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Abstracts

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Chair: Georg Reiser
Co-chair: Klaus Reymann

The role of increased mtDNA copy number in experimental ischemic stroke

Ahola, S.[a], Strbian, D.[a,b], Abo-Ramadan, U.[b], Tatlisumak, T.[b], Lindsberg, P.[a,b] and Suomalainen, A.[a]
[a]*Research Program of Molecular Neurology, Biomedicum-Helsinki, University of Helsinki, Finland*
[b]*Department of Neurology, Helsinki University Central Hospital, University of Helsinki, Finland*

Mitochondrial DNA (mtDNA) provides the cell with a set of respiratory chain enzyme subunits, which contribute to oxidative ATP production. Decreased mtDNA amount has been linked to variable multisystem disorders of childhood and adult age, but whether increased amounts of mtDNA affect cellular well-being is an open question. In a myocardial infarction model, increased mtDNA copy number in mice overexpressing human TFAM protein led to significantly improved survival rate together with decreased left ventricular dilatation and dysfunction. Mitochondrial helicase Twinkle is one of the essential proteins for mtDNA maintenenance and overexpression of Twinkle increases the mtDNA copy number by 1.5–3-fold in mice. Here, we utilized these Twinkle-mice to study the possible beneficial effects of increased mtDNA amount for cerebral ischemia. The mice underwent focal cerebral ischemia by suture middle cerebral artery occlusion (MCAO). Twinkle overexpressor mice and their wild-type littermates were subjected to either 45 minutes of ischemia ($n = 12$ per group) or to sham surgery ($n = 5$ per group). Cerebral blood flow measurement by laser-Doppler flow-metry confirmed proper ischemia and reperfusion. Mice were imaged by magnetic resonance imaging (MRI) immediately, at 24, and 72 hours after MCAO. The major endpoints were mortality, functional outcome, and the lesion size by means of MRI and TTC staining. The functional outcome was scored after 24, 48 and 72 hours as assessed by neurological scoring, beam walk test, and tail raising test. Animals were sacrificed 72 hours following MCAO and brain slices were stained with TTC to visualize the final lesion sizes. The analyses of survival and tissue consequences of the ischemia are in progress.

Functional and histological improvement after acute subdural hematoma in rats treated with hypertonic/hyperoncotic solution and surgical evacuation of blood

Alessandri, B., Jussen, D., Papaioannou, C., Heimann, A. and Kempski, O.
Institute for Neurosurgical Pathophysiology, Johannes Gutenberg-University Mainz, Germany

Traumatic brain injury is often associated with acute subdural hematoma (ASDH). Treatment of ASDH consists mainly of surgical evacuation of the hematoma in order to reduce pathophysiological processes. It is conceivable that early preoperative improvement of microcirculation with hypertonic/hyperoncotic treatment (HHT) can improve survival rates. The present study investigated the benefit of treatment with hypertonic/hyperoncotic solution on functional and histological outcome as supportive therapy accompanying surgical intervention. In a rat model of ASDH ($n = 50$, Sprague-Dawley rats) 400 μl autologous venous blood was infused subdurally at a rate of 50 μl/min. Thirty minutes after subdural blood infusion, the rats re-

ceived either HyperHAES (7.2% saline/ 6% hydroxyethyl starch) or vehicle (NaCl 0.9%) intravenously, followed by surgical evacuation of the hematoma 1 hr after ASDH induction in those rats scheduled for surgical treatment. We explored acute effects of HHT on blood variables, ASDH-induced changes of intracranial pressure (ICP), and cerebral perfusion pressure (CPP), and investigated the chronic effects of HHT, surgical blood clot evacuation, and the combination of both on functional and histological outcome following ASDH (12 days). HHT expectedly raised the serum sodium concentration and lowered hematocrit. ASDH increased ICP and decreased CPP in all groups. HHT improved CPP by reducing ICP. All treated groups showed a better recovery with respect to neurological function and neuronal cell death compared with the vehicle treated ASDH group. HHT with surgical evacuation or HHT alone improved functional and histological outcome slightly more than surgical evacuation alone. In this rat model, HHT led to a decrease of ICP after ASDH. This significantly improved functional and histological outcome were improved significantly after blood evacuation alone. The combination of evacuation of subdural blood and early HHT improved histological outcome further but not significantly, which was due to the strong effects of single treatments and a ceiling effect of the combined treatment in this model.

Neuroprotection after acute subdural hematoma in rats: The potential danger of systemic and subdural application of erythropoietin

Alessandri, B.[a], Wähman, M.[a], Rahimi, M.[a], Raabe, A.[b], Vatter, H.[b], Heimann, A.[a] and Kempski, O.[a]
[a]*Johannes Gutenberg-University, Institute for Neurosurgical Pathophysiology, Mainz, Germany*
[b]*Johann Wolfgang Goethe-University, Department of Neurosurgery, Frankfurt a.M., Germany*

Traumatic brain injury (TBI) is often associated with acute subdural hematoma (ASDH). Treatment of ASDH) consists mainly of surgical evacuation of the hematoma in order to reduce intracranial pressure and mortality. The hemopoetic cytokine Erythropoietin (EPO)has been shown to be neuroprotective in various animal models of TBI. The present study investigates cute effects of EPO on intracranial pressure, cerebral perfusion pressure, tissue oxygen concentration (ptiO2) and cerebral blood flow as well as the neuroprotective potential of EPO applied either systemically or, after removal of the blood clot, Subdurally. In a rat model of ASDH ($n > 5$/group, Sprague-Dawley rats) 400 μl autologous venous blood was infused subdurally at a rate of 50 μl/min. Twenty (20) minutes after subdural blood infusion, rats received i.v. either NaCl, 200, 2000 or 20'000 IU EPO systemically. Brain tissue concentration of EPO was determined 1 hour after ASDH. In a second set of animals, NaCl or 20 mIU were applied subdurally after blood clot removal. Brains were removed 48 hours after ASDH for histological evaluation. Ipsilateral brain tissue concentration of EPO rose to: 1.7 ± 0.2, 11.7 ± 0.2. 88.4 ± 4 and 672 ± 63 mIU/ml tissue in the NaCl, 200, 2000 and 20'000 IU group, respectively. EPO had no effects on the time-course of ptiO2 and CBF during 60 min monitoring after ASDH. EPO at 20'000 IU increased ICP significantly during the last 10 mn of monitoring. Measurement of lesion volume 48 hours after ASDH revealed a significant neuroprotective effect of 200 and 2000 IU EPO (12.5 ± 0.4; 9.6 ± 0.5 mm^3) when compared to the NaCl group (16.6 ± 0.3), whereas 20'000 IU was neurotoxic (20.2 ± 0.7). Subdural application of 20 mIU after removal of blood reduced lesion damage by almost 60%. EPO was neuroprotective in a model of ASDH. Hover, high EPO concentrations in brain tissue included an ICP increase and led to adverse effects on lesion growth. Very low EPO concentrations are therefore needed if EPO is applied subdurally after blood volume removal. Thus, save concentration ranges should be evaluated for the use of EPO in TBI patients.

Mesenchymal stem cells as oligodendrogenic inductors: A potential mechanism in brain repair

Aigner, L., Bogdahn, U., Rivera and F. J.
Clinic of Neurology, University of Regensburg, Germany

Adult stem cells reside in different tissues and organs of the adult organism. Among these cells are MSCs that are located in the adult bone marrow and NSCs that exist in the adult central nervous system (CNS). In transplantation experiments, MSCs demonstrated neuroprotective and neuroregenerative effects that were associated with functional improvements. The underlying mechanisms are largely unidentified, but immunmodulation, secretion of neurotrophic factors and interactions with endogenous progenitors might be involved. Here, we reveal that the interactions between adult MSCs and NSCs, mediated by soluble factors,

induce oligodendrogenic fate decision in NSCs at the expense of astrogenesis. This was demonstrated (a) by an increase in the percentage of cells expressing the oligodendrocyte markers GalC and myelin basic protein, (b) by a reduction in the percentage of glial fibrillary acidic protein (GFAP)-expressing cells, and (c) by the expression pattern of cell fate determinants specific for oligodendrogenic differentiation. Thus, it involved enhanced expression of the oligodendrogenic transcription factors Olig1, Olig2, and Nkx2.2 and diminished expression of Id2, an inhibitor of oligodendrogenic differentiation. Currently, the identity of the oligodendrogenic activity is under investigation. This work might have major implications for the development of future transplantation strategies for the treatment of degenerative diseases in the CNS.

