SUMMARY OF DOCTORAL THESIS

Aged nervous system response to epileptic seizure and stroke.

Immunohistochemical, cellular and molecular analysis.

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Introduction

Old age is associated with an enhanced susceptibility to stroke and poor recovery from brain injury, but the cellular processes underlying these phenomena are uncertain. Therefore studying the basic mechanism underlying functional recovery after brain ischemia in aged subjected it is of considerable clinical interest. Potential mechanisms include neuroinflammation, changes in brain plasticity-promoting factors, unregulated expression of neurotoxic factors, or differences in the generation of scar tissue that impedes the formation of new axons and blood vessels in the infarcted region. Available data indicate that behaviorally, aged rats were more severely impaired by ischemia than were young rats, and they also showed diminished functional recovery. Further, as compared to young rats, 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. 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. Reduced transcriptional activity in the healthy, contralateral hemisphere in conjunction with an early upregulation of DNA damage related genes and the early induction of proapoptotic genes in the periinfarct area of aged rats are likely to account for poor neurorehabilitation after stroke in aged rats. Finally, the regenerative potential of the rat brain is largely preserved up to 20 months of age but gene expression is temporally displaced, has lower amplitude, and is sometimes of relatively short duration. Most interestingly, it has recently been shown that the human brain can respond to stroke with increased progenitor proliferation in aged patients opening the possibilities to utilize this intrinsic attempt for neuroregeneration of the human brain as a potential therapy for stroke. Given the heterogeneity of stroke, a universal anti-inflammatory solution may be a distant prospect, but probably neuroprotective drug cocktails targeting inflammatory pathways in combination with thrombolysis may be a possibility for acute stroke treatment in the future.

Epilepsy is, also, the most frequent neurodegenerative disease after stroke (but the exact etiology of seizure development is unknown With continued debate over the
functional significance of adult neurogenesis, identifying an *in vivo* correlate of neurogenesis has become an important goal*. Neurogenesis persists throughout life in the adult mammalian dentate gyrus and is influenced by seizure activity. Kindled seizures are widely used to model epileptogenesis, but the molecular mechanisms underlying the attainment of kindling status are largely unknown. Previously we showed that achievement of kindling status in the Sprague-Dawley rat is associated with a critical developmental interval of 25 ± 1 days which is coincident with the time required for maturation of newly born neurons. Subsequent studies on neurogenesis in the adult hippocampus have revealed that at 3 weeks the newly generated neurons have an intrinsic excitability (Tashiro et al., Nature 2006). Therefore we hypothesized that after 25 days following stimulation of neurogenesis the brain is highly susceptible toward further stimulation by PTZ (pentylenetetrazole). By quantitative immunohistochemistry and confocal 3D-image reconstruction analysis using tissue from this new model, we now report that convulsive seizure leads first to a non-specific, transient increase in the number of proliferative cells that are proliferating cell nuclear antigen-, BrdU- and doublecortin-immunopositive. However, repeated convulsive seizures with a periodicity of 25 days led to an enrichment of newly generated neurons, which were BrdU-positive in the dentate gyrus and temporal cortex at day 25 post-seizure. At the same time, there is a massive increase in the number of neurons expressing the migratory marker, doublecortin, at the boundary between the granule cell layer and the polymorphic layer in the dorsal hippocampus and at the lateral ventricle-corpus callosum boundary, some of which are positive for BrdU. By gene profiling and real-time PCR we show that a number of neurogenesis-related genes are downregulated in the hippocampus of kindled rats. Moreover, drugs known to enhance neurogenesis like L-NAME increased the seizure susceptibility at 25 days whereas drugs known to inhibit neurogenesis like temozolomide, reduced the seizure susceptibility at at the critical interval. Paradoxically, prolonged temozolomide treatment increased seizure susceptibility during the critical period by increasing the number of abnormal neurons suggesting that both increased neurogenesis and abnormal neurogenesis may lead to increased seizure susceptibility. This model may be can be used as an in vivo correlate of neurogenesis to study basic questions related to neurogenesis and neurogenic mechanisms of epilepsy.
Materials and methods

