

RESEARCH REPORT 2000/2001

FAN GMBH AND IFN PROJECT GROUP NEUROPHARMACOLOGY

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Summary

The main topics of the Project Group Neuropharmacology in close collaboration with the FANgGmbH are "cerebral ischemia" and "phenomena of brain plasticity" investigated both under physiological and under pathophysiological conditions.

For this aim we use different approaches. The method of extracellular field potential recordings in both acutely isolated and cultured hippocampal slices allow us to test potential neuroprotective drugs after oxygen/glucose-deprivation (OGD) *in vitro*. With different Ca²⁺ sensitive dyes we try to elucidate mechanisms of hypoxic/hypoglycemic cell death in cultured hippocampal slices. This preparation also enables us to study mechanisms of regeneration after ischemic and traumatic injury, e.g. by proliferation of endogenous stem cells. With different approaches *in vivo* we investigate the underlying mechanisms of neuronal death after ischemia, we test new neuroprotective strategies targeting at ion transporters and proteases, and we elucidate the possibilities of CNS regeneration after an ischemic insult.

In order to study mechanisms of learning and memory we use the model of long-term potentiation (LTP) in acutely isolated hippocampal slices. These experiments are partially performed in combination with measuring intracellular, dendritic Ca²⁺ concentration changes during LTP-induction with different fluorescent dyes by confocal laser scan microscopy, thereby characterizing which Ca²⁺ sources might be involved in LTP.

Zusammenfassung

Schwerpunktt Themen der Projektgruppe Neuropharmakologie in enger Zusammenarbeit mit dem Forschungsinstitut Angewandte Neurowissenschaften gGmbH sind die „Zerebrale Ischämie“ und „Phänomene der Hirnplastizität“ unter physiologischen und pathophysiologischen Bedingungen.

Für diese Fragestellungen nutzen wir unterschiedlichste Vorgehensweisen. Die Registrierung extrazellulärer Feldpotentiale von sowohl akuten Hirnschnitten als auch Schnittkulturen ermöglicht uns die Prüfung neuroprotektiver Wirkstoffe nach einer Hypoxie/Hypoglykämie *in vitro*. Mit verschiedenen Ca-sensitiven Farbstoffen versuchen wir am Modell der Hypoxie/Hypoglykämie an Hippokampus-Schnittkulturen Mechanismen des Schlaganfall-

bedingten Zelltodes aufzuklären. Dieses Präparat ermöglicht uns auch Mechanismen der Regeneration, z.B. durch Proliferation endogener Stammzellen, nach ischämischen und traumatischen Verletzungen aufzuklären. Wir untersuchen die dem Zelltod zugrundeliegenden Mechanismen nach Ischämie auch mit verschiedenen *in vivo*-Ansätzen, u.a. prüfen wir neuartige neuroprotektive und neuroregenerative Strategien durch Beeinflussung von Iontentransportern und Proteasen.

Zur Untersuchung der Mechanismen von Lernen und Gedächtnis nutzen wir das Modell der Langzeitpotenzierung (LTP) an akut isolierten Hippokampuschnitten. Bei diesen Versuchen wird die Änderung der intrazellulären, dendritischen Ca^{2+} Konzentration bei LTP-Induktion mittels schneller konfokaler Laser-Scanmikroskopie hinsichtlich der beteiligten Ca^{2+} -Quellen untersucht.

1. Characterization of tetanus-induced dendritic Ca^{2+} waves in hippocampal CA1 neurons

T. Jäger

An activity-dependent intracellular Ca^{2+} increase represents a key signal for the activation of mechanisms involved in synaptic long-term plasticity. We found that local 100 Hz stimulations of the Schaffer collateral fibres, usually used for the induction of synaptic long-term potentiation, elicits an intradendritic Ca^{2+} rise that propagates as a wave within dendrites of hippocampal CA1-neurons (Fig. 1). These waves are detectable by unmasking a biphasic Ca^{2+} signal due to inhibition of action potential generation. The second component of the biphasic Ca^{2+} signal, propagates within dendritic structures at short distances (Jäger et al., 2001). Pharmacological investigations with the metabotropic glutamate receptor (mGluR) group I receptor antagonist, 4-CPG, elucidated the involvement of mGluR-mediated Ca^{2+} release in the generation of the Ca^{2+} wave. With an increase in the concentration of NMDA-receptor antagonists during the wash-in period, the first component decreased gradually whereas the second was completely inhibited at a threshold. Drugs affecting the functionality of internal Ca^{2+} stores, like ryanodine and cyclopiazonic acid, have only minor effects on the activity-dependent evoked Ca^{2+} signal. These substances cause a broadening of the post-tetanic Ca^{2+} signal components and an increase of the decay time constants.

