Comments and Opinions

Restoring Neuronal Function After Stroke by Cell Replacement
Anatomic and Functional Considerations

Marcel Dihné, MD; Hans-Peter Hartung, MD; Rüdiger J. Seitz, MD

Background and Purpose—A major challenge to effective treatment after stroke is the restoration of neuronal function. In recent years, cell-based therapies for stroke have been explored in experimental animal models, and the results have suggested behavioral improvements. However, the anatomic targets of a cell-based stroke therapy and the relationship of cell grafts to post stroke reorganization are poorly understood, which results in difficulties defining strategies for neuronal substitution. Given that stroke causes a variety of secondary changes at locations beyond the infarct lesion, overcoming these difficulties is even more important.

Summary of Review—We describe which brain structures and cell types are candidates for substitution and how new neuronal functionality could be implemented in a damaged brain by capitalizing on current concepts of post stroke plasticity. (Stroke. 2011;42:2342-2350.)

Key Words: repair ■ regeneration ■ neuronal cell replacement ■ stem cells ■ stroke

Neurons Die After Stroke, Causing Functional Deficits
Stroke is the second leading cause of death worldwide.1 With the exception of thrombolysis, which affects only approximately 3% of all patients with stroke, there is no approved therapy for acute stroke. Early stroke mortality varies between 8% and 50% by country,2 and patients who survive a stroke must hope for spontaneous or rehabilitation-supported functional recovery.3 A full 50% of patients with stroke have lasting disabilities. Of these, approximately 30% are institutionalized in nursing homes or assisted-living environments, and >20% depend on assistance for daily activities.3 The functional impairments due to stroke include disturbances in motor activity, speech, sensation, vision, and cognition. The heterogeneity in impairments derives from differences in lesion size and location, which can affect the cerebral cortex, subcortical structures such as the basal ganglia, or white matter and brain stem regions.

The etiology of ischemic stroke includes a temporarily or permanently disturbed blood supply (typically after a thromboembolic occlusion of the cerebral arteries), which results in a rapidly developing pan-necrotic area due to focal ischemia leading to acute energy failure in neurons (the most sensitive), astrocytes, oligodendrocytes, and endothelial cells.4,5 The penumbra is an area of misery perfusion and moderate energy deprivation that surrounds the necrotic area (Figure 1). Depending on the speed of artery revascularization and tissue reperfusion, the ischemic tissue damage may be limited, which allows for more or less efficient perilesional reorganization and neurological recovery.6 Given that behavioral impairments result from the loss of neuronal function, the most direct and complete recovery from established stroke lesions should result from the functional replacement of lost cells, neurons, and connections.

True Neuronal Substitution Requires Specific Anatomic and Functional Profiles
Stroke lesions usually involve a variety of neuroanatomical structures that contain a diversity of cell types with complex connectivity patterns. Presumably, micro- and macroanatomical conditions and connectivity will never reach their original conditions after stroke lesions have been established. Nevertheless, rebuilding basic anatomic structures and fiber connections could provide a regenerative neuronal scaffold.

To achieve this rebuilding, functionally important structures that are damaged by cerebral infarction need to be identified in experimental animal models and, later, individual patients. The location and size of the infarct and remote secondary tissue damage need to be identified through high-resolution, 3-dimensional MRI. Retrograde axonal degeneration occurs most frequently after a stroke,7 which affects brain areas that are remotely situated from the primary insult (Figure 2). Techniques such as diffusion tensor imaging can be applied to visualize secondarily degenerating fiber tracts.8 Recently, this technology has resolved anatomic structures of <1 cm², which,9,10 although limited, will be helpful for identifying the target repair site.

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After site identification, neuronal substitution must consider the site’s specific original neuronal phenotypes, which include not only the anatomic features, but also the functional properties and adaptive processes after stroke, which should be implemented by regenerative strategies.

