

REGENERATION OF TISSUE AND FUNCTION AFTER CEREBRAL ISCHEMIA IN THE AGED RATS: NEW THERAPEUTIC STRATEGIES

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Abstract

Old age is associated with an enhanced susceptibility to stroke and poor recovery from brain injury. Therefore find therapeutic strategies aimed at improving functional recovery after brain ischemia in aged subjects is of considerable clinical interest. Recent results show that: (i) compared to young rats, middle aged rats develop a larger infarct area, as well as a necrotic zone characterized by a higher rate of cellular degeneration, and a larger number of apoptotic cells; (ii) in both old and young rats, the early intense proliferative activity following stroke leads to a precipitous formation of growth-inhibiting scar tissue, a phenomenon amplified by the persistent expression of neurotoxic factors; and (iii) the regenerative potential of the rat brain is largely preserved up to 20 months of age but gene expression temporally displaced, has a lower amplitude, and is sometimes of relatively short duration.

Keywords: stroke, aging, cytotogenesis, therapy, neurogenesis.

REGENERAREA TISULARĂ ȘI FUNCȚIONALĂ DUPĂ ISCHEMIA CEREBRALĂ LA ȘOBOLANI VÂRSTNICI: NOI STRATEGII TERAPEUTICE

Rezumat

Vârsta înaintată este asociată cu o susceptibilitate crescută la accidente vasculare cerebrale și la recuperare deficitară. De aceea este necesar să se găsească strategii terapeutice de recuperare funcțională după ischemia cerebrală la vârstnic. Rezultate recente arată că: (i) comparativ cu șobolanii tineri, cei de vârstă medie dezvoltă arie de infarct și de necroză mai mare, cu o rată crescută de degenerare celulară și de celule apoptotice; (ii) la șobolanii tineri și vârstnici, activitatea proliferativă intensă precoce după accidentul vascular cerebral conduce la formarea precipitată de țesut cicatricial, care inhibă creșterea, fenomen amplificat de expresia permanentă a factorilor neurotoxici; (iii) potențialul regenerativ al creierului de șobolan este preservat în bună parte până la vârsta de 20 de luni, dar expresia genică este temporal deplasată, are amplitudine scăzută și este de durată mai scurtă.

Cuvinte cheie: accident vascular cerebral, citogeneză, neurogeneză, îmbătrânire, terapie.

BRAIN ISCHEMIA IN AGED ANIMALS

Age-related brain injuries, including stroke, are a major cause of physical and mental disabilities. Therefore studying the basic mechanism underlying functional

recovery after brain stroke in middle aged subjected it is of considerable clinical interest.

Stroke models using aged animals are clinically more relevant than stroke models in young animals

Aging is associated with a decline of locomotor, sensory and cognitive performance in humans [1] and

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animals [2-4] part of which are due to age-related functional decline of the brain. Studies of ischemia in experimental animals have demonstrated the neuroprotective efficacy of a variety of interventions, but most of the strategies that have been clinically tested failed to show benefit 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 insult. Indeed, 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 [5].

Although it is well known that aging is a risk factor for stroke and Alzheimer's disease [6-9], the majority of experimental studies of stroke have been performed on young animals, and therefore may not fully replicate the effects of ischemia on neural tissue in aged subjects [10-13]. In this light, the aged post-acute animal model is clinically most relevant to stroke rehabilitation and dementia cellular studies, a recommendation done by the STAIR committee [14] and more recently by the Stroke Progress Review Group [15].

Stroke models for aged rats

Over the past 10 years suitable models for stroke in aged rats have been established. All are based on the middle cerebral artery occlusion (MCAO). MCAO has been produced with permanent [10,12,16-18] or transient occlusion for 30-120 min using a thrombus [10] through intraluminal filament occlusion [15,19,20] or a hook attached to a micromanipulator [11], or by occlusion of distal branches of the MCA [21], while long-term hypoxia-ischemia could also be induced by unilateral common carotid artery occlusion [22].

Selective MCA occlusion in aged rodents

The nearly universal approach of causing ischemia in young rats with filamentous models of occlusion fails to account for the unique physiology of an aged animal, as well as utilizing a method of ischemia that is facile and reproducible, but differs greatly from the classic cause of clinically relevant ischemia, a thrombus.

