortical neurons for 7 days. Dendritic complexicity and axonal outgrowth were quantified using MAP2 (dendritic marker) and SMI312 (axonal marker) immunofluorescence and automated high-throughput analysis. As compared to untreated controls, the presence of hNPCs significantly promoted dendritic branching (**c**), total dendritic length (**d**), axonal outgrowth (**e**), and area of influence per individual neuron (**f**). Neutralization of factors significantly reduced the effects of hNPCs on dendritic branching (**c**; TSP1, TSP2), total dendritic length (**d**; TSP1, TSP2, VEGF, ROBO-Fc), axonal length (**e**; TSP1, TSP2, VEGF, ROBO-Fc), and area of influence (**f**; TSP1, TSP2, VEGF, ROBO-Fc). Representative photomicrographs and digital reconstructions (Reco) of neurons co-cultured with (hNPC) and without (Vehicle) hNPCs stained for MAP2 (**g**) and SMI312 (**h**). Scale bar: 50 µm, Mean ± SEM; *$p < 0.05$, **$p < 0.01$. *Data partially published in Andres et al. Brain 2011 Jun;134(Pt 6):1777-89. doi: 10.1093/brain/awr094, with kind permission from Oxford University Press*

like BMSCs, UCBCs, ATSCs, and ESCs as well, might have distinct action profiles (for review see *Andres et al*. [110]).

In another study, we investigated the effects of grafted murine NPCs on microglial activation, proliferation and phagocytosis [111]. VEGF secreted by NPCs was identified to mediate potent effects after grafting NPCs in mice. Thus, neural precursor cells are not only influenced by surrounding microglia, but also regulate microglia functions and activity. This might also play an important role in stroke and needs to be addressed in further studies.

Intravascular, i.e. intraarterial or intravenous administration of NPCs, is another feasible approach for cell transplantation in stroke. The advantage of this technique is the widespread distribution of the grafted cells in larger ischemic areas, which is not accomplishable by means of focal stereotactic transplantation. NPCs are recruited across the BBB by mechanisms similar to transendothelial homing of immune cells, including endothelial attachment and rolling along the endothelial surface. This process is facilitated by the integrin very large antigen-4 (VLA-4) expressed on immune cells as well as NPCs, which supports tethering and rolling on flow on vascular cell adhesion molecule 1 (VCAM-1). Expression of VCAM-1 is upregulated on the endothelial surface after stroke, leading to transendothelial recruitment of immune cells from the systemic circulation into the ischemic brain parenchyma. We have previously demonstrated that enrichment of NPCs by fluorescence activated cell sorting for VLA-4 and intracarotid delivery promoted cell homing to the area of stroke in mice and improved behavioral recovery [112]. In a second step, the interaction between CC-chemokine ligand-2 (CCL2) in the perivascular space and its receptor CC-chemokine ligand receptor-2 (CCR2) expressed on the plasma surface of NPCs was identified to be critical for targeted homing of intravascularly delivered NPCs [113]. Blocking CCR2 or using NPCs derived from CCR2 knock out animals led to a significant reduction of transendothelial migration as shown by bioluminescence and immunohistochemical studies. On the other hand, increasing the expression of chemokine receptors on the cellular surface by chemical pretreatment of the cells with brain derived neurotrophic factor (BDNF) augmented the transendothelial migration [114]. According to the temporal profile of adhesion molecule upregulation and chemokine expression after stroke the are ideal therapeutic windows for intravascular cell delivery. In an ischemic reperfusion rat stroke model we have demonstrated that intraarterial cell injection was most efficient 48 h after the ictus [114].

Recent clinical studies demonstrated the feasibility of transplantation of modified BMSCs (SB623) with improvement in clinical outcome 1 year following stable, chronic stroke [115].

4 Cell-Free Treatment Strategies

The demand of therapeutic options complementing thrombolysis and the better understanding of the endogenous repair mechanisms after stroke have favored the development of interventions based on cell transplantation. The observation that neurovascular plasticity is crucial in brain recovery has brought different cell types directly involved

in angiogenesis and neurogenesis in the focus. In the regenerative scenarios following the ischemic insult, in addition to NSCs and NPCs, endothelial progenitor cells (EPC) are of particular importance being both targets and effectors. This is not surprising in view of the functional and anatomical coupling of vascular and neuronal cells (which is particularly evident in the neurogenic niche). Indeed, preclinical studies have confirmed that NPCs transplanted in stroked rodents are capable to generate neurons and promote angiogenesis. Similarly, EPC not only integrate in the brain vasculature, but also display neuroprotective actions. Another feature that makes EPCs suitable for therapy is their capacity to be readily recruited to the ischemic site.