Neuronal Substitution in Lesions of the Cerebral Cortex and Adjacent White Matter

Anatomic and Developmental Considerations

In mice, monkeys, and humans, the cerebral cortex develops from the anterior components of the neural tube, specifically the dorsal and ventral telencephalon, which exhibit a common 6-layer structure, which contains specific cell types and configures specific connections.11 These layers develop through temporal commitment and the specification of embryonic neural stem cells. Neurons born in the embryonic ventricular or subventricular zone migrate radially to their target positions. The ganglionic eminence, which is located in the embryonic basal telencephalon, is composed of neuronal precursors that migrate tangentially toward their destinations within the 6 cortical layers. A variety of transcription factors and secreted cues guide cortical development by ensuring the correct local phenotypes, positions, and connections.12 The concerted action of developmental factors results in layer-specific, corticocortical, horizontal projections13 and vertical connections to the striatum, thalamus, brain stem, and spinal cord.14 Cortical neurons can be classified by their transcription factor profiles and their neurotransmitter (eg, glutamate or γ-aminobutyric acid) or connection type (projection or interneurons).

The thalamus originates from the posterior embryonic forebrain, and its cell specification is profoundly influenced by the middiencephalic organizer during development.15 The thalamus consists of different subnuclei with unique functional properties. One of these, the ventrobasal complex (the primary somatosensory nucleus), is reciprocally connected to the somatosensory cortex (S1). The involvement of the S1 in motor function is strongly supported by horizontal connections between primary motor and sensory areas.13 During embryonic development, specific guidance cues, including ephrin and the Eph genes, are involved in configuring the ventrobasal–S1 system.16 This system is of fundamental, functional importance because it transfers sensory information from the spinal cord and the trigeminal nerves to cortical areas. Other thalamic nuclei such as the ventrolateral thalamic nucleus and the lateral geniculate nucleus are connected to the primary motor cortex (M1) and primary visual cortex (V1), respectively.

Notably, delayed secondary neuronal degeneration occurs remotely at the ventrobasal after cortical S1 damage.7 Because thalamic nuclei are indispensable for somatosensory, motor, and visual functions,17 isolated cortical repair appears to be insufficient for restoring function. This finding is also true in the context of cortical connections with the striatum and brain stem. Therefore, although we focus on corticothalamic connections, we emphasize that there are analogous connections to consider. In summary, (1) distinct brain structures such as the cerebral cortex and thalamus are characterized by specific compositions and site-specific neuronal phenotypes; (2) connectivity within and between distinct brain structures is tightly regulated; (3) tissue architec-
ture provides the basis for functionality; and (4) prerequisites for structuring the site-specific cellular code and tissue architecture are predominantly provided during development.

**Functional Considerations**

Cortical plasticity has been observed in young and adult animals during physiological and pathological conditions. Under physiological conditions, cortical plasticity occurs in adult rodents and primates throughout life within visual-, somatosensory-, and motor-related areas and involves behavior- and learning-dependent variations in the cortical representation of peripheral organs. Neurophysiological and neuroanatomical changes in macroscopic projections of corticothalamic and horizontal axons are all morphological correlates of cortical plasticity, which illustrates that normal adult learning enables rewiring processes.

Different types of plasticity have been observed after cortical ischemia, including the reappearance of damaged cortical representations in areas adjacent to the focal–ischemic area. Neurophysiological and neuroanatomical changes have been found in areas that are both adjacent and remote to the cortical ischemia (e.g., the contralateral cortex). In adjacent areas, the facilitation of long-term potentiation and synaptic plasticity occurs, as demonstrated by a sequential increase in markers for axonal sprouting and synaptogenesis. First, the expression of the growth cone marker growth-associated protein 43 and other growth-associated markers has been observed in rodents between 3 and 14 days post cortical infarction and was followed by increased expression of the synapse marker synaptophysin until 60 days postinfarction. Positive signals for axonal sprouting have been found to be sequentially replaced by negative factors such as Nogo, chondroitin sulfate proteoglycan, ephrin-A5, semaphorin-3A, and neuropilin-1 to balance the initial reaction. Most growth inhibitory genes have been found to be upregulated several weeks post infarction, which not only prevents aberrant connectivity, but also limits reorganization. Quantitative measurements of cortical connection patterns have demonstrated that new projections (e.g., from contralateral cortical areas to ipsilesional cortical areas and the striatum) are induced after stroke.