Animals

Eighteen hours prior to surgery, male 3-month-old (young) and 18-month-old (aged) Sprague-Dawley rats were fasted but allowed free access to water to minimize variability in ischemic damage that can result from varying plasma glucose levels. MCAO was performed as described below. Survival times for the study were: 3 days (n=8 young; n=7 aged rats) and 14 days (n= 10 for young; n=10 for the aged rats).

All experiments were approved by the University Animal Experimentation Ethics Board as meeting the ethical requirements of the German National Act on the Use of Experimental Animals.

Reversible occlusion of the middle cerebral artery

Blood flow through the middle cerebral artery was transiently interrupted in deeply anesthetized rats as previously described. The right middle cerebral artery was slowly lifted with a tungsten hook attached to a micromanipulator until blood flow through the vessel was completely stopped for 90 minutes. Both common carotid arteries were then occluded by tightening prepositioned thread loops. After 90 minutes, the middle cerebral artery and the common carotid arteries were re-opened, allowing full reperfusion of the brain.

Administration of pentylenetetrazole (PTZ) and Temozolomide (TMZ)

In order to investigate our hypothesis that extent of neurogenesis suppression in repetitive seizure can reduce the severity of crises, the rats from this group, were treated i.p. with a subconvulsant dose of PTZ at a concentration of 30 mg/kg on day 0, 25 and 50. Immediately after crisis, all rats was subjected to temozolomide treatment (25 mg/kg per day, i.g.) for 7 days following first and second PTZ injection. The animals were sacrificed at day 25 after the last PTZ injection (after 75 days).

RNA isolation

Total RNA was isolated from the microdissected tissue using TRIZol reagent (Invitrogen life technologies, Germany) as described by the manufacturer, followed by DNase 1 (Ambion) digestion and further purified using RNeasy Mini extraction kit (Qiagen, Hilden, Germany).
cDNA Array Assay

To analyze gene regulation, we employed commercially available defined oligo microarrays containing known rat genes arranged in three categories: stem cells hypoxia signalling pathway, and apoptosis cDNA microarrays. These arrays contain 258, 96, and 96 known genes respectively (SuperArray, Bethesda, MD) and were processed according to the manufacturer’s instructions.

Real Time Semi-Quantitative PCR

For real-time PCR, 2µg of total RNA was reverse-transcribed using random hexamers and the reverse transcription reagents (Superarray, Bethesda, MD). PCR reaction was set up by mixing 10ng of cDNA, rat primers (Superarray), Master mix (Superarray), and SYBR Green I (Molecular Probes).

EEG recordings

Qualitative EEG record analysis was performed by visual examination of the record with attention paid to generalized and intermittent focal epileptic activities. Epileptic activities were distinguished by non-epileptic activities based on change in waveform morphology, amplitude, and frequency and the associated absence or presence of behavioral change (according with Racine scale).

BrdU/DCX labeling

Neuronal phenotype: Sections were double-immunolabeled with guinea pig anti-DCX antibodies (1:4000, Chemicon, PA) and rat anti-BrdU antibodies (1:2000; Serotec, UK). The antigen-antibody complexes were visualized with donkey anti-guinea pig Cy2-conjugated antibodies (1:2000) and donkey anti-rat Rhodamine-conjugated antibodies (1:3000), respectively.

Microscopy

For light microscopy, a Nikon Eclipse microscope (Duesseldorf, Germany) was used. Images (768 x 1024 pixels) were captured electronically using a CCD camera (Optronics). The digital images were arranged and labeled using Adobe Photoshop, and printed with a Kodak XLS 8000 digital printer. For a group of micrographs, the camera settings for exposure, gain, and contrast enhancement were the same. Confocal analysis of sections was performed using a Nikon Eclipse microscope equipped with a laser device from Visitech (Munich, Germany). 3-dimensional reconstruction of overlapping
antigens was achieved by taking a sequence of confocal images that were spaced 0.1 µm apart across a 25 µm-thick section. The resulting images were loaded into the 3-D analysis software (Leika, Germany).