Our results point to the coincidental involvement of NMDA-receptor- and mGluR-mediated Ca^{2+} increase for the generation of the synaptically-driven Ca^{2+} waves.

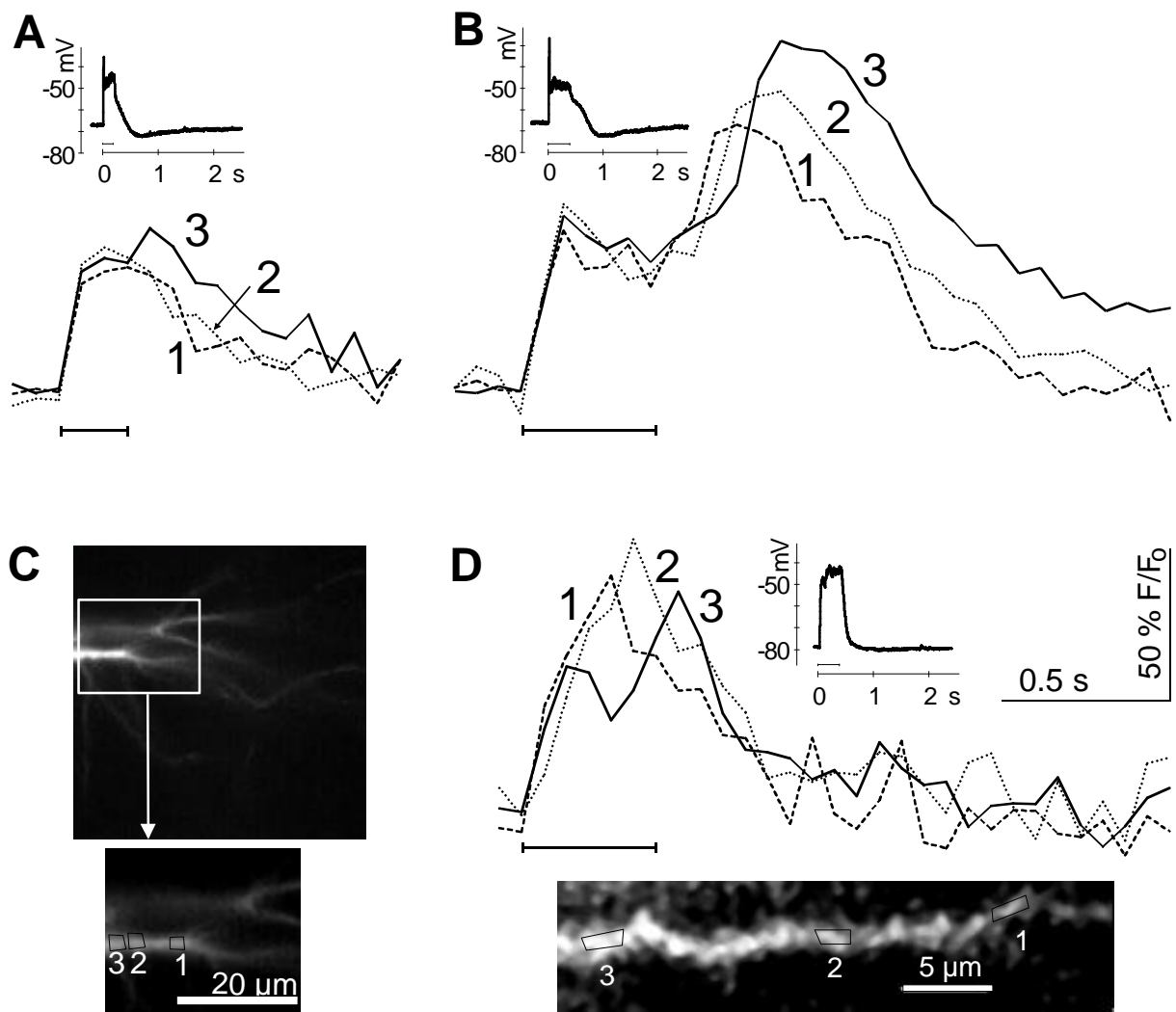


Fig. 1: (A) Induction of intradendritic Ca^{2+} waves in dendrites of hippocampal CA1 neurons. A 200 ms tetanization (100 Hz, PW 0.2 ms) evokes a fast increase in $[\text{Ca}^{2+}]_i$, but fails to elicit a second slow component of the Ca^{2+} transients. (B) Whereas, a 400 ms tetanization (100 Hz, PW 0.3 ms) elicits a second component of the Ca^{2+} transients that propagates along the dendrite. Note that the second component of the Ca^{2+} transients reach maxima of fluorescence changes (F/F_0) along the dendrite at different time-points, although the initial components of the Ca^{2+} transients remain comparable. (C) Representation of CA1 dendrites and analyzed regions of interest (squares), whose Ca^{2+} transients are shown in (A and B) (lower image). The lower image is a magnification of the box marked region in the upper image. The fluorescence change upon the 400 ms tetanization (100 Hz, PW 0.3 ms, B) can be seen at the Internet site.

(D) A second CA1 neuron and its Ca^{2+} transients upon a 400 ms tetanization (100 Hz, PW 0.3 ms). The delay of the second component of the Ca^{2+} signal in the third region demonstrates the propagation of the Ca^{2+} wave. Below the Ca^{2+} transients in the dendrite and the investigated regions (numbered squares) are shown.

The transients in A, B and D (— ; - - - - ; ····) represent the Ca^{2+} changes at a chosen region of interest, indicated by the numbers and visualized in the according image (in C [for A and B] and in D). The small insets in A, B and D characterize the tetanus-induced membrane depolarization, recorded intracellularly. The small horizontal lines under the membrane potential traces and under the Ca^{2+} transients indicate the time of tetanization.

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2. Involvement of metabotropic glutamate receptor activation in hypoxic/ hypoglycemic and ischemic injury

P. Henrich-Noack, U. H. Schröder, C. Sabelhaus

According to the excitotoxicity hypothesis changes in glutamate homeostasis is one of the key factors in the complex hypoxic/hypoglycemic events. Besides ionotropic glutamate receptors one could expect that also mGluRs are involved in mechanisms of necrosis and apoptosis. Previous experiments of this group have demonstrated that antagonists of mGluRs can protect neurons in an electrophysiological *in vitro* model. Recently we were able to demonstrate, that also agonists of group III mGluRs as (R, S)-4-phosphonophenylglycine exert protection in hippocampal slices (Sabelhaus et al., 2000).

