REVIEW

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Enabling brain plasticity and neurological recovery in the ischemic brain: effect of age and vascular risk factors as confounders

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Abstract

Cerebral plasticity and neurological recovery can be stimulated in the ischemic brain by exogenous pharmacological and cell-based treatments. Neurons, neuroblasts and endothelial cells synergistically interact with each other as a regenerative triad, creating an environment in which neurological recovery takes place. Developmental genetic programs are reactivated. Brain neurons and capillary cells are enabled to sprout, and glial cells support plasticity processes. Until now, the large majority of studies were performed in young, otherwise healthy animals, which lack the risk factors and co-morbidities associated with human stroke. Recent behavioral, histochemical and molecular biological studies have shown that restorative brain responses may differ between young and old animals, and that they are also modulated by vascular risk factors, such as hyperlipidemia and diabetes, which are highly prevalent in ischemic stroke. We claim that age aspects, vascular risk factors and co-morbidities should more intensively be examined in future experimental studies. Confounding effects of age, risk factors and co-morbidities should carefully be considered in clinical proof-of-concept trials.

Keywords: neuronal plasticity, angiogenesis, ageing, hyperlipidemia, diabetes.

Introduction

Over more than two decades, therapeutic efforts in the stroke field have focused on the promotion of neuronal survival, which failed to succeed in clinical trials in humans until now [1, 2]. From experiences with neuroprotection therapies, it may be concluded that the stimulation of survival alone is without prospect, as long as no successful remodeling of brain tissue takes place. Indeed, studies during the last years have shown that profound remodeling processes take place in the brain subsequent to ischemic stroke [3, 4]. This has invigorated hopes that we may become able to promote stroke recovery by means of pharmacological or cell-based therapies. Promising results from experimental studies have led to clinical trials, the results of which are currently awaited.

Remodeling of ischemic brain tissue involves interactions between neurons, glial and microvascular cells that create a microenvironment, in which neurological recovery may ensue. Neurons and brain capillaries sprout. Neuronal outgrowth enables the formation of functional axons and synapses in the brain both over long (*e.g.*, along pyramidal tract; [5, 6]) and short (*e.g.*, within motor cortex; [7, 8]) distances, thus allowing for the restitution of neuronal networks that were damaged by the stroke event. In the process of brain remodeling, proliferating microvascular cells play a supportive role, enabling the migration of neural precursor cells and promoting the remodeling of neurons and glial cells *via* secretion of growth factors [3]. This rearrangement of cell-cell interactions is followed by the restitution of a functional blood-brain barrier, leading to the recuperation of brain homeostasis [9].

The interactions between neurons, glial cells and microvascular cells are finely tuned. They involve mutual cell communication via release of growth factors and physical cell-cell interactions across the extracellular matrix that is itself subjected to remodeling processes after stroke [10]. In view of the complexity of these systems, taking into consideration both the structural and functional heterogeneities of brain structures and the heterogeneities of ischemic strokes both regarding their size, etiology, and localization [4], the development of neurorestorative therapies is a true challenge. In recent years, increasing insights were obtained into factors complicating neurorestorative therapies. Importantly, it was noted that neurorestorative processes in the adult brain may substantially differ based on associated risk factors and comorbidities, and ageing processes also influences them. In this minireview, we highlight some of these confounding factors, pointing out ways how future study failures may be circumvented. As a matter of fact, minireviews cannot replace the need for more thorough literature assessments. For this purpose, the reader is referred to more detailed works of these authors, in which some of the here presented concepts have been presented in more detail [3, 4, 9].

Role of age for stroke development and recovery

Age is the principal nonmodifiable risk factor for stroke. The incidence of stroke increases significantly with age in both men and women, with half of all strokes occurring in people over 75 years, and one third in people over 85 years [11]. In addition, there is an age-dependent increase in conversion of ischemic tissue into infarction suggests that age is a biological marker for the variability in tissue outcome in acute human stroke [12]. Aging is associated with a decline of locomotor, sensory and cognitive performance in humans and animals. Neuroprotection studies in young animals have demonstrated the efficacy of a variety of pharmacological interventions. Yet, all strategies that have clinically been tested in larger trials failed to show benefits in aged humans. One possible explanation for this discrepancy between experimental and clinical studies may be the role that age plays in the recovery of the brain from stroke insults. In this light, the aged post-acute animal model is clinically most relevant to stroke rehabilitation, as recommended by the STAIR Committee and more recently by the *Stroke Progress Review Group* [13]. In aged rats, reversible focal cerebral ischemia stroke can be reliably induced by transient occlusion of the middle cerebral artery (MCA) using a hook attached to a micro-manipulator [14].