We have developed a method for improving the precision and accuracy of clot placement within the middle cerebral artery (MCA) of rats, utilizing a micro-catheter and laser Doppler flowmetry. This approach abandons the use of filamentous occlusion, and improves upon the methods of nonselective thrombus injection into the internal carotid artery. Selective injection of the thrombus reduces the size of clot needed to achieve stable occlusion with minimal failed embolizations and a low percentage of early recanalizations (both of which can easily be excluded from data analysis by utilizing strict criteria for ischemia and reperfusion determined with laser Doppler flowmetry, should 100% consistent ischemic strokes be

needed for study). Infarctions were consistent in both size and distribution within the MCA perfusion territory. Selective embolization in aged animals (n=10) resulted in substantially larger infarctions than those seen in aged animals (n=10) following non-selective embolization ($P<0.05$), or young animals (n=10) subjected to filamentous occlusion ($P<0.001$) (Fig. 1). Clots were localized to the MCA by direct examination at 0, 60 and 120 minutes post-embolization (n=14). All aged animals surviving 24 hours exhibited moderate to severe functional deficits, with selectively occluded animals having a higher mean score (selective=14, nonselective=9, suture=7) on the modified neurologic severity scale ($P=0.002$). Furthermore mortality rates of 33% and 37% were seen for the selective and non-selective ischemia, respectively, while 0% mortality was seen with suture occlusion. This model provides a highly reproducible method for embolization of the MCA and reliable reperfusion with rt-PA [23].

Aging alters BBB integrity in selective MCAO

We examined the effects of age on stroke progression and outcome by assaying the association between blood-brain barrier (BBB) disruption, neuronal damage, and functional recovery (Fig. 2). Using middle cerebral artery occlusion (MCAO), young (3 months) and aged (18 months) rats were assessed for BBB disruption at 20 min post-MCAO, and 24 h post-MCAO with tissue plasminogen activator induced reperfusion at 120 min. Results showed that BBB disruptions in aged rats occurred early and increased nearly two-fold at both the 20 min and 24 h time points when compared to young animals (Fig. 3). Neuronal damage in aged rats was increased two-fold as compared to young rats at 24 h, and differed qualitatively in the degree of inflammatory cell infiltration, hemorrhage and edema. No difference in neuronal damage was observed between young and aged rats at 20 min. Results indicate that aged rats suffer larger infarcts with increased BBB disruption, greater neuronal damage, and reduced functional recovery. Moreover, BBB disruption at 20 min post-MCAO in aged rats, in the absence of significant neuronal damage, suggests a potential mechanism by which alteration in BBB functional integrity during stroke contributes to a greater degree of subsequent neuronal injury [24].

Aged ischemic rats have higher mortality rates but not necessarily larger infarcts

Generally, the mortality rate in aged rats is higher than that of young rats in particular if the occlusion is done by an embolus (47% vs. 9%) [18]. The intraluminal filament method or photothrombosis cause, by comparison, lower (20-24%) post-stroke mortality rates [11,15,16,20].

In humans, there is no difference in infarct size with age [25,26]. Data on the infarct size in aged rats is contradictory suggesting that aged rats do not have necessarily larger infarcts. Some studies found an age-

related increase in cerebral infarct size [17,19,20], whereas others did not [11,18,27-29].

Aged ischemic animals recover more slowly than young animals and to a limited extent

Aged individuals recover less well from stroke [25] and rehabilitation aims at improving the physical and cognitive impairments and disabilities of patients with stroke. Therefore studies on behavioral recuperation after stroke in aged animals are necessary and welcome.

Various experimental settings have been used to assess the recovery of sensorimotor functions, spontaneous activity and memory after ischemia in aged rats [13,15,27,29]. Overall, the results indicate that aged rats have the capacity to recover behaviorally after cortical infarcts albeit to a lower extent than the young counterparts [12,13,15,20,27,29]. It should be kept in mind, however, that before stroke aged rats are already impaired as compared to young animals and showed significant decreased performance in some tests like spontaneous activity [27] and Morris water-maze [30]. A summarizing time course of recovery of aged vs. young rats is shown in Fig. 4. More specific, first, all rats had diminished performance on the first post-surgical day, part of which was attributable to the surgery itself (Fig. 4). Second, aged rats started recovery after a delay of 3-4 days, depending on the difficulty of the testing. Similar findings have been reported recently for post-stroke recovery of senescence-accelerated prone mice [31]. Third, the extent of recovery was also dependent on the complexity and difficulty of the test. For example, aged rats had difficulties in mastering complex tasks such as neurological status (that measures a complexity of motor, sensory, reflex, and balance outcome), rotarod or adhesive removal test (that is a measure of somatosensory dysfunction) and Morris water maze [18,27,30], but not simpler tasks such as foot-fault test score and corner test score. Fourth, the performance level in aged rats also depends on the infarct size, i.e. functional impairments in the group with the largest infarcts (20% tissue loss) were more severe than the functional impairments in the rats with 4% tissue loss [15].