Astroglial protein S-100 as tissue-marker of hypoxic-ischemic damage after perinatal asphyxia in lambs

Alonso-Alconada, D.[a], Alvarez, A.[a], Alvarez, F. J.[b], Goñi-de-Cerio, F.[a], Mielgo, V. E.[b], Rey-Santano, M.[b], Caballero, A.[c], Valls-i-Soler, A.[b,d] and Hilario, E.[a]
[a]*Department of Cell Biology & Histology, Leioa, Spain*
[b]*Research Unit on Pediatrics, Leioa, Spain*
[c]*Department of Neuroscience, Leioa, Spain*
[d]*Department of Pediatrics, Leioa, Spain*

Objetive: To evaluate the efficacy of protein S-100 (a biochemical marker) as tissue-marker of brain damage after hypoxic-ischemic injury. *Methods:* Fetal lambs with a developmental age of 133 days (term: 145 days) were randomly assigned to three different experimental groups; Healthy group: without asphyxia and with mechanical ventilation for 3 hours ($n = 5$). 0 hours post-Partial Cord Occlusion Group (0h-PCO group): sacrificed after 60 minutes of asphyxia. ($n = 5$). 3 hours post-Partial Cord Occlusion Group (3h-PCO group): after 60 minutes of asphyxia and mechanical ventilation for 3 hours ($n = 5$). Asphyxia was induced by partial occlusion of umbilical blood flow during 60 minutes. Brains were fixed by perfusion with 4% paraformaldehyde and divided in different regions: frontal and parietal cortex, basal nuclei and hippocampus. These regions were disaggregated and immunolabeled for astroglial protein S-100. Samples were analyzed by flow citometry and immunolabeled cell percentage was evaluated. Data were compared by one-factor ANOVA ($p < 0.05$). *Results:* 0h-PCO group did not show differences of astroglial protein S-100 levels in comparison to the control in brain regions studied (parietal cortex: 58.4 $\pm$ 16.9% vs. 62.4 $\pm$ 9.3%; frontal cortex: 51.2 $\pm$ 15.7% vs. 59.2 $\pm$ 15.7%; hippocampus: 42.1 $\pm$ 13.5% vs. 58.4 $\pm$ 5.4%; striatum: 48.4 $\pm$ 20.2% vs. 54.6 $\pm$ 9.4%, respectively). However, in all brain regions, the astroglial protein S-100 levels were significantly decreased in the 3h-PCO group in comparison to both previous groups (parietal cortex: 11.5 $\pm$ 7.2%; frontal cortex: 8.4 $\pm$ 1.6%; hippocampus: 8.2 $\pm$ 7.5%; striatum: 10.7 $\pm$ 4.5%). *Conclusion:* Our results suggest that astroglial protein S-100 determined as early as 3 h after hypoxic ischemic injury could be used as a tissue-marker of asphyctic event. *Grants:* University of Basque Country (EHU06/99), Basque Government (IT-287-07) and Ministry of Health (FIS PI06/0908, FIS PI06/0839).

The effect of Sonic hedgehog and Wnt-7a on neural stem cell differentiation *in vitro* – an electrophysiological and immunohistochemical analysis

Anderova, M.[a,c], Prajerova, I.[a,b], Honsa, P.[a] and Chvatal, A.[a,b,c]
[a]*Department of Cellular Neurophysiology – Laboratory of Neurobiology, Inst. Exp. Med., ASCR, Prague, Czech Republic*
[b]*Department of Neuroscience, 2nd Medical Faculty, Charles University, Prague, Czech Republic*
[c]*Center for Cell Therapy and Tissue Repair 2nd Medical Faculty, Charles University, Prague, Czech Republic*

Sonic hedgehog (Shh) and Wnt-7a are secreted morphogens involved in neurogenesis in the adult brain. GFP-labeled P0 mouse neural stem cells expressing either Shh (Shh/GFP) or Wnt-7a (Wnt-7a/GFP) were used to study their effect on neural stem cell proliferation and differentiation *in vitro.* Cells expressing only GFP (WT/GFP) were used as a control. Electrophysiological characterization using the patch-clamp technique and immunohistochemical analysis were carried out 8 days after the induction of differentiation by retinoic acid. In WT/GFP cells three distinct cell populations were identified. Large flat cells with a cell-body diameter of 40 μm formed an underlying layer (43.6%), expressed GFAP and nestin, and displayed passive, time- and voltage-independent K+ currents, with an average membrane potential (Vm) of -87 mV and input resistance (IR) of 61MOhms. The second population

of cells (38.5%) with a triangular cell-body (diameter 25 μm) expressed predominantly NG2-proteoglycan and displayed passive, time- and voltage-independent K+ currents together with an inwardly rectifying current activated by hyperpolarization. Their mean Vm and IR were -88 mV and 276 MegaOhms, respectively. The third group of cells (18%) with a cell-body diameter of 15 μm (termed neuron-like cells) were MAP-2, DCX or β-III tubulin positive with a mean Vm of -77 mV and IR of 876 MOhms. They displayed voltage-dependent KA, KDR and TTX-sensitive Na+ currents (INa). In Shh/GFP cells the number of GFAP-positive cells (42.8%) was not significantly different from those in WT/GFP cells, nor were their passive membrane properties. The number of NG2-positive cells increased to 48.4% and their passive membrane properties were similar to those in controls. The number of neuron-like cells was reduced to 8.8% and, moreover, INa currents were not detected. In Wnt-7a/GFP cells the number of large flat cells was reduced to 6% and the number of triangular cells decreased to 8%, while the number of neuron-like cells significantly increased to 77%. All three cell sub-populations identified in Wnt-7a/GFP cells showed increased IR and decreased Vm, and, furthermore, in neuron-like cells the INa current was significantly increased. Based on our immunohistochemical data we can conclude that both Shh and Wnt-7a increase the expression of neuronal markers in differentiated neural stem cells compared to WT/GFP cells. However, electrophysiological analysis revealed an increased incidence of neuron-like current patterns only in Wnt-7a/GFP cells.

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Anxiety as a consequence of local ischemia and its correction by Afobazole

Ashrafyan, K. and Balasanyan, M. G.
Yerevan State Medical University, Armenia

The aim of the investigation is to evaluate anxiolytic effect of Afobazole after local ischemia in new model of stroke where local ischemia was performed after long-term restriction of movement activity – hypokinesia (HK) since it is known that HK causes chronic ischemization of cerebral tissue. White inbred male rats were used. Control animals were housed in groups of eight. As a model of HK, all experimental animals were kept individually in narrow cages for 45-days. Anxiety was evaluated by elevated plus-maze (EPM). The acute local cerebral ischemia was performed by the ligation of middle cerebral artery (MCA). Afobazole (1 mg/kg, i.p.) was administered during 12 days after ligation of MCA twice daily. The data obtained showed that ligation of MCA after HK 45 days causes anxiogenic-like effect in rats. They displayed less time spent on $[F(1,6) = 0.41,\ P < 0.5]$ and percentage of entries into the open arms $[F(1,6) = 0.03,\ P > 0.5]$ of EPM. Psychopharmacological study of Afobazole has revealed that Afobazole had no visible effects in control rats: both time spent on and percentage of entries into the open arms of EPM were insignificantly changed. However, Afobazole treatment during 12 days after local ischemia displayed significant anxiolytic-like effect. It affected the following behavioral measures: increases were observed for the percentage of open arm entries $[F(1,6) = 0.25,\ P > 0.1]$, the time spent in the open arms $[F(1,6) = 1.642,\ P < 0.5]$. In conclusion, results obtained in the present study suggest that Afobazole evidently displays anxiolytic-like effect in animals with anxiety caused by local ischemia. Moreover, new model of local ischemia increases the relevance of laboratory studies and laboratory findings may provide cues regarding the clinical use of Afobazole.

Blockade of bradykinin receptor B1 but not bradykinin receptor B2 provides protection from brain edema and cerebral infarction

Austiant, M.[a], Braeuninger, S.[a], Pesquero, J. B.[b], Bader, M.[c], Stoll, G.[a], Renné, T.[d] and Kleinschnitz, C.[a]
[a]*Department of Neurology, University of Würzburg, Germany*
[b]*Departamento de Biofisica, Universidade Federal de São Paulo, Brazil*
[c]*Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany*
[d]*Institute for Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Germany*

Background: Brain edema is detrimental in ischemic stroke and its treatment options are limited. Kinins are highly active proinflammatory peptides which are released during inflammation and tissue injury including cerebral infarction. The cellular effects of kinins are mediated by two different receptors (B1 and B2 receptor [B1R, B2R]) and comprise induction of edema formation by increased vascular permeability and release of proinflammatory mediators. *Methods and Results:*

We analyzed the effect of B1R and B2R deficiency on infarct size, edema formation and inflammatory processes after transient middle cerebral artery occlusion (tMCAO) in mice. B1R null mice developed significantly smaller brain infarctions and less neurological deficits compared to wildtype controls ($16.8 \pm 4.7\,\mathrm{mm}^3$ versus 50.1 ± 9.1 mm^3, respectively; $p < 0.0001$). This was accompanied by a dramatic reduction of brain edema in the ischemic hemisphere as measured by extravasation of Evan's blue tracer ($6.7 \pm 1.6\,\mathrm{mm}^3$ versus 81.7 ± 17.8 mm^3, respectively; $p < 0.0001$) and less postischemic inflammation within the infarcted tissue. Pharmacological blockade of B1R likewise salvaged ischemic brain tissue (15.0 ± 9.5 mm^3 versus 50.1 ± 9.1 mm^3, respectively; $p < 0.01$) and improved neurological outcome in a dose-dependent manner even when B1R inhibitor was applied 1 hour *after* tMCAO. In contrast, B2R deficiency or inhibition did not confer neuroprotection and had no effect on the development of tissue edema. *Conclusions:* These data demonstrate that blocking of B1R can diminish brain infarction and edema formation in mice and may open new avenues for acute stroke treatment in humans.