Studies of animals housed in enriched environments suggest the existence of a critical period of increased neural plasticity that extends from 14 to 30 days post stroke onset in rats. These data, along with the previously discussed data on genes upregulated after stroke and recent clinical findings, suggest that the brain opens a post stroke window of plastic modification during which plastic modifications are supported. In humans, this window may be larger, and many patients with stroke improve for months after the insult. However, late improvements might be predominantly due to behavioral compensation rather than neuronal rewiring.

Interestingly, synchronous low-frequency (0.1 to 2 Hz) electrophysiological activity has been observed in areas that are recruited for neuronal rewiring after the infarct, namely perilesional and contralateral cortical areas. This “regenerative,” synchronous neuronal network activity is particularly seen after stroke, but not after mechanical lesions, which suggests a specific post stroke plasticity. The suppression of synchronous cortical activity blocks axonal sprouting, which illustrates that these electrophysiological changes are indispensable for anatomic rewiring. Spontaneous activity in cortical circuits has a similar slow, rhythmic frequency during development, which suggests that developmental and regenerative mechanisms after stroke exhibit analogous features. Together, these data demonstrate that cortical ischemia produces growth-supportive alterations in functional neuronal network activity.

**Perspective I**

**Cell Therapy Must Generate Lesion-Specific Neuronal Phenotypes**

The anatomic and developmental data reviewed suggest that distinct brain regions consist of a defined composition of different neuronal cell types, which are specified during embryonic development. Restorative approaches after established stroke lesions should consider this diversity and specificity to allow for functional improvements. A general advantage of differentiating neural populations from immature precursors in vitro is the possibility of controlling the differentiation process through extrinsic factors and morphogens that largely recapitulate in vivo development. Until now, embryonic stem (ES) cells have been considered to be the most suitable source of stringently differentiated cell types with a phenotype-specific identity. Neural populations derived from murine and human ES cells have already been shown to differentiate into neurons after transplantation into adult animal stroke models. They have even shown mature electrophysiological activity and improved functional outcome. Recently, it was demonstrated that ES cell-derived neural populations could generate dorsolateral cortical phenotypes by antagonizing sonic hedgehog signaling. Significantly, even cortical layer-specific phenotypes were generated, including GABAergic and glutamatergic neurons. After transplantation into neonatal or embryonic mice cortices, these cells showed an area-specific projection into corresponding thalamic nuclei (M1 to ventrolateral thalamic nucleus and S1 to ventrobasal). Recapitulation of in vivo development has also been demonstrated in experiments that used self-organized, 3-dimensional, aggregate cultures of ES cell-derived neural populations. A cortex-like apicobasal polarization was observed in these aggregates, and the selective generation of rostral or caudal cortical tissue was possible. The next challenge is to generate neurons situated in retrogradely degenerated areas in vitro. To produce ES cell-derived thalamic neurons, factors of the midiencephalic organizer will need to be recruited. Striatal differentiation can be achieved by ventralizing rostrally differentiated ES cells with sonic hedgehog (Figure 3).
Given that induced pluripotent stem cells appear to circumvent the ethical problems and limitations of human ES cells at the same time as preserving their broad capability, they potentially represent a powerful future cell source. This cell type can be generated from adult human somatic cells (e.g., dermal fibroblasts) and offers the possibility of producing disease-specific, autologous, functional neural cell grafts; however, initial experiments have produced low efficiencies for induced pluripotent stem cell technology. Human induced pluripotent stem cells belong to a primed pluripotent state, which is characterized by low single-cell clonogenicity and stability in tissue culture; however, recent data have demonstrated that human induced pluripotent stem cells can be converted to a naïve pluripotent state, which offers single-cell clonogenicity similar to murine ES cells and is more stable than the primed pluripotent state. However, it remains to be seen whether induced pluripotent stem cell technology can overcome other problems such as acquired genetic modifications and aberrant reprogramming of DNA methylation.