Benefits of neurorestorative therapies

Neurorestorative therapies can be instituted over extended time windows in the stroke recovery phase. In rodents, beneficial effects on neurological recovery were reported even when treatments were initiated days, weeks or months after stroke [5, 15–20]. In sharp contrast to neuroprotection, the efficacy of neurorestorative therapies does not depend on the successful tissue reperfusion. Thus, neurorestorative therapies are efficacious even under conditions of permanent focal cerebral ischemia [16, 18, 21–23]. In view of the long therapeutic window, considering that tissue reperfusion is not a requirement, neurorestorative therapies can potentially be of benefit for all stroke patients.

Neuronal plasticity

Subsequent to the anterograde Wallerian degeneration of injured axons, the surviving proximal axonal segments (*i.e.*, the axon stumps) reorganize along the infarct rim [23, 24] or grow out behind the site of injury [5, 25]. In the pyramidal tracts, the stroke event *per se* was shown to promote axonal sprouting both in ipsilateral [5, 25] and contralateral [8, 22, 25, 27] to the stroke. Endogenous responses are enhanced by pharmacological and cell-based restorative therapies, which promote the outgrowth of fibers originating from the contralesional motor cortex that traverse the midline in order to reach neurons denervated by the stroke [5, 16, 18, 21, 22, 25–28].

Importantly, axonal growth responses are relatively uniform for different types of treatment, namely growth factors (*e.g.*, erythropoietin, vascular endothelial growth factor [VEGF]) [5, 25, 27], neutralizing antibodies directed against the axonal growth inhibitor NogoA [18, 21], neurostimulants (*e.g.*, amphetamine) [28], and cell-based therapeutics, such as neural precursor cells (NPC) [16]. Contralesional plasticity as a basis for neurological recovery was observed in rats [16, 18, 21], mice [5, 22, 25] and macaque monkeys [29]. Stroke-related neuronal plasticity was evident not only in young, but also in aged animals [18, 30, 31]. Thus, evidence for axonal remodeling as a correlate of restorative therapies is solid in the experimental setting.

Neurogenesis

Stroke induces cell proliferation within the sub-ventricular zone, migration of newly born immature neurons into peri-infarct tissues and long-term survival and maturation into a small number of cells with a mature neuronal phenotype and ultrastructural evidence for synapses [32–37]. Post-stroke neurogenesis occurs in close association with the vasculature. Newly born immature neurons can be found associated with blood vessels after stroke [35, 36]. Xenotransplants of stem/progenitor cells also home to the ischemic tissue and associate with blood vessels after stroke [38, 39].

In peri-infarct cortex, newly born neurons migrate into the vicinity of the stroke lesion and form a tight physical association with blood vessels in the first week after stroke, in a neurovascular niche, in peri-infarct cortex. This vascular/neuroblast association occurs with blood vessels that are actively remodeling after stroke, and undergoing angiogenesis. Pharmacological blockade of angiogenesis after stroke significantly reduces the number of immature neurons that are present in peri-infarct cortex, by almost 90% [35].

Angiogenesis

Axonal sprouting and neurogenesis occur in common areas of peri-infarct tissue after stroke together with angiogenesis, forming a unique regenerative triad that supports neural repair. Angiogenic growth factors are released by neurons and glial cells that induce endothelial proliferation and sprouting [3, 40–42]. Acting on VEGF receptor-2 (VEGFR2) that is highly abundant at the tip of new-formed capillaries, VEGF induces endothelial proliferation, and concurrently upregulates the trans-membrane ligand Delta-like (Dll)-4, which signals to microvascular cells in the capillary stalk, downregulating VEGFR2 on the stalk cells *via* interaction with its receptor Notch-1, thus preventing uncontrolled capillary growth [43, 44]. This lateral inhibition, which is a key principle underlying structural development during ontogeny, is recapitulated following ischemia [45–47].

In adolescent mice, VEGF promotes angiogenesis in the ischemic brain [48], elevating regional cerebral blood flow (CBF) during subsequent ischemic episodes, thus stabilizing energy metabolism and preventing secondary brain infarction [49]. Interestingly, whereas angiogenesis is most pronounced near evolving brain infarcts [50, 51], axonal plasticity that is also induced by VEGF has been observed contralateral to the stroke following proximal, *i.e.*, intraluminal MCA occlusion [25, 27]. Hence, brain capillary sprouting and long-distance axonal plasticity may spatially dissociate from each other in the ischemic brain.