Neurobiology of tissue recuperation after stroke in middle aged animals

Poor recovery may reflect the combination of more aggressive activation of factors leading to infarct progression (neuronal degeneration, apoptosis, phagocytosis), factors impeding tissue repair (astroglial scar, neurite inhibitory proteins) and neurotoxic factors.

At the same time, the response of factors promoting brain plasticity and growth may be less responsive. Growth promoting factors include growth-associated proteins, GAP43 and CAP23, the growth-promoting transcription factor c-jun, the growth-promoting cell guidance molecule L1 and the CDK5 inhibitor p21, microtubule-

associated proteins MAP1B and MAP2, immature neurons marker doublecortin and stem cell marker, nestin [32-35]. Pathogenesis of tissue damage is due mainly to inflammatory interactions involving cytokines, chemokines and leukocytes and neurotoxic factors like the C-terminal fragment of β -amyloid (A- β) [11,29,33,36-38]. One of the main findings is that both timing and magnitude of these factors is dis-regulated in the post-ischemic middle aged rat brain (Fig.4).

The regenerative potential of the brain appears to be competent up to 20 months of age

To explore the potential of older animals to initiate regenerative processes following cerebral ischemia, we studied the expression of the juvenile-specific cytoskeletal protein, microtubule associated protein 1B (MAP1B), the adult-specific protein, microtubule-associated protein 2 (MAP2), and the axonal growth marker, β III-tubulin in male Sprague-Dawley rats at 3 months and 20 months of age.

Focal cerebral ischemia, produced by reversible occlusion of the right middle cerebral artery, resulted in vigorous expression of both MAP1B penumbra of 3 month- (Fig. 5A) and, to a lesser extent, 20 month-old rats (Fig. 5B) at 14d following the stroke [32,33]. Similarly, MAP2 protein and mRNAs were upregulated in the periinfarcted area at almost the same levels both in young (Fig. 5C) and middle aged (Fig. 5D). Somewhat lower levels of expression were noted for the axonal growth marker, β III-tubulin, in the periinfarcted area of middle aged rats (Fig. 5F) as compared to young rats (Fig. 5E). Collectively, these results suggest that the regenerative potential of the brain at the structural level is competent up to 20 months of age.

Recent studies confirm that mechanisms for self-repair in the young brain also operate in the middle aged brain. For example, stroke causes increased numbers of new striatal neurons despite lower basal cell proliferation in the subventricular zone in the middle aged brain [39,40]. However, despite conserved proliferative activity in the subventricular zone, the number of neurons that reach the injury site is quite modest, as was shown recently for doublecortin-positive neurons in the infarcted area of middle aged rats [41]. One possible explanation is that lateral ventricle-derived nestin-positive cells do not pass the corpus callosum barrier, and therefore cannot contribute to generation of neurons in the neocortex. Indeed, current evidence indicates that the great majority of newly formed cells in the adult brain are non-neuronal [42-44].

Recent studies also indicate that the molecular profile of growth-promoting genes is very different between middle aged and young adult during the sprouting response to lesions to the CNS. Middle aged individuals activate most growth-promoting genes at later time-point following stroke than do young adults. This includes a

delayed induction of GAP43, CAP23 and the growth-promoting transcription factor c-jun. The growth-promoting cell guidance molecule L1 and the CDK5 inhibitor p21 are actually down-regulated during the axonal sprouting process in middle aged individuals compared with a robust and early upregulation of these two molecules in young adults [34,35].

Few neuroprotectants are effective in middle aged rodents

A major goal of clinical research is to limit the infarct size. One major line of investigation has involved the hypothesis that infarct size is determined by the degree of excitotoxicity. This line of reasoning is based on the observation that excessive concentrations of glutamate can lead to neuronal death.

The failure of multiple clinical trials to demonstrate any neuroprotective efficacy of several glutamate or N-methyl-D-aspartate (NMDA) receptor antagonists has led investigators to search for other potential causative mechanisms. Good candidates are antagonists to the N-methyl-D-aspartate (NMDA) receptor antagonists like MK-801 and the AMPA receptor antagonists, NBQX. However, both MK-801 and NBQX were found to be less effective neuroprotectants in middle aged than in young rats [45]. Nevertheless, a more recent study showed that treatment of middle aged rats with sildenafil, a phosphodiesterase type 5 inhibitor used to enhance cGMP-mediated relaxation of pulmonary vasculature, improves functional recovery following stroke in both young and middle aged rats. This treatment may exert its effects by promoting brain plasticity through enhancement of angiogenesis and synaptogenesis [20].