**Results**

*Basal transcriptional activities in the cortex of sham-operated young and aged animals.*

Since baseline differences between gene expression in young and old control rats might affect levels found after infarction, we first summarize the principal findings in control animals. Changes in the mRNAs levels for two major enzymes responsible for reactive oxygen species (ROS) scavenging, catalase (CAT) and superoxide dismutase (SOD) were significantly decreased in the brains of aged rats (Table 1), which is indicative of a reduced capacity to remove radicals from the aging brain. Fatty acid-binding protein 7 mRNA, was also increased in the aged rat brain.

**Differentially regulated genes in the post-ischemic rat brain**

We found sixty one genes (13.8%) that were differentially regulated in the post-ischemic rat brain (Fig.1). Of these, 28 genes (6.1% of the total number of genes) were upregulated. Within the upregulated genes, 11 genes were increased in both age groups, while 9 were upregulated only in the young rats and 8 only in the old. Twenty genes representing 4.5% of the regulated genes were downregulated. Thirteen genes showed both up and down regulation in the two age groups. The cumulative number of genes that were up- or down-regulated 1.5-fold for each time point is shown for the ipsilateral (periinfarct, pi) sensorimotor cortex and the contralateral (cl) sensorimotor cortex. From these data, it can be inferred that at day 3 post-stroke the major age-specific transcriptional effects in the periinfarcted area were (i) differential regulation (both up- and downregulation) of apoptotic genes, and (ii) a 50% decrease in the number of regulated stem cell-related genes. At day 3 post ischemia, the contralateral, healthy hemisphere of young rats is much more active, at transcriptional level, than that of the aged rats, especially at the level of stem cell-coding genes. At this time point, age-specific transcriptional events were (i) absence of regulation of hypoxia signaling-related genes, and (ii) downregulation of apoptosis-related genes. At day 14 post-stroke we noted
a persistent downregulation of stem-cell related genes in the contralateral (healthy) sensorimotor cortex of aged rats.

**Verification of microarray data by real-time semi-quantitative PCR**

To confirm the microarray data, real-time quantitative PCR was performed. We randomly selected bone morphogenetic protein receptor type 2, brain fatty acid binding protein, cadherin 5, fibroblast growth factor 1 receptor, inhibin beta, insulin-like growth factor 1 receptor, integrin beta 5 and intercellular adhesion molecular 5 from the stem cell related cDNA array. We also selected chromogranin A, uncoupling protein 2, glutathione peroxidase1, superoxide dismutase2, catalase, growth associated protein 43, tubulin alpha1, vascular endothelial growth factor A from hypoxia signaling pathway. In addition, we chose caspase 7 and growth arrest and DNA-damage-inducible 45 alpha from the apoptosis array. The expression pattern of these genes from real-time PCR matched well with the expression profile obtained by microarray analysis.

**Seizure susceptibility of kindled animals is associated with selective cytogenesis in brain**

In kindled rats, by 25 days post-seizure, the BrdU-positive cells became restricted to the temporal neocortex and hippocampus. Many positive cells were detected in the leptomeninges, and these are, most-likely, circulation-derived BrdU-labeled cells that enter the brain via leptomeningeal blood vessels; many of these cells are still in a mitosis-like state.

**Temozolomide treatment significantly decreases the susceptibility of seizure by inducing a dose- and time-dependent depletion of DCX positive**

Our results showed that temozolomide treatment for 7 days resulted in a significant decrease in the number of fully seized animals (grade 4 and 5 on Racine scale) after the second PTZ subconvulsive dose on day 25. Interestingly, the number of fully seized animals were increased after third PTZ in temozolomide treated animals compare with control group.