Influence of growth factors or TGFbeta-1 on neurogenesis and microglial reaction after Endothelin-1-induced ischemia

Baldauf, K. and Reymann, K. G.
Leibniz Institute for Neurobiology (*IfN*), *Project Group Neuropharmacology, Magdeburg, Germany*

Many investigators have shown that a lot of cells, generated after an ischemic event, die during the first weeks and despite an ongoing enhanced neurogenesis, repair and survival in the affected regions is low. We followed two strategies, first, to enhance proliferation and second, to prevent cell death. We applied EGF/FGF over a period that has been shown to be regenerative and we tried to prevent cell death and protect newly generated cells with TGFb1 at a time where the most of them die. As a model, we used the Endothelin-1 induced ischemia. We analyzed infarct size, BrdU/NeuN double labeling for an influence of this treatment on neurogenesis and additionally the reaction of inflammatory cells to our TGFb1 treatment. In our model, we found no promotional or detrimental influences on infarct size and additionally no significant changes in neurogenesis 4 weeks after ischemia. Our results showed a tendency to an increase of the number of Ox42-positive and BrdU/Ox42-positive cells in TGFb1 treated animals in striatum and cortex in non-ischemic animals. Contrary in the striatum, cortex and lateral ventricle on the contralateral side and in the dentate gyrus we found a significant down regulation of Ox42 positive cells after treatment with TGFb1 in ischemic animals. The same result was obtained, when we counted cells directly in the destroyed parts of the cortex.

Investigating the behavioral recovery of stroke lesioned rats using the staircase test

Baldauf, K., Braun, H. and Reymann, K. G.
Leibniz Institute for Neurobiology (*IfN*), *Project Group Neuropharmacology, Magdeburg, Germany*

The functional recovery of stroke damaged animals is an important aspect for testing the effectiveness of any pharmacological treatment. We applied the so-called staircase test in order to investigate the functional recovery of stroke-lesioned rats in the context of a transplantation study. Animals were trained two weeks before ischemia (5 days per week). Training was performed twice per day for 15 min. Two days before starting the training session the feed was reduced to 5 pellets per animal. The individual staircase test was performed by positioning the rat for 15 min into the staircase device and counting afterwards the rest of the pellets on each side of the staircase. For each rat a baseline was determined taking into account the last 5 tests before ischemia. Performance deficits were detectable over 4 weeks. The intra-parenchymal transplantation 1 week after fMCAO induces additional deficits on the contralateral limb. In transplantation controls, we did not observe any spontaneous recovery during 4 weeks. The dietary treatment reduces the motivation of animals to grab the pellets. Finally, there is a tendency that animals partially lose their trained ability to grab the pellets after 5–7 days without training. *Conclusion:* The staircase test allows the detection of functional deficits after stroke, however frequently test have to be performed, the dietary treatment reduces the overall motivation of rats to grab the pellets and it is unclear, whether or not cell transplantation is able to overcome the reduced performance.

The role of pannexins in cerebral ischemia

Bargiotas, P.[a,b], Monyer, H.[b] and Schwaninger, M.[a]
[a]*Institute of Pharmacology, University of Heidelberg, Germany*
[b]*Clinical Neurobiology, University of Heidelberg, Germany*

Pannexins (Px) are known to form functional homomeric (Px1) and heteromeric (Px1/Px2) hemichannels with distinctive properties. Recently published data support the involvement of pannexins in cerebral ischemia. During *in vitro* ischemia, large-conductance channels with biophysical properties of pannexins, open and lead to ionic dysregulation and cell death. Moreover, Px1 seems to play an important role in processing and release of IL-1β, which is an essential mediator of brain damage during ischemia. In the current study, we investigated the effects of pannexin deletion on ischemic brain damage *in vivo*. Px1-/-, Px2-/-, and Px1-/-Px2-/- mice and their wild-type littermates underwent left middle cerebral artery occlusion (MCAO). Double deletion of both PX1 and Px2 (Px1-/-Px2-/-) significantly reduced infarct size whereas Px1-/- and Px2-/- showed a trend to smaller infarcts. The neurological deficit was evaluated before and after MCAO with the corner test. Px2-/- and Px1-/-Px2-/- mice showed a significant smaller deficit than the wild-type littermates. Our data show that pannexin hemichannels have a detrimental effect during ischemia. Apparently, there is some redundancy between Px1 and Px2 in ischemic pathophysiology. Pannexin hemichannels may be an important new pharmacological target to prevent neuronal death in stroke.

Oxygen-glucose deprivation reveals two astrocytic populations in the cortex of GFAP/EGFP mice: *In situ* quantification of cell volume changes

Benesova, J.[a], Hock, M.[d], Butenko, O.[a,b], Prajerova, I.[a,b], Anderova, M.[a,c] and Chvatal, A.[a,b,c]
[a]*Department of Cellular Neurophysiology, Institute of Experimental Medicine, ASCR, Prague, Czech Republic*
[b]*Department of Neuroscience, Charles University, 2nd Medical Faculty, Prague, Czech Republic*
[c]*Center for Cell Therapy and Tissue Repair, Charles University, Prague, Czech Republic*
[d]*Department of Epithelial Physiology, Institute of Physiology, ASCR, Prague, Czech Republic*

Astrocytic volume changes during ischemia/reperfusion *in situ* were quantified in the cortex of 30–40-day-old GFAP/EGFP mice using confocal microscopy combined with 3D-reconstruction. Twenty minutes of oxygen-glucose deprivation (OGD) revealed the presence of two distinct astrocyte populations: low response (LR) and high response (HR) astrocytes. In LR-astrocytes, OGD led to a small volume increase of $\sim$10%; subsequent 40-minute reperfusion led to a complete volume recovery. In HR-astrocytes, OGD evoked a marked volume increase of 41%; reperfusion led within the first 20 minutes to an additional volume increase with no apparent volume recovery. Using the patch-clamp technique in the whole-cell configuration, changes in membrane potential during OGD and reperfusion confirmed two differently responding populations of astrocytes: one group of cells showing a marked depolarization from -82.8 mV to -54.5 mV with very small Vm recovery during reperfusion, while in the second group of cells only small changes in Vm were observed. Conversely, a pH shift from 7.4 to 6.8, achieved either by the reduction of HCO3- and the elevation of CO2 in the artificial cerebrospinal fluid (ACF) or by lactate addition into the ACF (ACFpH 6.8 or ACFlactate), led to a volume decrease of 15–20% in all astrocytes; two differently responding populations of astrocytes were not detected during acidification. ACF-pH 6.8 application caused only a small depolarization from -86 to -83, while the application of ACFLactate led to a subtle membrane hyperpolarization from -83 mV to -85 mV, and depolarization was observed during reperfusion. As AQP4 and Kir4.1 channels take part in astrocytic swelling, immunohistochemical analysis was employed to examine differences in their expression that might underlie the dissimilar responses of HR- and LR-astrocytes to OGD. Immunostaining for Kir4.1 revealed strongly and weakly Kir4.1-positive cells, while no diversity was found in AQP4 immunoreactivity. The levels of intracellular taurine, an osmotically active amino acid participating in regulatory volume decrease, also markedly differed in the cortical astrocytes. As chloride movement plays an important role in astrocytic volume regulation, its contribution to cell volume changes evoked by OGD was studied using inhibitors of chloride channels (DIDS, NPPB, Cd2+, tamoxifen), the Na-K-Cl cotransporter (bumetanide) and the K-Cl cotransporter (DIOA). Our data show the presence of two populations of astrocytes in the cortex of GFAP/EGFP mice, based on membrane potential and volume changes during OGD, and also the essential role of chloride movement in volume reg-

ulation. Immunohistochemical analysis suggests that the diverse expression of Kir4.1 channels as well as differences in taurine levels might contribute to the distinct ability of astrocytes to regulate their volume. Supported by GACR305/06/1316, GACR305/06/1464, AVOZ50390512, LC554, 1M0538.