To focus on the influence of mGluRs on cerebral ischaemia *in vivo* we established a 2-vessel occlusion-model of transient global ischaemia in gerbils, in which the lesion occurs selectively in the CA1 layer of the hippocampus. Surprisingly, results obtained from this model differ markedly from our *in vitro* findings, for instance we did not see pronounced neuroprotection when we applied different mGluR antagonists and the mGluR III. A neuroprotective effect was observed only with (S)-4C3HPG, which acts as an antagonist at group I mGluRs and as an agonist at group II mGluRs (Noack et al., 2001). The receptor group(s) or subtype(s) responsible for the neuroprotective effect is/are not yet identified.

Funding: LSA 2507A/0086H

3. Sodium-dependent ion exchangers as novel targets for neuroprotective drugs

U.H. Schröder, J. Breder, C. F. Sabelhaus

Cerebral ischemia and, under in vitro conditions, hypoxia/hypoglycaemia lead to a massive influx of Ca^{2+} und Na^+ into neurons, resulting in damage and subsequent death. So far most studies focussed on the ion channels involved whereas the participating ion exchangers received little attention. Interface-type organotypic hippocampal slice cultures prepared from 10 day old Wistar rats and acutely isolated slices from adult rats were employed to monitor neuroprotective compound actions in the short-term (electrophysiology on cultures and adult slices) and long-term range (Propidium iodide [PI] staining of the nuclei of deceased cells 24 h after oxygen/glucose deprivation - OGD). The $\text{Na}^+/\text{Ca}^{2+}$ exchanger couples Na^+ to the Ca^{2+} gradient and may thus contribute to neuronal damage by pumping Ca^{2+} into the neuron during ischemia. The selective $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor KB-R7943 significantly improves recovery of population spike amplitudes in rat slice cultures and reduces PI staining after OGD. Our data suggest that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, operating in reverse mode, contributes to hypoxia/hypoglycemia-induced injury in CA1 neurons (Breder et al. 2000). The broad spectrum Na^+/H^+ -exchange inhibitor harmaline, which blocks all exchanger isoforms found in the hippocampus, also caused neuroprotection in organotypic cultures in the short- and long-term range, but not in acutely isolated slices (Breder et al. 2001, submitted). Neuroprotection occurred even when the compound was only applied after the insult, indicating that the underlying mechanism may involve neuronal acidification, which in turn may downmodulate Ca^{2+} -activated destructive enzymes as long as the intracellular Ca^{2+} concentration after the insult is high. Initial studies with more isoform-selective inhibitors (EIPA, SM20220) suggest that neuroprotection via this mechanism can not only be achieved for juvenile but also for adult CA1 neurons.