Glial cells

Besides controlling water shifts within the brain, astrocytes remove excitatory neurotransmitters (*e.g.*, glutamate) and electrolytes (*e.g.*, potassium) from the extracellular space, thus controlling neuronal excitability and plasticity [50]. Subsequent to ischemia, astrocytes secrete lipoproteins, namely apolipoprotein-E, into the perivascular space, thus inducing the ATP-binding cassette transporters, namely the drug efflux transporter ABCB1 [51, 52], contributing to the maintenance of brain homeostasis.

In addition to these roles in maintaining tissue micro-environments, astrocytes and oligodendrocytes release growth factors, proteases and proteoglycans into the extracellular space that specifically regulate neuronal outgrowth. Under physiological conditions, chondroitin sulfate proteoglycans released by astrocytes and oligo-dendrocytes create a growth-repulsive microenvironment for axons and dendrites [53, 54]. Upon focal cerebral ischemia, these proteoglycans are downregulated in areas exhibiting axonal growth at 2–3 weeks after the stroke [55]. Therapeutic interventions promoting axonal plasticity, such as VEGF, further decrease the proteoglycan levels [25, 56, 57].

Molecular signals associated with brain remodeling

Signals associated with brain development

In recovery from stroke, the adult CNS tissue re-activates pathways implicated in brain development. Most genes are persistently upregulated after stroke [58]. Some genes were transiently upregulated (10–14%) or upregulated in a delayed fashion (21–28%). A minority of genes were downregulated (2%). Several genes persistently upregulated both in young and old rats (*Aldh1a2*, *Cdk5rap2*, *Crabp2*, *Rac2*, *Rbp1*, *Mafb*, *Nr2f2*) are closely associated with brain development. Of note, during the acute stroke phase *Cdk5rap2* displayed twice the levels in young rats compared with aged rats, while *Mafb* revealed twice the levels in aged rats compared with young rats. Several genes implicated in extracellular matrix (ECM) and cytoskeleton remodelling (*Fbln1*, *Grn*, *Klk6*, *Ninj1*, *Ppgb*, *Tubb6*) were persistently upregulated, while *Vcan* was transiently upregulated. *Adam8* was transiently upregulated in young rats only, while *Ppgb* showed delayed upregulation in young but persistent upregulation in aged rats. Other genes that were up-regulated in both age groups were *Hbegf* and *Racgap1*. *Cebpb* and *Wnt6*, which play pivotal roles in CNS development and plasticity, were upregulated late in young rats only [58].

Signals associated with neurogenesis and neuronal plasticity

Many genes associated with neurogenesis, axonal, dendritic and synaptic plasticity were either persistently downregulated (47%) or showed delayed downregulation (12%) both in young and old rats [58]. Notably, many neurogenesis and brain plasticity-related genes (32%) displayed a delayed pattern of downregulation. However, a gene-by-gene analysis revealed many important age-related differences. Downregulated genes related to synaptic plasticity included *Arc*, *Neurod1*, *Neurod2*, *Nr4a1*, *Nr4a2*, *Rasl10b*, *Rnf39*, *Tpm3*, *Trim9*, *Ube2b* and *Ube3a*. Most of them (*Neurod1*, *Nr4a1*, *Nr4a2*, *Rasl10b*, *Trim9* and *Ube2b*) recovered by day 14 after stroke in young but not aged rats. In the latter group, these genes remained permanently downregulated or did not change at all. Some others were transiently down-regulated (*Neurod2*) or downregulated with delay (*Tmp3*, *Ube3a*) in aged rats only. Genes promoting axonal plasticity were also regulated. These included *Camk2n2*, *Cntn4*, *Ntng1*, *Sema6a*, *Sept2* and *Slitrk1*. Of these, *Cntn4* and *Ntng1* did not change in aged rats but were persistently downregulated in young rats, while *Camk2n2* and *Slitrk1* were transiently downregulated in both age groups. Very few genes (*Sema6a*, *Sept2*, *Ilr6a*) were moderately up-regulated in both age groups. *Ilr6a* is a gene expressed by neuronal precursor cells. *Pou3f1*, which is required for the maintenance of myelination, was downregulated in young but not aged rats. In the contralateral sensori-motor cortex, there were 12 genes (2.7%), which were upregulated and 6 (1.3%) which were downregulated at day 3 post stroke in young rats [14]. On day 14, the number of upregulated genes decreased from 12 to 3 (0.7%), while the number of downregulated genes did not change. In general, the contralateral sensorimotor cortex of the aged rats was transcriptionally inactive in post-ischemic rat brains, especially in the first week post-stroke. Compared to young rats on day 3, aged rats had six-fold fewer upregulated genes [14]. By day 14, the number of upregulated genes did not change, while the number of downregulated genes increased to 8 (1.8%). Nevertheless, aged rats showed a robust increase in the expression of cyclin-dependent kinase inhibitor 1B (Cdkn1b), which inhibits the activity of cyclin-CDK complexes and plays a role in cell cycle control [14].