Multimodal evaluation of autologous bone marrow cell therapy of stroke in a novel large animal model of focal cerebral ischemia

Boltze, J.[a,b], Förschler, A.[c], Barthel, H.[b,d], Nitzsche, B.[a], Boltze, C.[a], Reischauer, A.[e], Lobsien, D.[f], Hoffmann, A.[g], Sabri, O.[d], Emmrich, F.[a,b] and Gille, U.[g,h]
[a]*Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany*
[b]*Translational Centre for Regenerative Medicine, University of Leipzig, Germany*
[c]*Department of Neuroradiology, University Hospital rechts der Isar, Munich, Germany*
[d]*Department of Nuclear Medicine, University of Leipzig, Germany*
[e]*Institute for Veterinary Pathology, University of Leipzig, Germany*
[f]*Center for Diagnostic Radiology, University of Leipzig, Germany*
[g]*Institute for Veterinary Anatomy, University of Leipzig, Germany*
[h]*VITA 34 Intl. Inc., Leipzig, Germany*

Introduction: Efficiency of experimental bone marrow (BM) cell therapies of stroke has been shown in rodents. However, transfer to clinical application requires close-to-practice large animal models. We evaluated benefit of autologous BM cell transplantation in a novel sheep model of focal cerebral ischemia allowing control of lesion size and subsequent functional deficits. *Material and Methods:* 30 adult rams were subjected to permanent middle cerebral artery occlusion (MCAO) for stroke induction. 24 hours after MCAO, 15 animals received 4.0 to 5.1x10E6 autologous mononuclear BM cells per kilogram bodyweight. 15 sheep served as controls. Functional outcome was continuously observed by behavioral phenotyping. Lesion size development was monitored by MRI and PET performed at days 1, 14 and 42 before brains were removed for further histological investigation. *Results:* In BM cell treated animals, functional improvement was enhanced as compared to control animals ($p < 0.01$) while control animals suffered from moderate to severe motor and sensory dysfunctions like ataxia, absent startle reflexes and spatial hemineglect for the entire observation period. MRI investigations showed similar lesion size in both groups at day 1 ($p = 0.59$), but reduction of lesion size and hemispherical atrophy in cell treated rams 42 days upon MCAO ($p < 0.01$). These findings could be confirmed by 15O-water- and 18F-Desoxyglucose PET ($p < 0.05$). No tumor formation was observed upon BM cell administration. *Conclusion:* Autologous BM marrow administration 24 hours following stroke is safe and effective in sheep. BM cell administration might be used as a novel treatment option in fighting stroke in upcoming clinical trials.

Imaging new structural and functional circuits in adult sensorimotor cortical regions recovering from stroke

Brown, C. E., Aminoltejari, K., Erb, H., Winship, I. and Murphy, T. H.
Department of Psychiatry, Brain Research Center University of British Columbia, Vancouver, Canada

Previous macroscopic imaging studies have suggested that a key component of stroke recovery is the transfer of function from damaged brain areas to surviving peri-infarct regions. Although new areas of activation have been reported, how sensory information flows in and out of these regions on a millisecond time scale, and the structural basis of these new functionally responsive areas, are not understood. Here, using *in vivo* voltage sensitive dye imaging combined with analysis of dendritic spine plasticity and tract tracing, we define new principles and pathways by which reorganized cortical networks operate. Several weeks after stroke in the adult forelimb sensorimotor cortex, forelimb-evoked responses re-emerged in adjacent peri-infarct motor and hindlimb areas, and appeared to recruit medial-posterior cortical domains. Interestingly, the functional re-mapping of the forelimb representation was driven, not by an increase in peri-infarct motor and hindlimb responsiveness per se, but rather an increase in the amount of time these areas spent processing forelimb related sensations. Structurally, reorganized areas exhibited high levels dendritic spine turnover and were the recipients of both new inputs from medial-posterior cortical areas, and made new functionally enhanced outputs to the striatum. Collectively, our findings indicate that the re-mapping of a sensory representation during behavioral recovery from

stroke is associated with profound changes, not only in fine synaptic structure or long-range input and output connections, but changes in the fundamental manner in which these areas processes sensory information.

When the brain goes diving: Neuroglobin and hypoxia tolerance of the seal brain

Burmester, T.[a], Mitz, S. A.[a], Ramirez, J.-M.[b], Hankeln, T.[c], Reuss, S.[d], Blix, A. S.[e] and Folkow, L. P.[e]
[a]*Institute of Zoology and Zoological Museum, University of Hamburg, Germany*
[b]*Department of Organismal Biology and Anatomy, University of Chicago, USA*
[c]*Institute of Molecular Genetics, University of Mainz, Germany*
[d]*Department of Anatomy and Cell Biology, University of Mainz, Germany*
[e]*Department of Arctic Biology, University of Tromsø, Norway*

Diving mammals survive repeated exposure to severely hypoxic conditions in their everyday life. The hooded seal (*Crystophora cristata*) can dive to more than 1,000 m depth and stay submerged for about 1 hour. During the dive, arterial oxygen tension drops to levels that are fatal to terrestrial mammals. It is unknown, however, how the seal brain tolerates hypoxia resulting from long-duration dives. Intra- and extracellular recordings from isolated neocortical slices of adult hooded seals showed that seal neurons display a remarkable tolerance to hypoxia. We further compared the localisation in neuroglobin in rat and seal brains. Neuroglobin is an oxygen-binding protein in the nervous tissue of man and other vertebrates, which is distantly related to myoglobin. Although its exact function is still elusive, there is strong evidence that neuroglobin is linked to the oxidative energy metabolism. Immunofluorescence studies showed that in the hypoxia-sensitive rat, neuroglobin is exclusively localised in neurons throughout all brain regions. By contrast, neuroglobin is predominantly expressed in glial cells (astrocytes) of the seal brain. The hooded seal thus is the first mammal in which the expression of neuroglobin was found to be not restricted to neurons. We further showed that cytochrome c (which is a marker for mitochondria) is mainly located in rat neurons, confirming the notion that neurons of terrestrial mammals largely rely on aerobic metabolism. In seals, however, mitochondria are most predominant in astrocytes. We therefore hypothesise that the neurons of the seal brain are hypoxia tolerance because they largely rely on anaerobiosis, whereas glial cells employ aerobic metabolism.

Role of the WNT antagonist Dickkopf-1 in the neuronal degeneration in experimental animal models of cerebral ischemia

Busceti, C. L.[a], Biagioni, F.[a], Mastroiacovo, F.[a], Cappuccio, I.[b], Battaglia, G.[a], Fornai, F.[a,b], Melchiorri, D.[a], Aronica, E.[d], Caricasole, A.[e], Bruno, V.[a,b] and Nicoletti, F.[a,b]
[a]*I. N. M. Neuromed, Pozzilli, Italy*
[b]*Department of Human Physiology and Pharmacology, University "La Sapienza", Rome, Italy*
[c]*Department of Human Morphology, University of Pisa, Italy*
[d]*Department of Neuropathology, University of Amsterdam, The Netherland*
[e]*SienaBiotech, Siena, Italy*

Dickkopf-1 (Dkk-1) is a secreted glycoprotein that acts as an extracellular inhibitor of the canonical Wnt/β-catenin/TCF/LEF pathway by interacting with the Wnt co-receptors, low-density lipoprotein-related protein receptors, type 5 and -6 (LRP5/6). Inhibition of the canonical Wnt pathway produces a series of intracellular events that may be detrimental to neurons, including a reduced availability of free β-catenin which regulates gene expression, and an increased phosphorylation of the tau protein. Both events are driven by a disinhibition of glycogen synthase kinase-3® (GSK-3®), an enzyme negatively regulated by the Wnt pathway. We found a substantial induction of Dkk-1 in vulnerable neurons (e.g. pyramidal cells of the hippocampal CA1 region) of gerbils or rats subjected to transient global brain ischemia. Induction of Dkk-1 preceded neuronal death and was associated with an increased expression of p53, a sensor of DNA damage. A likely scenario is that the DNA damage produced by the ischemic insult increases p53 levels, and that p53 acts as transcriptional activator of the Dkk-1 gene, thus amplifying neuronal damage. We have also found that Dkk-1 is induced in the perifocal region of (i) rats subjected to focal brain ischemia induced by local infusion of endothelin-1 in the territory of the middle cerebral artery; and (ii) in mice subjected to focal brain ischemia induced by cauterization of the middle cerebral artery. Remarkably, the extent of infarct volume was

reduced in rats treated with lithium ions (which inhibit GSK-3β) and in mutant mice homozygous for a hypomorphic allele of Dkk-1 (*doubleridge* mice). We also studied the involvement of Dkk-1 in *in vitro* models of excitotoxic neuronal death. We found that Dkk-1 is induced in cultured cortical neurons challenged with toxic concentrations of N-methyl-D-aspartate. This induction is associated with a reduction of nuclear levels of β-catenin, and consistently precedes neuronal death. Moreover, both Dkk-1 antisense oligonucleotides and lithium ions protected cultured neurons against excitotoxicity. Taken collectively, these data suggest that DKk-1 is a marker of excitotoxic death and contributes to the cell death cascade following cerebral ischemia.

MALDI-MS-imaging in a mouse model of focal ischaemia

Carswell, H. V. O.[a], Goodwin, R. J. A.[b], Cobb, S. R.[c], McCabe, C.[d] and Pitt, A. R.[a]
[a]*Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK*
[b]*Sir Henry Wellcome Functional Genomics Facility, Institute of Biomedical and Life Sciences, University of Glasgow, UK*
[c]*Division of Neuroscience and Biomedical Systems, Institute of Biomedical and Life Sciences, University of Glasgow, UK*
[d]*Division of Clinical Neuroscience, University of Glasgow, UK*

Introduction: Herein we used a novel approach to proteomics, namely MALDI (matrix assisted laser desorption / ionisation mass spectroscopy) imaging, to map changes in tissue sections in mice after middle cerebral artery occlusion (MCAO). Although in its infancy, MALDI imaging has the potential to resolve and identify markers directly from tissue sections thereby enabling the markers in the MALDI spectra to be localized in the anatomical substrate with significantly enhanced resolution compared with the traditional proteomic analysis of homogenates. *Methods:* Adult male Dax-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita region on X-chromosome, gene-1) knock-out mice, which have endogenous overexpression of aromatase, underwent permanent MCAO. 24 hours later 10 μm thick coronal snap frozen sections were thaw mounted on a conductive surface, and further prepared by a number of washing steps followed by manual pneumatic spray coating with sinapinic acid at optimized concentration and acidity. Analysis was performed on a range of instruments (Bruker and Applied Biosystems) with imaging resolutions down to 50 um. Data collection was optimized and high-throughput validation of markers investigated. *Results:* In this study, biomarker profiles were identified in the mass range up to 30kDa that allowed the localization of anatomical structure in brain and classification of stroke-induced changes compared to controls. Well in excess of 100 target proteins were identified, one of which was ATP synthase subunit e. Immunohistochemistry is currently being performed to confirm identifications. *Conclusion:* MALDI imaging is becoming a powerful method for imaging tissue for markers that define particular regions, and has great potential as a method for mapping changes after stroke.