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Colaboration: K.-P. Huang, NIH Bethesda, USA

4. Role of ion channels in ischemia

U.H. Schröder, J. Breder, C. F. Sabelhaus, S. Busse

The hippocampus is highly susceptible to hypoxic/ischemic injury. The sustained influx of Ca^{2+} and Na^+ and the efflux of K^+ during such events eventually lead to pronounced neuronal damage,

especially in the CA1 region. Many ion channels have been reported to be involved in these ion movements but their role in ischemia-induced neurodegeneration is still not completely clear. Using acutely isolated hippocampal slices from adult and organotypic hippocampal slice cultures (OSC) from juvenile rats we investigated if the activation or inhibition of cation channels influences neuronal recovery after OGD. Recovery of the synaptically evoked population spike in the CA1 region in acutely isolated slices and propidium iodide staining after 24 h in OSC were taken as measures of neuronal viability. The AMPA-receptor antagonist NBQX and the Ca^{2+} -channel blocker nimodipine did not influence neuronal viability. In contrast, the Na^{2+} -channel blocker tetrodotoxin significantly reduced damage when present during and the NMDA-receptor antagonist MK-801 also when present after an insult (Breder et al. 2000). Initial studies indicate that the activation of K^{+} -channels, in particular the ATP-regulated- K^{+} -channel, may also reduce hypoxic/ischemic injury after an insult. Our experiments with the novel ATP-regulated- K^{+} -channel opener Y-26763 suggest that the underlying mechanism does not involve alterations in mitochondrial function, although isoforms of ATP-regulated- K^{+} -channel are present in the plasma membrane as well as in mitochondria. The timing of activation of cation channels may thus be critical for neurodegeneration and K^{+} -channels may be a promising target to reduce injury after ischemic insults.

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5. Intracellular ion measurements during ischemia-like conditions in hippocampal slice cultures

M. Martínez-Sánchez

During oxygen and glucose deprivation (OGD) neurons develop several dysfunctions like depolarization of the cell membrane, a decrease in the ATP level and a intracellular increase of calcium and sodium concentrations. The disruption of intracellular ion homeostasis is considered a major factor in the cellular injury developed during ischemia/reperfusion. The critical mechanisms by which sodium and calcium concentrations are upregulated under ischemic conditions are still unclear at present. *In vitro* studies using hippocampal slices and/or isolated neuronal cultures have provided controversial results.

Using organotypic hippocampal cultures, the dynamics of calcium and sodium during and after ischemia, are being investigated in our group by fluorescence microscopy. We use calcium-sensitive dyes (Fura-2, Mag-fura-2 and Fura-FF) and the sodium-binding benzofuran isophthalate (SBFI) to define calcium and sodium dynamics respectively, during

OGD/reperfusion in pyramidal neurons maintained in an organotypic hippocampal culture. Pharmacological manipulations using Ca^{2+} channel blockers and glutamate antagonists have been performed to investigate the contribution of mitochondrial, plasma membrane and/or endoplasmic reticulum efflux mechanisms in the ischemic pathophysiology (Martinez et al., 2001).

In vitro ischemia-like conditions induce a large increase in the intraneuronal Ca^{2+} and Na^{+} concentration. In both cases, this increase is time-dependent on the hypoxic/hypoglycemic severity and in most of the cases reverse to basal levels during reperfusion. Pharmacological approaches addressed to elucidate the origin of calcium deregulation during ischemia have revealed a major component of calcium influx from the extracellular space under hypoxic/hypoglycemic conditions. Activation of the ionotropic glutamate, mainly NMDA receptors together with the contribution of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (acting in the reverse mode) are the main mechanisms responsible for the ischemic Ca^{2+} rise. Release of calcium from internal stores through IP_3 -sensitive receptors in the endoplasmic reticulum, contributes partially to the rise in intracellular calcium concentration induced by OGD.