Confounders for neurorestorative therapies

While the requirements for brain recovery have thoroughly been assessed in recent years, the over-whelming majority of studies published were conducted using otherwise healthy adolescent rats and mice, which potentially raises problems in a typical age-related disease, in which patients exhibit high loads of vascular risk factors and co-morbidities. Age itself, similarly as risk factors and vascular diseases have recently been shown to modulate restorative brain responses, with may result in the loss of therapeutic actions or even evoke detrimental actions that outweigh the therapeutic response. For obvious reasons, such observations have high impact for the translation of treatments to human patients, in which such bystander effects carefully need to be taken into account. In the following, observations made in aged animals and animals exhibiting vascular risk factors are summarized.

Compromised brain remodeling associated with age

Aged rats respond to plasticity-promoting therapies. Improved neurological recovery associated with preservation of pyramidal tract axons ipsilateral to the stroke and enhanced pyramidal tract sprouting contralateral to the

stroke were found in 25-month-old or 12-month-old ischemic rats treated with neutralizing NogoA antibodies [18], pharmacological compounds [31] or BMSC [30]. Although neurological recovery was successful, dendritic and synaptic plasticity of hippocampal CA3 and CA1 pyramidal and dentate gyrus granule cells were not influenced by NogoA antibodies in aged (24 months) rats, yet improvements in spatial memory were present [59].

Specific aspects related to aging were not only observed for neuronal sprouting, but also for neurogenesis and angiogenesis. The number of proliferating NPC in the SVZ and SGL were lower in the brain tissue of 15-month-old than 3-month-old rats, both under physiological and ischemic conditions [60]. Although the *de novo* generation of neurons in the ischemic striatum was very similar in both groups, neurogenesis was decreased in the dentate gyrus of 15-month-old rats when exposed to focal cerebral ischemia [60]. It has been proposed that decline in neurogenesis in old animals is related to a reduced expression of VEGF receptor-2 on the surface of NPC [61]. Although existing evidence is limited to a rather small number of studies, the preserved neurological recovery in old animals argues against specific age limits for neurorestorative treatments. Despite this, age aspects need to be controlled in clinical proof-of-concept studies.

Subtle alterations of growth factors, but intact brain remodeling associated with arterial hypertension

Stroke patients exhibit a high prevalence of vascular risk factors. Three out of four stroke patients suffer from arterial hypertension [62]. Half of the patients exhibit hypercholesterolemia, and one out of four patients is diabetic [62]. Experimental studies poorly mimic comorbidities, since experiments are mostly performed in animals that are otherwise healthy. The consequences of vascular risk factors for brain remodeling are incompletely understood. In spontaneously hypertensive rats, subtle reductions in brain concentrations of neurotrophic factors and their receptors (namely BDNF, neutrophins-3/4, TrkA, TrkB) have been noted in the dentate gyrus [63]. In focal cerebral ischemia, these alterations did not result in major disturbances of contralesional axonal sprouting in response to neutralizing NogoA antibody treatment, enabling neurological recovery in a way very similar to non-hypertensive rats [21]. This preserved response to NogoA antibodies suggests that growth factor abnormalities may not be clinically relevant. Whether this conclusion is true for conditions of prolonged arterial hypertension, which in humans causes cerebral microangiopathy, remains to be shown.