During the past decade, a multitude of cell replacement studies have been performed in experimental stroke models, and some initial clinical trials have been conducted. Mesenchymal stem cells from the umbilical cord have induced some functional recovery after stroke. However, their general route of transdifferentiation into neural phenotypes in vitro does not seem to recapitulate functional and site-specific in vivo development, and their neuronal differentiation and local survival at the lesion site is poor. Accordingly, these cell types frequently have been transplanted intravenously, which provides increasing evidence that their main mode of action includes systemic immunomodulatory and neuroprotective mechanisms. Human fetal neural stem cells have been shown to differentiate into neuronal phenotypes and promote functional recovery after experimental stroke, but the exploitation and in vitro cultivation of adult human neural stem cells is particularly difficult. It remains to be seen whether adult human neural stem cell technology can produce a sufficient number of adequate neuronal cells.

**Perspective II**

**Tissue Engineering Could Help to Rebuild Functional Anatomy**

New cortical assemblies interface with existing assemblies in accordance with the original efferent projections (including projections to the thalamus). The feasibility of axonal growth from transplanted stem cell-derived neurons over long distances within the brain, specifically from cortical sites to deep-brain structures, has been shown in the growth-permissive brains of embryonic and postnatal animals. However, the adult brain is a more restrictive environment. First, the formation of an astroglial scar, which is common to different brain pathologies, inhibits axonal outgrowth through inhibitory extracellular matrix molecules such as chondroitin and keratan sulfate proteoglycans. Second, intrinsic transcription factors and extrinsic guidance cues used for correct neuronal positioning and patterning of macro- and microanatomical fiber connections as well as cellular features such as radial glia are expressed exclusively during development in a chronologically and spatially regulated manner.

For neuronal patterning after extended structural damage to be considered reasonably accurate, it must go beyond the simple transplantation of stem cell-derived populations into the ischemic core or penumbra. Neurons that are transplanted into a stroke cavity without any structural support do not receive sufficient signals from the damaged adult brain to know where to position their cell bodies or send their axons. Therefore, tissue engineering methods may be crucial for providing anatomic organization to stem cell-derived neurons that are otherwise randomly arranged after transplantation. Thus far, different kinds of biodegradable scaffolds have been...
shown to improve graft survival, and coating them with extracellular matrix molecules has improved neuronal differentiation and neurite outgrowth.\textsuperscript{60} Transplantation of human ES cell-derived neural populations that were seeded in Matrigel scaffolds has improved functional outcomes after stroke.\textsuperscript{61} Park and colleagues\textsuperscript{62} even cultured neural stem cells on whole complexes of synthetic, biodegradable, porous polymer scaffolds. After transplantation into mice, these complexes filled the infarct cavity with a new parenchyma-like structure. The neural stem cell–scaffold complex fixed transplanted cells in space, served as a permissive substrate for neurite growth, exhibited reciprocal graft-to-host neuronal connections (even to contralesional cortical sites), reduced mononuclear cell infiltration and astroglial scarring, and supported vascularization.

Future development of scaffolds might consider specific anatomic conditions.