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6. Immunophilines and global cerebral ischemia

W. Schmidt

The Project Group Neuropharmacology has recently studied immunophilines in the brain and their role in pathophysiological processes after cerebral ischemia. Immunophilins are members of a highly conserved family of proteins all of which have cis-trans peptidyl-prolyl isomerase (PPI) enzymatic activity that is involved in protein folding processes. The prototypic members of the immunophilin family, cyclophilin A and FK506 binding protein 12 (FKBP12), were discovered by their ability to bind and mediate the immunosuppressive effects of the drugs, cyclosporin A, FK506 (Tacrolimus) and rapamycin. The discoveries that immunophilins are present in the nervous system and that they are enriched far more in the brain than in the immune system introduces a new level of complexity in the regulation of neuronal function. FKBP12 associated with FK506, in neurons binds to serine/threonine protein phosphatase 2B (calcineurin), inhibiting its phosphatase activity and leading to neuroprotective effects after cerebral ischemia.

Different experimental models of brain ischemia were established to study neuroprotective effects of FK506. Using transient occlusion of both common carotis arteries for the induction

of global ischemia *in vivo* we found that FK506 in a concentration of 10 mg/kg administered both prior to ischemia and after reperfusion was highly neuroprotective for CA1 hippocampal pyramidal cells (Schmidt et al., 2000). In contrast to the strong neuroprotective effect of FK506 in the model of global ischemia we did not observe any protection by this drug when organotypic hippocampal slices in cultures were exposed to oxygen/glucose deprivation. The reason for this discrepancy still remains unclear. Moreover, we studied the temporal pattern and the cellular distribution of FKBP12 and caspase 1 and 3 using immunohistochemical techniques in the hippocampal formation after global ischemia in gerbils (Schmidt et al., 2000).

Four to six hours after the insult and parallel to the degeneration of neurons FKBP12 and caspase 1 and 3 are upregulated in perikarya of CA1 pyramidal cells whereas days a few later they are partially localized in micro- and astroglial cells. After ischemia and a survival time of 7 days almost all neurons in the CA1 region were degenerated, whereas FK506 was highly neuroprotective to CA1 pyramidal cells.

From our data we conclude that FKBP12 may be involved in the pathophysiology of neuronal degeneration following global ischemia. These results suggest, that FK506 and possible inhibitors of immunophilins have clinical potential for the treatment of stroke and ischemia.

Taken together, *in vivo* models, but not *in vitro* models of brain ischemia represent a useful tool for studying the mechanisms underlying FK506-induced neuroprotection against brain ischemia.

Funding: Europäischer Sozialfond, FANgGmbH

7. Expression of Protease Activated Receptors in the brain and their role in ischemia

M. Riek, W. Schmidt, P. Henrich-Noack

Very recent data from our laboratory have shown that Protease-Activated Receptors (PARs) might be involved in the pathophysiological cascade following transient global cerebral ischemia (Striggow et al., 2000), but might also be involved in mechanisms underlying the "preconditioning effect" (i.e. a weak challenge induces a protection for a following, normally deteriorious ischemic insult). *In vitro* (organotypic hippocampal slice cultures), application of thrombin in the picomolar range lead to an increased survival after oxygen-glucose-deprivation, however, after applying high doses of thrombin (micromolar concentration), this protease per se (even without additional hypoxia/hypoclycaemia) caused cell damage. For our *in vivo* studies we used the model of transient global cerebral ischemia in gerbils. By applying