Endothelin-1 in the subventricular zone of adult mice

Castañeda, M., López Vicchi, M., Suburo, A. M.
Facultad de Ciencias Biomédicas, Universidad Austral, Pilar, Argentina

Endothelin isoforms (ET-1, -2, and -3) signal through receptors ETR-A and ETR-B. ET-1, the best known isoform, is a potent mitogen in many cells, including astrocytes. Since adult neural precursor cells (NPCs) share phenotypical characteristics with astroglia and express glial fibrillary acidic protein (GFAP), we studied endothelinergic molecules in the subventricular zone (SVZ). Cells from this neurogenic niche, located along the lateral wall of the lateral ventricle (LWLV), proliferate and migrate towards the olfactory bulb along the rostral migratory stream (RMS). *Methods:* Male C57Bl/6 mice (6–8 weeks old) were euthanatized and fixed by paraformaldehyde perfusion. Brains were cryosectioned and immunolabeled with antibodies recognizing ET-1, ET1+ET2+ET3 (pan-ET), ETR-A, ETR-B, GFAP and prominin-1, an NPC marker. Antibody binding was detected by immunoenzymatic or immunofluorescence procedures. Granulocyte-Colony Stimulating Factor (G-CSF) or devascularization of cortical areas M1, M2 and S1 were used to enhance NPC proliferation and migration. Experimental groups and their corresponding controls were fixed 5 days after surgery or G-CSF treatment. *Results:* In normal mice, ET-1 and pan-ET immunoreactivities were found in cells and processes along the LWLV and the RMS. ETs also appeared in a few cells within the corpus cal-

losum (CC), dorsal to the RMS. The SVZ showed no ETR-A immunoreactivity, but exhibited low levels of ETR-B. ET-1 and ETR-B immunoreactivities increased after G-CSF treatment or ipsilateral devascularization. Under these conditions, the RMS enlarged laterally beneath the CC and included many cells expressing ET-1, pan-ET-1 and ETR-B immunoreactivities. Cells with the same immunoreactivities also increased within the CC, being most numerous at the cingulum. ETR-A was undetectable in these regions. Every level of the SVZ and RMS showed co-localization of ET-1 and ETR-B with the NPC markers prominin-1 and GFAP. *Conclusions:* The SVZ contained cells with ET and ETR-B immunoreactivities. Endothelinergic cells increased after neurogenesis activation, suggesting a relationship to NPC lineages. Co-localization of ET-1 and ETR-B with characteristic NPC markers supports this hypothesis. Endothelinerg signals could regulate proliferation, migration and differentiation of NPCs, and might also be important for repair of cortical injuries.

Modulation of the activity of the mitochondrial BK-channel and of the permeability transition pore by hypoxia

Cheng, Y.[a], Gu, X. Q.[b], Bednarczyk, P.[c] and Siemen, D.
[a]*Department of Neurology, University of Magdeburg, Germany*
[b]*Department of Pediatrics, University of California, San Diego, USA*
[c]*Nencki Institute of Experimental Biology, Warsaw, Poland*

During stroke-induced hypoxia, the disorder of mitochondrial function in neuronal tissue not only results in an energy deprivation, but also plays a role in neuronal apoptosis. The permeability transition pore (PTP), a mitochondrial ion channel with large conductance (up to 1.3 nS). When open, it is involved in cytochrome c release finally leading to apoptosis. The mitochondrial K-channel of the BK type (BK-channel) is thought to act cytoprotective. We therefore studied the BK-channel and PTP under hypoxia and their interaction. Hypoxia was induced by bubbling with nitrogen for least for 1 hour or by addition of 1 mM dithionite. The oxygen concentration of the hypoxic solution induced by bubbeling with N2 was measured by an oxygraph to be 21 ± 2.7 nmol/ml as compared with 222 ± 19 nmol/ml in the normal solution. We studied the BK-channel and PTP by means of the patch-clamp technique. It was found that the open probability (Po) of the BK-channel was increased under hypoxia. As it was suggested that the PTP is controlled by the BK-channel, we also studied the PTP under hypoxia and found a decreased Po. Further, we measured the mitochondrial membrane potential using fluorescent safranine O. More Ca2+ was required to open the PTP under hypoxia and less Ca2+ was required when the BK-channel selective inhibitor iberiotoxin (100 nM) was present. This result supports our hypothesis that an activated BK-channel keeps the PTP closed helping to protect neurons from apoptosis.

Protein SUMOylation and KAR function in ischemia

Cimarosti, H., Isherwood, M., Webster, R. and Henley, J.
Department of Anatomy, School of Medical Sciences, University of Bristol, UK

In experimentally induced global and focal ischemia and in the brains of hibernating squirrels, where blood flow is severely reduced, protein SUMOylation is hugely increased. During ischemia massive glutamate release leads to excessive glutamate receptor activation and consequent excitotoxic cell death. We showed recently that SUMOylation of the kainate-type ionotropic glutamate kainate receptor (KAR) subunit GluR6 is involved in agonist dependent internalisation. We therefore investigated if SUMOylation and subsequent internalistion of GluR6 may play a neuroprotective role an *in vitro* model of brain ischemia based on oxygen-glucose deprivation of organotypic hippocampal slice cultures. Firstly, we investigated the temporal pattern of protein SUMOylation that follows such an insult, and we are now testing the hypothesis that increased levels of SUMO conjugation confer neuroprotection. Characterization of the changes in levels of SENP-1, the enzyme responsible for deconjugation of SUMO has also been possible. We are currently probing blots for GluR6 to examine the degree to which this receptor subunit is SUMOylated because of OGD. Furthermore, we plan to transduce a number of hippocampal slices with Sindbis virus, engineered to induce overexpression of SUMO or SENP-1. Using propidium ioidide staining, we will be able to directly assess the effect of increased/decreased SUMOylation on the viability of cells following OGD exposure. With this study, we hope to gain insight into the mechanisms involved in

cerebrovascular diseases. Support: Marie Curie Fellowships.

Exposure to a low dose of nicotine causes severe oxidative stress

Ciobica, A.[a], Hritcu, L.[a], Artenie, V.[a] and Padurariu, M.[b]
[a]*Alexandru Ioan Cuza, University, Department of Molecular and Experimental Biology, Iasi, Romania*
[b]*T. Popa University of Medicine and Pharmacy, Iasi, Romania*

Nicotine has been reported to be therapeutic in some patients with certain neurodegenerative diseases and to have neuroprotective effects in the central nervous system. However, nicotine administration may result in oxidative stress by inducing the generation of reactive oxygen species in the periphery and central nervous system. There is also evidence suggesting that nicotine may have antioxidant properties in the central nervous system. The antioxidant properties of nicotine may be intracellular through the activation of the nicotinic receptors or extracellular by acting as a radical scavenger in that it binds to iron. The possibility that nicotine might be used to treat some symptoms of certain neurodegenerative diseases underlies the necessity to determine whether nicotine has pro-oxidant, antioxidant or properties of both. In the present study we evaluated the effects of nicotine treatment (0.3 mg/kg g.c., i.p., SIGMA, 7 continuous days administration), on the antioxidant enzymes activity. We assessed the activity of superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA) in the prefrontal and temporal cortex homogenates after 7 days of continuous nicotine administration. The exposed animals had decreased levels of superoxid dismutase and glutathione peroxidase after nicotine treatment. The level of malondialdehyde was increased. These biochemical evidences suggested that exposure to a low dose of nicotine caused severe oxidative stress.