Despite the evidence that intravenously transplanted EPCs engraft in the brain capillaries of stroked rats [116], it is now clear that soluble factors released by transplanted cells promote cell viability by providing trophic support, modulating local immunity and activating tissue remodeling processes, including angiogenesis. Thus, the paracrine actions of transplanted cells are not considered any more as 'bystander effects', but present with major tissue regenerative activities that precede the eventual differentiation and replacement of injured cells (if occurring at all). This concept and the presence of limitations inherent to cell transplantation such as the poor efficacy due to extensive death of grafted cells, microemboli, and tumor formation. have inspired a new type of therapeutic strategies designed on the administration of cell-secreted factors also referred as secretome. Indeed, using a rat hindlimb ischemia model, we have demonstrated that the EPC secretome in the form of conditioned medium (EPC-CM) has the same or even superior therapeutic capacity than transplanted cells [117]. These observations have been extended in a rat model of stroke [118]. In addition, we could demonstrate that intraventricular infusion of EPC-CM significantly increased the number of DCX-positive neuronal precursors in the SVZ of adult naïve rats (Fig. 7.3).

For the translation into clinical practice, the effectors of the diverse secretomes and their mechanisms of action still need to be fully elucidated [119]. We and others have recently reported that the EPC secretome-induced effect on viability of rat brain microvascular ECs under standard culture conditions is mediated by PI3K/AKT and MAPK/ERK activation [120, 121]. Moreover, EPC-CM significantly protected rat brain microvascular ECs from an ischemic insult induced by an oxygen-glucose deprivation. Importantly, these effects seem to be mediated not only by growth factors such as BDNF [121], but also involve not yet identified lipidic factors [122].

5 Pharmacological Treatment

Small molecules, e.g. the neuroprotective and differentiation-inducing ergogenic amino compound creatine [123], and many others, can be systemically administered in order to promote endogenous repair processes after stroke or to improve the fate of grafted NPCs. However, this is usually limited by low penetration of the BBB, resulting in poor CNS bioavailability, and systemic side effects.

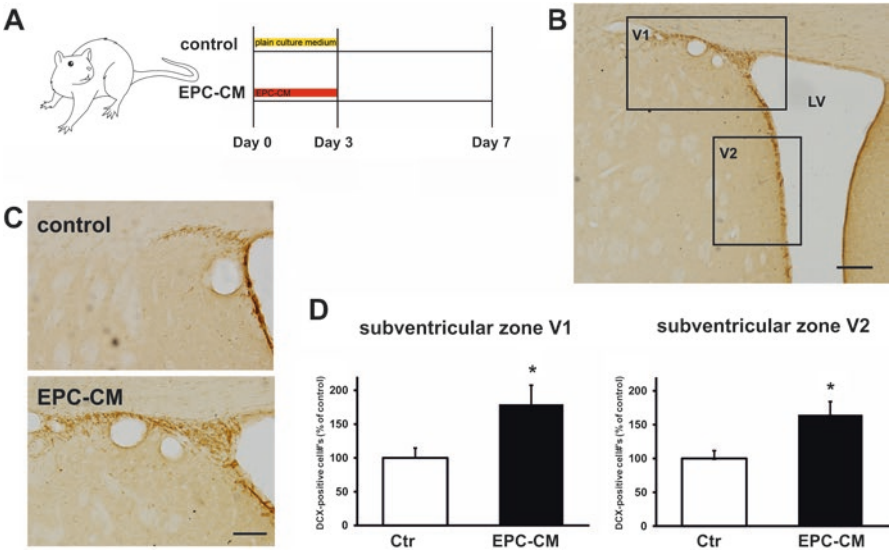

Fig. 7.3 Effects of endothelial progenitor cells-derived conditioned medium (EPC-CM) on number of doublecortin (DCX) positive neuronal precursors in the subventricular zone of adult rats. EPC-CM or control medium was infused into the right lateral ventricle (LV) by means of an Alzet pump for a period of 3 days. After 7 days, brains were removed and processed for immunohistological analyses (**a**). DCX-positive cells were examined in two regions of the subventricular zone (V1, V2) as indicated with *boxes* (**b**). The digitalized representative photomicrographs demonstrate a higher number of DCX-positive cells in V1 after EPC-CM treatment as compared to controls (**c**). Scale bars: 200 μm (**b**), 100 μm (**c**). Significantly higher DCX-positive cell numbers were observed in the EPC-CM groups (*filled bars*) as compared to controls (Ctr, *open bars*) in both the V1 and V2 regions (**d**). Values are expressed as percentage of controls and are given as mean ± SEM. *$p < 0.05$ vs. corresponding controls

Inhibition of tonic (extrasynaptic) gamma aminobutyric acid (GABA) signaling during the repair phase in the post-ischemic brain has been shown to promote functional recovery in mice, suggesting that GABA plays an important role in modulating brain repair [124]. Administration of N,N-Dimethyl-2-(6-methyl-2-p-tolylimidazo[1,2-a]pyridin-3-yl)acetamide (Zolpidem), a GABA agonist specific to the α-1 receptor subtype, was demonstrated to improve behavioral recovery [124].