A more general method of neuroprotection that is efficacious in young rats is ischemic pre-conditioning. However, the degree of protection was reduced in middle aged rats as compared to young rats [46]. A likely explanation is that the brains of middle aged animals showed a reduced stress response that is likely to act neuroprotectively to stroke [19].

Neurosteroids have been recently shown to be effective as neuroprotective agents for ischemic stroke. Treatment with physiological concentrations of estradiol decreases ischemic injury by almost 50%, compared to sham-operated controls, in both young and aging rats [47,48]. It is possible that the protective function of estradiol in this model is the suppression of apoptosis in the infarct area, resulting in enhanced neuronal survival in the penumbral region of the infarct [47,48].

The use of stem cells to replace neurons lost after stroke potentially offers a novel approach to treatments aimed at improving recovery of tissue and function [49,50]. Such a treatment might utilize the endogenous reserves of stem cells located in the subventricular zone or the subgranular zone of the hippocampus. One major

concern, however, with any therapy designed to boost neurogenesis following stroke is that the capacity of the organism to produce new neurons may be diminished in the hippocampus and olfactory bulb of middle aged animals [39,51-56]. A countervailing cause for optimism is that a variety of treatments, such as environment enrichment [57], administration of growth factors [39,58] and induction of epileptic seizures [59] can increase the production of new neurons in middle aged animals, although at a lower level than in younger animals. Even more encouraging is a recent study demonstrating the same degree of neurogenesis in the striatum of old and young animals [40]. **Even though this study reported lower levels of neuron production by middle aged animals in the subgranular and subventricular zones, the report of equivalent levels in the striatum indicates that the potential for self-repair following stroke persists in the middle aged brain.** While the use of the organism's own stem cells has many advantages, this technique is in its infancy, and the field still awaits an unambiguous proof of principle.

Another experimental approach that has received considerably more attention is the use of external sources of stem cells. **One important question is the type of cells that should be used.** Both fetal [60] and murine stem cell lines [61,62] have been used successfully as grafts to improve functional deficits after experimental stroke in the rat. Adult stem cells, such as those derived from human umbilical cord blood, have also proven efficacious [63-66].

The appropriate route of stem cell administration must also be determined. One approach is transplantation either into the lesioned hemisphere, the contralateral hemisphere, or both. Other possible targets for stem cell administration are the striatum [40,62], the cortical parenchyma, or the cerebral ventricles [61]. **Following unilateral stroke, the grafted stem cells appear to be attracted both to the site of damage and to the corresponding contralateral region, suggesting the existence of both local repair processes and those involved in plastic changes in contralateral motor pathways [61].**

An additional second route of administration of stem cells is via the circulation, either intravenously [63,67-69] or by injection into the carotid artery [70]. The field of stroke therapy using stem cells is a new but promising area and it is hoped that studies to be carried out in the near future may validate a general therapeutic approach.

AXONAL SPROUTING AFTER STROKE IN AGED RATS

Stroke induces a novel pattern of ischemic damage and neural repair in the aged vs. young adult brain. Using small cortical stroke in the barrel field of the rat, we have shown that there is no difference in stroke size, degree of apoptotic cell death between young and aged animals. There is also no difference in the number of surviving neurons

in the ischemic border zones between aged and young adults. However, aged animals have greater oxidative DNA damage and a reduced expression of the neuroprotective protein HSP70 in peri-infarct cortex [28]. These two findings indicate that peri-infarct tissue in aged individuals have increased cell injury and reduced neuroprotective responses. Peri-infarct tissue may thus be more susceptible to factors in stroke that lead to increased stress or cell injury, such as fever, changes in blood pressure or alterations in blood vessel collaterals. This vulnerability in peri-infarct tissue may explain the controversy between different experimental stroke studies, with some stroke studies finding greater stroke size in aged individuals and others finding no difference in stroke size between young and adult and aged animals. Minor variations in experimental technique between different stroke models, strains of animals or within laboratories may lead to changes in the stress and extent of cell death within peri-infarct cortex.