Porous 3-dimensional scaffold structures could harbor and macroanatomically pattern site-specific cell bodies to provide stability and promote survival (Figure 4A–B). Longitudinal channel-like scaffold structures that arise from the porous compartments could act as guide rails to protect and escort axons (Figure 4A–B). Conceivably, the specific coating of longitudinal channels with growth-promoting molecules such as the neural cell adhesion molecule L1 could support significant axonal outgrowth.\textsuperscript{63} Developmental guidance cues such as ephrin and Eph genes should be implemented in an analogous fashion.\textsuperscript{56} To allow functional signal conduction, axonal projections must be myelinated. For this, ES cells could be effectively differentiated into myelinating oligodendrocytes (using a separate approach\textsuperscript{64}) and seeded into the channel-like scaffold structures independent of brain area-specific neuron populations. This neuromatrix interface could then be neurosurgically positioned into the lesioned hemi-
sphere (Figure 4B–C). The insertion of large matrices into the infarct cavity using open brain surgery could be acceptable in situations in which severe focal brain damage has already occurred, but gentle techniques for large scaffold insertion that would protect intact host tissue would need to be better developed. After developing this procedure, it would be necessary to determine whether endogenous cortical areas would form horizontal reciprocal connections with the introduced cortical neurons and whether endogenous thalamic afferents would innervate the introduced thalamic neurons. Conceivably, synaptic graft–host interactions would be supported simply by the spatial proximity of appropriate structures after introducing the neomatrix interface. Graft–host interactions might also be supported by brain-derived neurotrophic factor, for example, which could then also be coated onto the porous scaffolds.

**Perspective III**

**Synchronous Activity of Transplanted Neuronal Populations Might Support Their Functional Integration**

Data on post stroke plasticity show that there is a remarkable demand and competition for cortical territory.18 Clearly, the brain is capable of transferring important functions to new neuronal assemblies.65 Assuming that these neuronal assemblies are available through cell therapy, an important question arises: During what stage of differentiation should stem cell-derived neural cells be transplanted? On the one hand, immature cells might show better survival (compared with more mature cell populations) and exhibit better detection of instructive environmental cues. On the other hand, this might be true predominantly for transplantation into embryonic or young adult brains, which already show a high degree of developmental plasticity.66 Adult brains, even with their aforementioned post stroke neuroplasticity, show comparatively few plasticity processes because important germinative structures are present solely during embryonic development.

Furthermore (and perhaps more importantly), synchronous low-frequency neuronal activity of compensatory endogenous brain areas have been shown to be a prerequisite for proper post stroke axonal sprouting. During development, electrophysiological activity19 and the expression of functional glutamate receptors on newborn neurons67 have been shown to play pivotal roles in shaping cortical anatomy and regulating neuronal survival. Consequently, transplanted neuronal populations should also exhibit synchronous neuronal activity to support synapse formation and host–graft communication. Interestingly, we and others have demonstrated that ES cell-derived neuronal populations can be differentiated into functional neuronal networks that synchronously oscillate at low frequencies.68,69 A prerequisite for oscillation was the terminal maturation of a dense network of glutamatergic and GABAergic neurons within neural aggregates.68 Because this feature may be essential for supporting their synaptic integration after transplantation, terminal neuronal maturation of grafted cells in vivo may be a critical factor in achieving low-frequency oscillation. Immature neural precursor cells are known to terminally differentiate only partially, even months after engraftment.39,70 Instead, transplanting young β-tubulin-positive, postmitotic neurons has already been accomplished in some lesion paradigms,71,72 and it appears plausible that such specifically committed populations could be transplanted and would rapidly mature into synchronously oscillating neuronal populations to elicit early functional graft–host interactions.