Zinc (Zn) homeostasis, which is integral to normal CNS functioning, might also be involved in regenerative processes after ischemia and other CNS disorders [125]. Zn ions (Zn^{2+}) have been shown to play a crucial role in the modulation of synaptic transmission as well as in cortical plasticity [126]. Zn is required for the mammalian brain development and physiology. Under normal circumstances, Zn is tightly bound to many proteins in the CNS, whereas ionic Zn is a major etiological factor in CNS damage or diseases due to its toxicity [125, 127–129]. As thus, intracellular Zn^{2+}-concentrations are tightly regulated, as proper homeostasis is critical in the maintenance of cellular processing [130, 131]. Excessive exposure to extracellular Zn^{2+} on the other hand damages neurons of the CNS. Namely, transient forebrain

ischemia in rats leads to an accumulation of chelatable, ionic Zn in degenerating CA1 neurons of the hippocampus, as well as in the cerebral cortex, thalamus, striatum, and the amygdala [127]. Interestingly, this accumulation precedes neurodegenerative processes, which could be prevented by the intraventricular injection of a Zn chelating agent, wherefore the early occurring toxic release of Zn^{2+} may be a key mechanism underlying selective neuronal cell death after ischemia [127]. As thus, administration of Zn^{2+}-chelating agents such as N,N,N′,N′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) could represent a potential cell-targeted therapy. TPEN significantly suppressed cell death, apoptosis, and neuronal glutamate release in primary cultured neurons undergoing a hypoxic-ischemic insult [132]. Moreover, there is a striking feature of a delayed rise in intracellular free Zn^{2+} in CA1 neurons just before the onset of histologically detectable cell death. Intrahippocampal injection of 1-naphthyl acetyl spermine, a selective channel blocker of GluR2-lacking alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors at *Schaffer* collateral to CA1 synapses 9–40 h after transient ischemia greatly reduced the late rise in intracellular free Zn^{2+} in postischemic CA1 neurons and afforded partial protection against ischemia-induced cell death. This receptor subtype appears to be an important therapeutic target for prevention of ischemia-induced neuronal death in humans [133].

Beside Zn^{2+}, nitric oxide (NO) is implicated in the pathogenesis of post-ischemic neuronal damage. The addition of exogenous NO or N-methyl-D-aspartate (NMDA) in order to increase endogenous NO leads to peroxynitrite (ONOO-) formation and consecutive Zn^{2+} release from intracellular stores in cerebrocortical neurons. Free Zn^{2+} in turn induces respiratory block, mitochondrial permeability transition, cytochrome c release, generation of reactive oxygen species, and p38 MAP kinase activation. This crosstalk between NO and Zn^{2+} dependent apoptotic signal transduction pathways may contribute to the delayed loss of neurons after ischemia [134].

Furthermore, we recently showed that Zn^{2+}-dyshomeostasis represents a major suppressor of axonal regeneration in the CNS, with Zn^{2+}-chelation (i.e. with TPEN) leading to persistent survival of many damaged neurons [135]. Thus, synaptic Zn^{2+} represents a previously unknown, critical suppressor of regeneration that might become a crucial player in neuroprotective and plasticity-enhancing strategies after stroke.

A greater understanding of the role of Zn^{2+} for cellular processes following CNS injuries where aberrant metal homeostasis is implicated in disease pathogenesis may therefore allow for the development of new potentially promising therapeutic approaches.

6 Conclusions

Ischemic stroke is the leading cause of severe long-term disability in the Western population, with very few therapeutic options. After an ischemic lesion, the neurovascular units are niches for NSCs and NPCs, supporting the regeneration potential,

and the brain is being reshaped by means of neuronal sprouting or by unmasking the existing, but functionally silent connections. *Kreisel et al.* proposed in 2006 a timeline classification of recovery processes after stroke, differentiating between five distinct stages: (1) hyperacute phase from the stroke event up to 6 h after; (2) acute phase lasting up to the fourth day after stroke characterized by secondary events; (3) subacute phase from the second day up to 2–3 weeks characterized by brain remapping and functional plasticity; (4) consolidation period lasting up to several months and being characterized by functional alteration; (5) chronic phase characterized with the tendency of the events to become static [136, 137]. Despite intense research efforts during the last decade, effective therapeutic agents that promote the repair phase of recovery are still missing. There is a high heterogeneity in the preclinical data, making it unable to be synthetized and translated to the next clinical level. Elucidating underlying mechanisms of endogenous repair processes and plasticity of the brain is critical for the development of new therapeutic strategies for stroke in humans.

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