Compromised brain remodeling associated with hyperlipidemia

Hyperlipidemia reduces angiogenesis [64] and promotes blood-brain barrier permeability [65]. These vascular changes involve multiple players, namely reduced endothelial NO synthase (eNOS) activity, excessive lipid peroxidation, and overactivation of matrix metalloproteinases (MMP)-2 and -9, calpain-1/2 and the small RhoGTPase RhoA [64, 65]. In rats subjected to focal cerebral ischemia, vitamin B_3 administration, which elevates high-density lipoprotein (HDL) and thereby reduces serum cholesterol, increased angiogenesis, the expression of VEGF and angiopoietin-1, and enhanced the phosphorylation (*i.e.*, activation) of eNOS and the angiopoietin-1 receptor Tie2, thus improving neurological recovery [66]. After focal cerebral ischemia in rats, vitamin B_3 supplementation enhanced white matter remodeling in the peri-infarct tissue, increased BDNF and TrkB levels and downregulated Nogo receptor levels [67]. These observations suggest that the pathophysiological sequelae associated with hyperlipidemia are amenable to therapeutic interventions, and that lipid-modulating strategies may particularly be promising to achieve this purpose.

As long as hyperlipidemia persists, brain responses to restorative therapies are nonetheless compromised, as suggested by a recent study, in which the effects of a cholesterol-rich diet-induced hyperlipidemia on VEGF-induced cerebral angiogenesis, post-ischemic regional CBF and recovery of the cerebral energy state were analyzed [68]. In this study, impaired angiogenesis was noticed following VEGF treatment in hyperlipidemic mice. The impaired angiogenesis was associated with blunted regional CBF responses and secondary breakdown of the energy state, once animals were subsequently exposed to focal cerebral ischemia [68]. Subsequent *in vitro* studies showed that upon exposure to low density lipoproteins (LDL), which mediate most of the detrimental effects of hyperlipidemia, VEGF's receptor VEGFR2 was internalized by endothelial cells and degraded via the late endosome in a syntaxin-16-dependent way [69]. As a consequence of the LDL exposure, VEGFR2 phosphorylation and downstream signaling were compromised. These data questioned the concept of therapeutic angiogenesis, which aims at restoring blood flow in advanced stages of atherosclerosis. Patients with advanced intracranial atherosclerosis particularly often exhibit hyperlipidemia [70, 71].

Compromised brain remodeling associated with diabetes

That impaired glucose control has detrimental effects for plasticity-promoting therapies was recently shown in rats suffering from streptozotocin-induced type I diabetes. Paradoxically, delivery of bone marrow stromal cells did not improve neurological recovery in diabetic rats, but increased mortality, blood-brain barrier leakage and brain hemorrhage [72]. Besides, excessive angiogenesis was noted in diabetic rats receiving bone marrow stromal cells that were associated with cerebral arteriole narrowing and neointima formation inside the internal carotid artery [72]. In histochemical studies, increased macrophage accumulation was noted in blood vessels of diabetic rats that had been treated with bone marrow stromal cells [72]. These abnormalities were attributed to an increased angiogenin expression in the brain and brain-supplying arteries of diabetic rats. The authors suggest that BMSC treatment should not be considered in diabetic patients.

In contrast to bone marrow stromal cells, delivery of vitamin B_3 was shown to enhance neurological recovery in streptozotocin-induced type I diabetes [73]. In line with the improved recovery, enhanced sprouting of motor

cortical axons across the midline in direction to the lesions-sided motor cortex was noticed. These results show that vitamin B_3 may be more suitable for the promotion of brain remodeling than bone marrow stromal cells in diabetes.

Consequences for translation to human patients

Over a long period of time, the lack of potential confounders in young, otherwise healthy laboratory animals was regarded as desirable in stroke research. Reproducible models inducing uniform stroke lesions were developed in genetically inbred animal strains (mostly rodents) allowing insights into post-ischemic remodeling and neurological recovery processes even in small numbers of animals. With these models, the therapeutic potential of neurorestorative treatments was identified. The recent insights that responses to neurorestorative therapies may differ depending on age, risk factors and co-morbidities undoubtedly raised the need for evaluating recovery processes under conditions more closely resembling those in human patients. This knowledge increases the complexity of research on neurorestorative therapies. Future studies should carefully mimic such conditions in preparation for clinical trials, in order not to ensure that the translation from bench to bedside not again ends up in study failures. Thus, patients with certain age or risk factor profiles, for example very old subjects or subjects suffering from poorly controlled diabetes, should possibly be excluded from clinical trials. Risk factors should rigorously be treated based on existing therapeutic guidelines. The overriding message of this minireview, however, is a strong credo in the potential of neurorestorative therapies. A robust substrate for restorative processes is present in the majority of stroke patients, even the elderly. This endogenous recovery potential offers itself for therapeutic use.

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