**Perspective IV**

**The Time Window for Cell Therapy, Graft Survival, and Secondary Graft Effects**

Transplantation of cells into acute stroke lesions appears difficult because graft survival is often poor.39 This limitation may be due to secondary inflammatory processes during the first days and weeks after stroke39 and/or multiple other factors such as the missing functional integration67 of new neurons or insufficient trophic support.51 In contrast, experiments in rodents using marker genes for axonal sprouting or synaptic plasticity23,24 and the behavioral–rehabilitative influence of enriched environments31 support the idea that functional improvements due to neuroplasticity are confined to a time window of 1 to 4 weeks after stroke onset. Recent clinical data have also shown the benefits of early rehabilitation.32,33 Therefore, from a functional point of view, neuronal cell grafts should be transplanted during the initial weeks after stroke to be placed in a receptive, plastic environment. To improve graft survival during this time, a rapid functional integration of grafted neurons into host neuronal networks appears to be important83 and should be supported by transplanting more mature neurons (see previously)71 and by tissue engineering.63,73

Angiogenesis also must be supported. Stroke induces angiogenesis in penumbral regions of the human brain involving different aspects of precursor proliferation, migration, and tube formation. It depends on several signals such as vascular endothelial growth factor, vascular endothelial growth factor receptor 2, angiopoietins 1 and 2, and their receptor, Tie2.75,76 Therefore, biodegradable tissue scaffolds should be coated with angiogenesis-promoting substances to support development of a new vasculature. Additionally, human ES cells can be differentiated into endothelial and mural cells, which generate blood vessel structures in vitro and improve cerebral blood flow, vascular density, and behavioral recovery after experimental stroke.77 Stem cell-derived vascular precursors that are incorporated into the tissue scaffolds might further enhance the generation of blood vessels and, consequently, the survival of neuronal cell grafts.

Importantly, cell therapy also can exert secondary, homeostatic effects on host cells,78 including the recruitment of endogenous progenitors,79,80 the induction of some repair processes,81,82 immunomodulation,56,83 and neuroprotection84,85 (Figure 5). In addition to the effects possibly exerted through paracrine signaling of intracerebral cell grafts after stroke,84,85 the dominant mechanism for local neuroprotection and improved behavior seems to be a systemic neuromodulatory or neurohumoral effect after stem cell therapy.56,83 These secondary cell therapy-related mechanisms are important for host recovery, but also for graft survival and func-
tional integration in a self-regulating manner. In combination, this may even enhance therapeutic efficacy.

Conclusions
The functional replacement of damaged neuronal circuits after stroke represents a unique benefit of cellular therapy. Specific, individual stroke-related anatomic and functional changes must be implemented in cell replacement strategies to most appropriately direct cellular properties toward a receptive environment. The route to the stem cell-based and true neuronal-regenerative approach that we have delineated will need to be explored first in animal models of focal cerebral ischemia (eg, the medial cerebral artery occlusion model in rats’). Given that lesion size and remote changes can vary considerably, the primary ischemic lesion and its afferent and efferent connections must be evaluated through high-resolution MRI to specify individual target structures and the degenerated neuronal network. Then, site-specific neuronal phenotypes, balanced in neurotransmitter-specific numbers, must be generated in vitro from immature precursors and grown on individual lesion site-adapted, biodegradable matrices that provide structural and trophic support as well as anatomic organization for neuronal cell bodies and axons. Next, the anatomically organized neuronal assembly should be differentiated in vitro toward mature and electrophysiologically active stages before being introduced into the ischemic brain for functional integration. Finally, the time window for neuroplastic functional integration is limited, because it covers only the first weeks after stroke onset. The post stroke neuroplastic environment allows for the functional integration of new neural populations. Overall, functional neuronal cell therapy for stroke must combine multiple anatomic and functional features of the donated cellular components and the recipient brain tissue to enable restoration of neuronal functionality in the surviving neuronal networks.

Disclosures
None.

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細胞補充療法による脳卒中後の神経細胞機能回復
—解剖学的および機能的検討

Restoring Neuronal Function After Stroke by Cell Replacement
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脳卒中によって梗塞病変以外の部位にもさまざまな二次的変化が生じることを考えると、こうした問題の克服はより一層重要であると考えられる。

レビューの要約: 本稿では、脳卒中後の可塑性に関する現行の概念を用いて、細胞補充療法の候補となる脳構造および細胞の種類と、損傷した脳に新たな神経細胞機能をもたらすための方法について論じる。

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