**Decreased rate of neurogenesis correlates with a decreased number of DCX positive cells**

Analysis of neurogenesis status assessed through cells expressing doublecortin (DCX, a marker of newly generated neurons) revealed that the hippocampal neurogenesis declined
substantially after second PTZ injection in temozolomide treated group compare with control group.

Dramatically decrease of hippocampal neurogenesis associated with neurodegeneration lead to increase the number and severity of crisis

In contrast after third PTZ injection, temozolomide treated animals had, unexpectedly, not significant difference in number of DCX positive cells in comparison with control group but the number and the severity of crisis was higher. Florojde stainind showed the presence of degenerated neurons was correlated with this events.

Conclusions

Aged rats recover poorly after unilateral stroke, whereas young rats recover readily, possibly with the help from the contralateral, healthy hemisphere. In this study we asked whether anomalous, age-related changes in the transcriptional activity in the brains of aged rats could be one underlying factor contributing to reduced functional recovery. We analyzed gene expression in the periinfarct and contralateral areas of 3 mo- and 18 mo-old Sprague Dawley rats. Our experimental endpoints were cDNA arrays containing genes related to hypoxia signalling, DNA damage and apoptosis, cellular response to injury, axonal damage and re-growth, cell lineage differentiation, dendritogenesis and neurogenesis. The major transcriptional events observed were:

(i) Early upregulation of DNA-damage and downregulation of anti-apoptosis-related genes in the periinfarct region of aged rats after stroke; (ii) Impaired neurogenesis in the periinfarct area, especially in aged rats; (iii) Impaired neurogenesis in the contralateral (unlesioned) hemisphere of both young and aged rats at all times after stroke; (iv) Marked upregulation, in aged rats, of genes associated with inflammation and scar formation.

These results were confirmed with quantitative real-time PCR. We conclude that reduced transcriptional activity in the healthy, contralateral hemisphere of aged rats in conjunction with an early upregulation of DNA damage-related genes and proapoptotic genes and downregulation of axono- and neurogenesis in the periinfarct area are likely to account for poor neurorehabilitation after stroke in old rats. Periodic stimulation of the brain by seizure-evoking pharmacological manipulations, performed within the developmental range of hippocampal neurogenesis, enlarges the population of newly
generated migratory neurons in the subgranular zone of the dentate gyrus. Since such neurons can contribute to the formation of new, recurrent excitatory circuits within the hippocampal formation, this neurogenesis may lower the threshold for seizure activity upon subsequent stimulation. Most importantly, both neurogenesis and neuronal degeneration contribute to the seizure susceptibility.

Our findings indicate that the aged brain has the capability to mount a cytoproliferative response to injury, but the timing of the cellular and genetic response to cerebral insult is accelerated in aged animals, thereby further compromising functional recovery. Elucidating the molecular basis for this phenomenon in the aging brain could yield novel approaches like long-term hypothermia, anti DNA damage, anti apoptotic or anti-inflammatory drugs treatment to neurorestoration in the elderly.
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Research activities

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2007- 2008 Automatisiertes immunhistologisches Analyse-Gerät (research assistant);

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**Prizes:**

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2009 *VentureCup-MV Ideenwettbewerb des Landes M-V*: Development of immunohistochemistry automatic analyzer for human and animal tissue;

2009 *National Romanian Society of Physiology*. Prize for the best oral communication of research results.

**Published books and papers**

**Books:**


**Papers:**


**Abstract:**


Conferences:


- 5-th International Symposium: Neuroprotection and Neurorepair: Cerebral Ischemia and Stroke. Magdeburg, Germany, 2008;

- 9th Congress of European Society for Clinical Neuropharmacology, Wien, Austria, 2009;

- 19th Meeting of the European Neurological Society (ENS) June 20-24, 2009; Milan, Italy;