Real-time imaging of neuroinflammation: gender effects on glial responses in cerebral ischemia

Cordeau Jr, P., Lalancette-Hébert, M., Weng, Y. C. and Kriz, J.
Department of Anatomy and Physiology, Laval University (*CHUL*), *QC, Canada*

Using a reporter mouse expressing luciferase gene under the transcriptional control of murine GFAP promoter (GFAP-luc mice) and biophotonic/bioluminescent imaging as tools, we developed a model-system for *in vivo* analysis of astrocyte activation/response in cerebral ischemia. The analysis of photon emissions from the brains of living animals revealed marked gender differences in astrocyte response to ischemic injury. The increase in GFAP signals was significantly higher in female mice in metestrus/diestrus period as compared to male transgenic mice. Similar results were obtained by quantitative immunohistochemistry. However, astrocyte activation/GFAP signals showed cyclic, estrus-dependent variations in response to ischemic injury. Physiologically higher levels of estrogen and application of pharmacological doses of estrogen during replacement therapy attenuated GFAP up-regulation after stroke. Interestingly, contrary to positive correlation between the intensities of GFAP signals and the size of the infarction in male mice, no correlation was observed in any of the experimental group in female GFAP-luc mice. Our results suggest that GFAP up-regulation in ischemic injury may have different functional significance in female and male experimental animals and may not directly reflect the extent of ischemia-induced neuronal damage in female GFAP-luc mice. Using novel live imaging approach, we demonstrated that early-phase brain inflammatory response to ischemia might be associated with gender-specific biomarkers of brain damage.

Cerebral ischemia induces mouse brain sialidase expression and activity resulting in altered synaptic glycoprotein sialyation

Costain, W. J., Rasquinha, I., Jones, C., Berin, P. and Wakarchuk, W. W.
Glycosyltransferase and Neuroglycomics unit, Institute for Biological Sciences, Ottawa, Canada

Central nervous system neurons are highly susceptible to ischemic damage. Cerebral ischemia is known to induce numerous alterations in gene and protein expression that are involved in mediating neuronal cell death and brain remodeling. Furthermore, cerebral ischemia induces dramatic and rapidly reversible structural changes in dendritic spine morphology that precede delayed post-ischemic neuronal death. This suggests that synaptic stress may initiate signals that propagate toward the cell body and instigate delayed cell

death through a process described as synaptic apoptosis. Extracellular and neuronal membrane glycoproteins are commonly modified with glycans that possess a terminal sialic acid residue. Here we have characterized the effect of cerebral ischemia on brain sialidase expression and activity. Additionally, we examined the effects of cerebral ischemia on the levels of synaptosomal glycoprotein sialyation. Samples were prepared from contralateral (CT) and ipsilateral (ISC) hemispheres from adult male C57 mice subjected to a transient (60 min) middle cerebral artery occlusion (MCAO) followed by 3–20 hrs of reperfusion. QPCR experiments revealed that the expression of Neu1 was significantly increased in ISC brain. Although no significant differences in Neu1 protein levels were detected, ISC brain sialidase activity was significantly increased. CT and ISC synaptosomal proteins were separated using 2D PAGE gels and transferred to nitrocellulose. Sialic acid specific biotin conjugated lectins (SNA I and MAL II) were used to identify synaptosomal sialo-proteins. Numerous sialic acid containing glycoproteins were detected in synaptosomes. Finally, cerebral ischemia induced numerous alterations in synaptosomal sialo-protein expression as well as glycosylation. The results presented here indicate that cerebral ischemia affects the levels of synaptic sialic acid containing proteins and that this effect is likely to be related to alterations in sialidase activity.

Systemic inflammation and biochemical and morphological alterations in the brain. Implication for neurodegeneration

Czapski, G. A.[a], Cakala, M.[a], Gajkowska, B.[a], Smialowska, M.[a] and Strosznajder, J. B.[a]

[a]*Department of Cellular Signalling, Department of Cell Ultrastructure, Medical Research Centre, Warsaw, Poland*

[b]*Department of Neurobiology, Institute of Neurobiology, Cracow, Poland*

Inflammatory reaction is a component of brain ischemia – reperfusion pathology and of the most neurodegenerative diseases. Our previous study carried out in Alzheimer's disease model indicated that systemic inflammation evoked by lipopolysaccharide (LPS) depending on dose and time of its application differently affected biochemical processes in the brain and memory in animals. LPS, a component of the wall of Gram-negative bacteria, is a commonly used for brain preconditioning to ischemia or to evoked systemic inflammation. However, the molecular alterations in the brain after systemic LPS administration are not fully understood. The aim of present study was to analyze the signaling cascades activated in the adult brain during mild systemic inflammatory reaction. Our results indicated that systemic inflammatory reaction evoked free radical cascade, lipid peroxidation, poly(ADP-ribose) polymerase (PARP) activation, β-NAD+ depletion and mitochondria failure in the brain. Systemic LPS administration increased inducible nitric oxide synthase (iNOS) expression and increased NOS activity in substantia nigra (SN) and midbrain. Moreover, the enhancement of TNFα and cyclooxygenase-2 (COX-2) was found in midbrain and hippocampus. Inhibitors of PARP, iNOS and cNOS prevented LPS-evoked molecular changes and inhibited translocation of apoptosis inducing factor (AIF) from mitochondria to nucleus. Electron-microscopic analysis revealed significant ultrastructural alterations: swelling of mitochondria, chromatin condensation, and deep invaginations of the nuclear membrane and the presence of many autophagolysosomes. These alterations demonstrate activation of apoptotic/autophagic processes in many neuronal cells of SN and hippocampus during systemic inflammation. Immunohistochemical analysis suggested a decreasing in tyrosine hydroxylase (TH)-immunoreactivity (ir) in SN neurons and in TH-ir fibres in medial forebrain bundle. In some mice, a decrease in the density of neuropeptide Y-ir neurons in CA regions of the hippocampus was observed (especially24h after LPS treatment). Our data indicated that mild systemic inflammation affects NO and arachidonic acid signaling cascades in the brain, that may influence proapoptotic processes.

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Overexpression of UCP2 protects thalamic neurons following global ischemia in the mouse

Deierborg, T.[a], Wieloch, T.[a], Diano, S.[b], Warden, C.[c], Horvath, T. L.[b] and Mattiasson, G.[a]

[a]*Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, Lund, Sweden*

[b]*Section of Comparative Medicine, Ob/Gyn and Neurobiology, Yale University School of Medicine, New Haven, USA*

[c]*Department of Pediatrics, Division of Clinical Nutrition, Endocrinology and Vascular Biology, and Section of Neurobiology, Physiology, and Behavior, University of California, Davis, USA*

Uncoupling protein 2 (UCP2) is upregulated in the brain following sublethal ischemia, and overexpression of UCP2 is neuroprotective in several models of neurodegenerative disease. We investigated if increased levels of UCP2 diminished neuronal damage following global brain ischemia by subjecting mice overexpressing UCP2 (UCP2/3tg) and wild-type littermates (wt) to a 12 min global ischemia. The histopathological outcome in the cortex, hippocampus, striatum and thalamus was evaluated at 4 days of recovery, allowing maturation of the selective neuronal death. Global ischemia led to extensive cell death in the striatum, thalamus and in the CA1 and CA2, and less pronounced cell death in the CA3 and DG hippocampal subfields. Histological damage was significantly lower in the ventral posterolateral and medial thalamic nuclei in UCP2/3tg animals compared to wt. These thalamic regions showed a larger increase in UCP2 expression in UCP2/3tg compared to wt animals relative to the non-protected DG. In the other regions studied, the histological damage was lower or equal in UCP2/3tg animals compared to wt. Consequently, neuroprotection in the thalamus correlated with a high expression of UCP2, which is neuroprotective in a number of models of neurodegenerative diseases.

MicroRNA: A new player in post-stroke brain damage

Dharap, A. and Vemuganti, R.
Department of Neurological Surgery, Clinical Science Center, University of Wisconsin, Madison, USA

Focal ischemia alters cerebral gene expression in a time dependent manner which is thought to mediate ischemic neuronal damage. Recent studies showed that microRNAs (miRNAs) which are non-coding small RNAs inhibit translation or degrade mRNAs specifically. We evaluated the cerebral microRNAome as a function of reperfusion time following transient middle cerebral artery occlusion in adult rats. Microarray analysis to estimate 238 rat specific miRNAs showed that normal rat cortex express $>$100 miRNAs at a moderate to high level. Following focal ischemia, 12 miRNAs altered rapidly (3 upregulated and 9 downregulated) by 3h of reperfusion. The miRNAs changed progressively with time up to 3 days of reperfusion. At 3 days, 60 miRNAs altered significantly (36 up- and 33 down-regulated). Approximately 50% of the miRNAs altered after MCAO showed $>$6 fold change at various reperfusion times compared to sham. The bioinformatics analysis using TargetScan showed correlation of several pro-inflammatory mRNAs known to be increased after ischemia with 4 down-regulated miRNAs indicating an inverse relationship. These studies show that miRNAs might play a role in controlling post-ischemic pathophysiological events by influencing the down-stream mRNAs. Understanding the functional significance of miRNAs might lead to the development of novel molecular therapies to minimize stroke-induced neuronal damage. Supported by NIH grant NS061071.