the thrombin-inhibitor hirudin we could increase significantly the number of surviving neurones in the CA1-region of the hippocampus after ischemia and we could negatively influence the thrombin-mediated preconditioning effect by hirudin in this model. However, in spite of these significant effects after targeting PARs, there had been no sufficient proof about the expression and localization of these receptors in the brain. In our current work we could show for the first time by immunohistochemistry with acutely isolated and cultured hippocampal slices, that in addition to PAR-1 also PAR-2, -3 and -4 are present in the adult rat brain (Striggow et al., 2001). We could see specific staining in cortex, thalamus, amygdala, striatum and - very important for our results from gerbil ischemia - specific staining for all 4 PAR-subtypes in the hippocampus. In further studies using RT-PCR techniques we could show interesting changes in gene-expression after transient global cerebral ischemia regarding PARs and their ligands: pro-thrombin-mRNA was significantly upregulated in the hippocampus after global ischemia in comparison to naive animals and sham-operated animals. There was no difference in expression of the protease Nexin-1 between ischemic animals and control groups. We also could not detect significant differences in the expression of PAR-1 -3 (sequence of PAR-4 is not yet sufficiently known) after 15 min of ischemia in comparison to naive and sham-operated animals.

Funding: LSA 2508, DFG Re 847/3-1

8. Immunohistochemical characterization of repair mechanisms in organotypic hippocampal slice cultures after hypoxia and traumatic injury.

A. Laskowski, M. Martínez-Sánchez, W. Schmidt

Both ischemic and traumatic brain injury leads to a massive loss of neurones in the CNS. Besides well known sprouting mechanisms, it is now accepted, that stem cells could play a central role in replacing neurones of mature brain tissues after cell damage. Nevertheless little is known about these repair mechanisms after ischemic and neurotraumatologic injuries yet. Therefore, using organotypic hippocampal slice cultures as an *in vitro* model, we examined the temporal profile of degenerative and regenerative processes induced by mechanical lesion of Schaffer collaterals or OGD including stem cell proliferation, differentiation and migration. After cultivating the slices and lesioning the Schaffer collaterals in the CA3-region, propidium iodide (PI) was used as a marker of neuronal degeneration since it recognises cells with leaky plasma membrans. Antibodies against neurofilament (NF) and growth associated

protein (GAP 43) were utilised for detecting degenerating and sprouting axons. Glial fibrillary acid protein (GFAP) and OX-42 antibody were applied in order to examine astro- and microglia marker, respectively. Stem or progenitor cells were marked by incorporation of bromodeoxyuridine (BrdU) and visualised by immunofluorescence with and BrdU-antibody. For recognising them as new neurones, we used double labelling of BrdU and β -III-tubulin as a marker for immature neurones. Image analysis of double immunofluorescent labelled pictures were taken by confocal laser scan microscopy.

PI staining revealed degenerative neurones not only at the lesion site but also at the projection area of Schaffer collaterals in the CA1 with a time dependent profile. Immunofluorescent labelling of NF and GAP 43 showed numerous growing fibres crossing the scar, already present one day after lesioning. These results indicate morphological changes such as sprouting and the appearance of growth cones occurring following mechanical lesion of the Schaffer collaterals (Laskowski et al., 2001).

GFAP- and OX-42 immunofluorescent images notified an alternation of astro- and microglia from quiescent to the activated form in the damaged tissue at the lesion. Reactive microglia show phagocytotic processes and thereby suggest a cleaning of the damaged area. Distribution- and time profile of BrdU-labelled cells co-stained with neuronal and glial markers are under investigation.

Funding: Europäischer Sozialfond, FANgGmbH, Graduiertenkolleg

9. Endogenous stem cell proliferation, differentiation and migration in the hippocampal formation of gerbils after global cerebral ischemia.

W. Schmidt

Neurogenesis caused by stem cells mainly in the hippocampal dentate area of adult mammalian brain may be a compensatory adaptive response to brain injury and could promote morphological and functional recovery after ischemia. Hippocampal stem cells from adult brain are capable to proliferate and differentiate into neurons and different glial cell types, but the mechanisms under which they are activated are still unclear. Using a model of global cerebral ischemia (2VO) in gerbils the present study was undertaken to determine whether global ischemia, which causes neurodegeneration selectively in CA1 pyramidal neurons, affects endogenous stem cells in the dentate area. Immunofluorescence together with confocal laser microscopy of anti-bromo-deoxyuridine (BrdU) antibodies after incorporation of BrdU into DNA during cell replication was used to demonstrate the proliferation of stem

cells. Applying different protocols of BrdU administration after global ischemia and multi-immunofluorescence with different cellular markers we studied the migration and differentiation of stem cells into the degenerated CA1 region.