On a cellular level there are at least two main processes of neural repair after stroke: post-stroke axonal sprouting and neurogenesis. Both processes contribute to neuronal reorganization in peri-infarct cortex. Axonal sprouting after stroke not only occurs within peri-infarct cortex [71] and between cortical hemispheres on the side of the infarct [72] but also from cortex contralateral to the infarct, into peri-infarct cortex, ipsilateral striatum, red nucleus and cervical spinal cord [73-76]. **Axonal sprouting** after stroke requires a molecular growth program [77-79] in which a neuron must respond to injury, then elaborate a growth cone, extend an axon and establish new synapses. This type of molecular growth program involves cytoskeletal reorganizing proteins, guidance and cell adhesion molecules, intracellular growth cone signaling molecules and specific transcription factors. Our studies indicate that the molecular profile of growth-promoting genes is very different between aged and young adult during the sprouting response. Aged individuals activate most growth-promoting genes at a greater delay after stroke than do young adults. This includes a delayed induction of GAP43, CAP23 and the growth-promoting transcription factor c-jun. The growth-promoting cell guidance molecule L1 and the CDK5 inhibitor p21 are actually down-regulated during the axonal sprouting process in aged individuals compared with a robust and early upregulation of these two molecules in young adults [34,35]. The environment of peri-infarct cortex also contains molecular systems that exert a powerful negative effect on axonal sprouting [80].

Among these systems, the aged brain has a unique upregulation of two molecules compared with the young adult: ephrin-A5 and myelin associated glycoprotein (MAG) [34]. These two molecules are important for axonal sprouting in cortical development or for the mediation of the switch in growth-inhibition during post-natal maturation. The unique upregulation of ephrin-A5 and MAG in the aged peri-infarct cortex suggest that these molecules may in part

mediate the age-associated decrease in axonal sprouting, and provide potential targets for neural repair after stroke.

NEUROGENESIS AFTER STROKE IN AGED RATS

Stroke induces cell proliferation within the subventricular 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 [81-85]. **Post-stroke neurogenesis appears to** divert migratory immature neurons from their normal path to the olfactory bulb, the rostral migratory stream (RMS) [84]. Recent research has begun to describe the cellular environments that may lead to post-stroke neurogenesis and immature neuron migration. In the ischemic striatum, immature neurons, identified through their staining for the microtubule-associated protein doublecortin, are found in association with astrocytes. Activated astrocytes in the ischemic striatum secrete stromal-derived factor-1 (SDF-1) and this induces immature neuron migration into this area [86]. SDF-1 induces neuronal migration during development in the hippocampus, cortex and cerebellum [87-89].

Post-stroke neurogenesis also occurs in close association with the vasculature. Newly born immature neurons can be found associated with blood vessels after stroke [84,85,90]. Xenotransplants of stem/progenitor cells also home to the ischemic tissue and associate with blood vessels after stroke [91,92]. In peri-infarct cortex, newly born neurons migrate into the region near the stroke site 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% [84]. Thus angiogenesis is causally linked to neurogenesis after stroke. This finding of a neurovascular niche for neurogenesis after stroke is supported by the many growth factors or pharmacological agents that appear capable of inducing both of these processes together, such as VEGF, erythropoietin, FGF2, statins and phosphodiesterase type 5 inhibitors [93-96].

Angiogenesis, neurogenesis and axonal sprouting occur in common areas of peri-infarct tissue after stroke and may form a unique regenerative triad that supports neural repair in this disease. Studies in stroke have defined specific receptor-ligand signaling systems that link angiogenesis and neurogenesis. As noted above, blocking angiogenesis severely reduces post-stroke neurogenesis. These angiogenic blood vessels in peri-infarct cortex secrete SDF-1 and angiopoietin-1 in the first week after stroke (Fig. 6). Administration of SDF-1 or ang-1 stimulates

neuroblast migration into peri-infarct cortex, and blockade of their receptors, CXCR4 and Tie2, blocks or disperses the migration of immature neurons after stroke [84]. This work identifies two intercellular signaling systems that mediate post-stroke neurogenesis within a neurovascular niche in peri-infarct cortex (Fig. 7). Erythropoietin (EPO) and VEGF are also in a position to mediate a neurovascular coupling of angiogenic blood vessels and migrating neuroblasts. EPO is induced in blood vessels and astrocytes in peri-infarct tissue after stroke [97]. This endogenous increase in post-stroke EPO production promotes post-stroke neurogenesis [94]. Pharmacological doses of EPO also promote angiogenesis and neurogenesis after stroke [93]. VEGF is induced in peri-infarct tissue after stroke and may be secreted by angiogenic blood vessels. VEGF receptor blockade downregulates post-stroke neurogenesis and exogenous VEGF promotes post-stroke neurogenesis [98]. VEGF is also produced by neurons and astrocytes in peri-infarct cortex [93,98] and is strongly bound to the extracellular matrix, so the exact cellular communication pattern within the VEGF system in stroke remains to be determined.

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