Carbamylated erythropoietin derivative is neuroprotective in a hypoxia model of mouse brain injury

Ding, J.[a], Lu, C.-Z.[b], Wang, X.[a] and Xiao, B.-G.[b]
[a]*Department of Neurology, Zhongshan Hospital, Fudan University, Shanghai, China*
[b]*Institute of Neurology, Fudan University, Shanghai, China*

Increasing evidences confirm that carbamylated erythropoietin derivative (CEPO) is a promising candidate of neuroprotection. In our study, we evaluated the neuroprotective capability of CEPO compared to erythropoietin (EPO) in a mouse model of cerebral hypoxia. The mouse was exposed to hypoxia (8% O2) in a special box for 0.5 h, 1.5 h, 3 h, and 6 h, respectively. The mouse under normoxic condition was used for controls. Y maze test demonstrated a time dependent learning ability deficit 1 week after hypoxia. The number of NeuN positive neurons in CA1 and CA3 was significantly decreased in 3h and 6h after hypoxia compared to the control group. After 6 h of hypoxia, EPO, CEPO and saline were i.p. injected into hypoxia mice, respectively. Y maze test showed that mouse learning ability persevered in CEPO group as well as in EPO group compared to the saline group, which was declined significantly in the 10 and 30 day after hypoxia. The NeuN positive neurons lost in CA1 and CA3 significantly lower in EPO and CEPO group compared to the saline group. Brdu/DCX double-stained cells in SVZ and DG area were higher in EPO and CEPO group than in saline group at 7 and 14 days after hypoxia,

indicating that EPO and CEPO could enhance the proliferation of adult neural stem cells in hypoxia mice. At 30 days after hypoxia, Brdu positive cells migrated into dentate gyrus, which was double stained with NeuN. The number of Brdu+ cells in corpus callosum which was double stained with F4/80 also higher in EPO and CEPO group than in saline group at 7 and 14 days after hypoxia. These results suggest that CEPO, Like EPO, enhances neurogenesis and differentiation in hypoxia brain injury. In addition, we observed that both CEPO and EPO might influence microglia proliferation in mouse hypoxia brain.

A role for heat shock transcription factor 1 in focal cerebral ischemia in mice

Duque Santos, S.[a], Lambertsen, K. L.[b], Finsen, B.[b] and Saraiva, M. J.[a]
[a]*Molecular Neurobiology Unit, Institute for Molecular and Cell Biology – IBMC, University of Porto, Portugal*
[b]*Medical Biotechnology Center, University of Southern Denmark, Odense, Denmark*

The Heat Shock Transcription Factor 1 (HSF1) is known as a master stress inducible regulator that rapidly modulates the synthesis of heat shock proteins (hsps) which in turn counteract particular stimuli. In brain ischemia both HSF1 activity and the influence of hsps have been reported.The mouse model knock-out for HSF1 is an invaluable tool in the study of HSF1 pathway(s) in brain injury and repair. We have studied the HSF1-KO brain, in conditions of cerebral ischemia induced by permanent middle cerebral artery occlusion. Infarct volumetric analysis showed a significant decrease in HSF1 $-/-$ infarct volume compared to HSF1 $+/+$ and HSF1 $+/-$. To exclude the possibility that this difference was due to a decreased volume of the cerebral cortex in HSF1 $-/-$ mice, we estimated the volume of unlesioned neocortex in HSF1 $+/+$, $+/-$ and $-/-$ brains. There was no difference in neocortical volume between HSF1 $-/-$ and HSF1 $+/-$ or $+/+$ meaning that the decreased infarct volume was a result of HSF1 abolishment. Next, microglial-macrophage CD11b expression was studied and as already reported in conditions of brain ischemia macrophage infiltration and microglia activation were observed in the infarct and in the peri-infarct areas. However, histological scoring of microglial-macrophage reactions indicated an increased macrophage infiltration in comparison to control mice. We also analyzed TNF-alpha expression and found an increase in this pro-inflammatory cytokine in HSF1 $-/-$ mice. Since TNF-alpha has been reported to have neuroprotective effects, the results indicate that HSF1 may influence cerebral infarction by modulating inflammatory pathways during brain ischemia. We aim in the future to identify other genes/proteins responsible for the observed decrease in infarct volume in the absence of HSF1.

Activated macrophages contribute to photoreceptor cell death

Ebert, S., Weber, B. H. F. and Langmann, T.
Institute of Human Genetics, University of Regensburg, Germany

Background: Retina dystrophies are characterized by photoreceptor degeneration through apoptotic mechanisms. Own experiments with Retinoschisin-deficient mice have identified microglia activation preceding apoptotic mechanisms in the retina. We hypothesize that activated microglia may exert cytotoxic effects and play an important role in photoreceptor cell death. *Methods:* RAW264.7 macrophages and BV2 microglia cells were activated for 3, 6, 24 and 48 hours with LPS (100 ng/ml) or IFNγ (20 ng/ml). 661 W photoreceptor cells were incubated for 3, 6, 24 and 48 h with the supernatant of LPS- and IFNγ-stimulated RAW264.7 and BV2 cells, respectively. Activation and NO-release of RAW264.7 and BV2 cells was quantified with a nitrite assay. The induction of apoptosis in 661W cells by macrophage products was investigated using lactate dehydrogenase (LDH) and Caspase 3/7 assays. Results. LPS and IFNγ strongly increased the production of NO in RAW264.7 cells (47 μM and 55 μM, respectively, compared to 2 μM in control media). Treatment of 661W cells with supernatants from stimulated RAW264.7 and BV2 cells caused increased cell death. The viability of 661W cells (LDH assay) incubated with macrophage-conditioned media for 48h was reduced to 25% (RAW264.7) and 20% (BV2) compared to cells grown in basal media. The activity of apoptotic Caspases 3 and 7 was increased 7-fold in 661W cells incubated with BV2-conditioned media for 48h and even 13-fold in 661W cells incubated with RAW264.7-conditioned media for 48 h. *Conclusion:* Our results suggest that activated macrophages and microglia release soluble products that induce cell death of cultured photoreceptor cells. The mechanisms of photoreceptor cell death directly involve apoptotic pathways.

Bcl-2 upregulation and neuroprotection in guinea pig brain following chronic simvastatin treatment

Eckert, G.[a], Franke, C.[a], Nöldner, M.[b], Abdel-Kader, R.[a], Johnson-Anuna, L. N.[c], Wood, W. G.[c] and Müller, W. E.[a]
[a]*Department of Pharmacology, ZAFES, Biocenter Niederursel, University of Frankfurt, Germany*
[b]*Dr. Willmar Schwabe Group, Karlsruhe, Germany*
[c]*Department of Pharmacology, University of Minnesota, School of Medicine and Geriatric Research, Education and Clinical Center, Veterans Affairs Medical Center, Minneapolis, USA*

The present study determined if chronic simvastatin administration *in vivo* would provide neuroprotection in brain cells isolated from guinea pigs after challenge with the Bcl-2 inhibitor HA 14-1 or the NO donor sodium nitroprusside (SNP). Bcl-2 levels were significantly increased in brains of simvastatin-treated guinea pigs while levels of the pro-apoptotic protein Bax were significantly reduced. The ratio of Bax/Bcl-2, being a critical factor of the apoptotic state of cells, was significantly reduced in simvastatin-treated animals. Cholesterol levels in the brain remained unchanged in the simvastatin group. Brain cells isolated from simvastatin treated guinea pigs were significantly less vulnerable to mitochondrial dysfunction and caspase-activation. These results provide new insight into potential mechanisms for the protective actions of statins within the CNS where programmed cell death has been implicated.

Combined therapeutic strategy using erythropoietin and mesenchymal stem cells potentiates neurogenesis following transient focal cerebral ischemia in the rat

Esneault, E.[a,b], Pacary, E.[a], Eddi, D.[a], Toutain, J.[a], Touzani, O.[a], Petit, E.[a], Schumann-Bard, P.[c], Roussel, S.[a] and Bernaudin, M.[a]
[a]*CI-NAPS UMR 6232, Université de Caen Basse-Normandie, Université Paris Descartes, CNRS, CEA, INSERM. CERVOxy group "Hypoxia and cerebrovascular pathophysiology", Centre Cyceron, Caen, France*
[b]*Porsolt and Partners Pharmacology, Le Genest Saint Isle, France*
[c]*Groupe Mémoire et Plasticité comportementale (GMPc), UFR des Sciences Pharmaceutiques, Université de Caen Basse-Normandie, Caen, France*

Many studies showed beneficial effects of either erythropoietin (EPO) or mesenchymal stem cells (MSC) treatments in cerebral ischemia. In addition to a neuroprotective role, EPO but also MSC favour neurogenesis and functional recovery. In attempt to improve further post-ischemic tissue repair, we investigated the effect of a systemic administration of MSC, in presence or not of EPO, on neurogenesis and functional recovery in a transient focal cerebral ischemia model in the adult rat. Twenty-four hours after ischemia, the rats were divided into 4 groups (PBS; MSC; EPO; MSC/EPO) and received a single intravenous injection of MSC (2.106 cells) and/or a repeated intraperitoneal administration of EPO (1000 UI/kg) during 3 days. The lesion volume, the MSC outcome, neurogenesis and functional recovery were assessed 51 days after ischemia. The results showed that cellular proliferation and neurogenesis were increased in the ipsilateral subventricular zone in the MSC/EPO group whereas no significant effect was observed in groups receiving MSC or EPO alone. MSC expressing neuronal or glial markers were detected in the ischemic hemisphere. These results suggest that EPO could act in a synergistic way with MSC to potentiate the post-ischemic neurogenesis. We thank H. Lundbeck A/S (Copenhague, Denmark) for their contribution to this project.