One month after global ischemia the number of proliferating stem cells increased in the dentate area as compared to sham operated animals. An increasing number of BrdU immunoreactive cells that express first the neuronal progenitor cell marker β III tubulin later the neuronal marker NeuN also appeared in the CA1 pyramidal layer, in which ischemia causes a loss of neurons (Fig. 2). Our data indicate an ischemia-induced enhanced neurogenesis in CA1 pyramidal layer that is caused by stem cells derived from dentate area (Schmidt and Reymann, 2001).

Funding: FANgGmbH

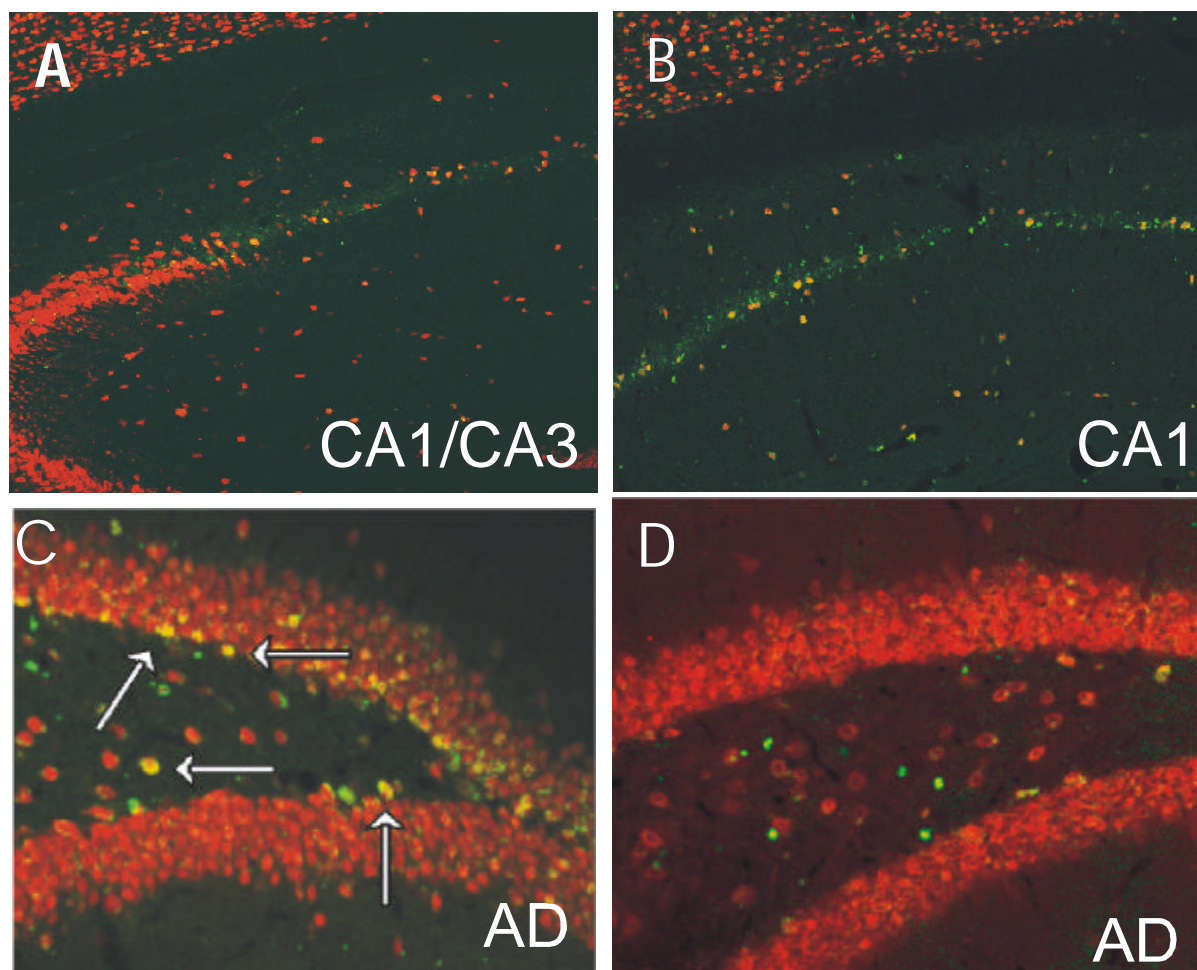


Fig. 2: BrdU (green) and the neuronal marker NeuN (red) labeling of the adult hippocampus of gerbils after global cerebral ischemia (A – C) and of control animal (D). Note newly generated neurons (yellow) in CA1 and area dentata.

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