Long-term hypothermia using H_2S greatly reduces infarct volume in aged rats after focal ischemia

Florian, B.[a], Buga, A.-M.[a], Walker, L.[a], Kessler, C.[a] and Popa-Wagner, A.[a]
[a]*Department of Neurology, University of Greifswald, Germany*
[b]*Yerkes National Primate Research Center and Department of Neurology, Emory University, Atlanta, USA*

Background and Purpose: In aged humans, stroke is a major cause of disability for which no neuroprotective measures are available. A viable alternative to conventional drug-based neuroprotective therapies is brain/body cooling, or hypothermia. In animal studies of focal ischemia, short-term hypothermia consistently reduces infarct size. Nevertheless, efficient neuroprotection requires long-term, regulated lowering of whole body temperature. Methods. Focal cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery in 17 month-old male *Sprague Dawley* rats. After stroke, the aged rats were exposed for 2 days to a mixture of air and a mild inhibitor of oxida-

tive phosphorylation, H2S, which resulted in sustained, deep hypothermia ($27.8 \pm 0.3\,^{\circ}$C). *Results:* Long-term hypothermia led to a 50% reduction in infarct size without obvious neurological deficits or physiological side-effects in aged rats. Conclusions. Prolonged, gaseous hypothermia is a simple and efficacious method to limit damage inflicted by stroke.

A re-examination of the capability of magnetic resonance imaging to observe macrophage infiltration into ischemic tissue

Farr, T. D., Seehafer, J. and Hoehn, M.
In-Vivo-NMR-Lab, Max-Planck-Institute for Neurological Research, Cologne, Germany

Stroke produces an inflammatory response partially characterized by blood borne macrophage infiltration of the injured tissue. Magnetic resonance imaging (MRI) of this phenomenon has been attempted using systemic venous administration of iron oxide for phagocytosis by macrophages. This technique has been used in models of photothrombosis and permanent middle cerebral artery occlusion (MCAO). However, results following transient MCAO were less promising; therefore, the aim of the present study was to establish a protocol compatible with that model. Male Wistar rats received 60 minutes of transient MCAO using the intraluminal filament technique. Animals were allowed to recover and MRI was performed at 2 ($n = 3$), 3 ($n = 3$), or 6 days ($n = 3$) post-MCAO. The scan package was composed of T2-, T2*-, and T1-weighted scans. At the conclusion of the imaging session animals were intravenously infused with 300 μmol Fe/kg of contrast agent, and imaged again the following day. Immediately afterwards, animals were perfusion fixed and tissue processed for histology. Prussian blue stain was performed to detect iron-containing macrophages, and immunohistochemistry for the macrophage marker ED-1, and the dextrane coating on the contrast agents, was also performed. Ischemic tissue was clearly visible in the T2-weighted images and quantitative maps. However, there was no area of observable signal change in any of the animals that could be attributed to the accumulation of iron laden macrophages in the T2*-weighted images or maps, or T1- weighted images, despite the clear presence of contrast agent in the blood. ED-1 immunohistochemistry revealed extensive macrophage accumulation in the infarcted tissue. However, Prussian blue positive macrophages were sparse and typically located in areas where small hemorrhagic transformations were thought to have occurred. Furthermore, no evidence of dextrane immunohistochemistry was observed in the brain. These results indicate that imaging the inflammatory response following transient MCAO is not straightforward. There is rising concern that non-specific accumulation of contrast occurs when it is administered near to the time of the insult, or when blood brain barrier breakdown and erythrocyte accumulation are present. Therefore, caution should be taken when evaluating experiments that rely on systemic administration of contrast agents to observe the inflammatory response. Acknowledgements: The authors wish to acknowledge Drs. Philippe Robert and Claire Corot (Guerbet) for supply of contrast agent. This work was supported by a grant from the StemStroke EU-FP6 program (LSHB-CT-2006-037526) and from the BMBF project “Stem cell based regeneration after stroke” (01GN0509).

Severe hypoxia reliably induces spreading depression in rat brainstem slices

Funke, F., Chaudhry, U., Kron, M., Dutschmann, M. and Müller M.
Zentrum Physiologie und Pathopysiologie, Georg-August-Universität Göttingen, Germany

Spreading depression (SD) reflects a concerted, massive depolarization of neurons and glia, which slowly spreads within the gray matter. Being associated with pathological conditions such as anoxia and ischemia, it has been well characterized in cortex and hippocampus. Little is known, however, about the susceptibility of the brainstem to SD. Exposing brainstem slices (400 μm) of neonatal rats (p5-13) to severe hypoxia, triggered hypoxic SD (HSD) within a few minutes as indicated by a sudden -10 to -25 mV shift in the extracellular DC potential; [K+]o rose to $\sim$50 mM. The DC potential deflections showed the characteristic profile and quickly recovered upon reoxygenation. Block of inhibitory synapses by 0.5 μM strychnine plus 20 μM bicuculline markedly hastened the onset of HSD. DC potential recordings from various brainstem nuclei in combination with monitoring of the intrinsic optical signal (IOS) showed that HSD was ignited preferably in the trigeminal nucleus. It then spread out medially and ventrally, also invading the hypoglossal nucleus, nucleus of the solitary tract, ventral respiratory group, and on several occasions also the contralateral hemisphere. As

judged from the IOS, HSD propagated discontinuously, and it occasionally arose from several spots, which might be due to the heterogeneous structure of the brainstem. Brainstem slices of adult rats (4–6 weeks) only produced slow, moderate DC potential changes that did not qualify as characteristic HSD episodes, mainly because they were not accompanied by an IOS. Extended durations of hypoxia, poisoning of glial cells with fluoroacetate or inhibition of inhibitory synapses by strychine plus bicuculline failed to facilitate the generation of HSD. However, upon cell swelling – induced by hypoosmolar solutions – characteristic HSD episodes could also be induced in adult slices. In conclusion, brainstem tissue is capable of generating HSD episodes, which may be of importance for pathological conditions such as basilar type migraine and brainstem infarcts. Against expectation, the susceptibility to the generation of HSD is highest in neonatal brainstem, whereas in adult brainstem facilitating maneuvers are required. This age preference may indicate a possible link of brainstem HSD and neonatal brainstem pathology such as sudden infant death syndrome.

Novel subcellular localization of two brain-specific proteins, p42$^{\mathbf{IP4}}$ (Centaurin-α1) and CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase) in mitochondria

Galvita, A.[a], Baburina, Y.[a,b], Grachev, D.[a,b], Krestinina, O.[a,b], Azarashvili, T.[a,b], Stricker, R. [a] and Reiser, G.[a]
[a]*Institute for Neurobiochemistry, Otto-von-Guericke University, Medical Faculty, Magdeburg, Germany*
[b]*Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, Pushchino, Russia*

In brain, p42IP4 (Centaurin-α1), mainly expressed in neurons, probably operates as dual receptor recognising the soluble inositol phosphate Ins(1,3,4,5)P4 (IP4) and the lipid PtdInsP3 (PIP3), two second messengers. As mitochondria make a significant contribution to Ca^{2+} homeostasis and contain phosphoinositides we suggest a possible localisation of p42$^{\mathrm{IP4}}$ in rat brain mitochondria (RBM). We have shown for the first time localisation of p42$^{\mathrm{IP4}}$ in isolated RBM. Mitochondrial localisation of p42$^{\mathrm{IP4}}$ was confirmed by immunoprecipitation from purified mitochondria from CHO cells transfected with p42$^{\mathrm{IP4}}$ (CHOt M) and by subfractionation of CHOt M. Detection of p42IP4 in the mitochondrial inner membrane indicates the existence of new functions for this protein. Previously, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) has been shown to be associated with mitochondria, but the exact role of CNP in mitochondria is still obscure. Opening of Ca^{2+}-induced permeability transition pore (PTP) in mitochondria is crucial in the control of processes leading to programmed cell death. The PTP is a multi-component protein aggregate in the mitochondrial membranes. We have shown the localization of CNP in both mitochondrial membrane fractions by sub-fractionation of RBM. The discovery of CNP in the inner membrane might indicate its role in the regulation of the major mitochondrial energy-transforming systems. We found association of CNP with functional complexes I-V of the inner mitochondrial membrane. Moreover, Ca^{2+}-induced PTP opening changed the CNP distribution among the respiratory chain complexes. These results might provide evidence that CNP is connected to the PTP complex operation.

Neuroprotection in hypoxia-tolerant mole rats: Potential role of respiratory globins

Gerlach, F.[a], Avivi, A.[b], Burmester, T.[c], Nevo, E.[b] and Hankeln, T.[a,*]
[a]*Institute of Molecular Genetics, University of Mainz, Germany*
[b]*Institute of Evolution, University of Haifa, Israel*
[c]*Institute of Zoology, University of Mainz, Germany*

[*]Presenting author.

The blind subterranean Israeli mole rat (genus Spalax) is a model for naturally occurring neuroprotection. Mole rats are able to survive extended periods of severe hypoxia in their underground burrows without neurological damage. We have asked whether respiratory proteins (globins) expressed in nerve cells contribute to this unusual hypoxia-tolerance.

Neuroglobin, primarily expressed in neurons of the CNS and PNS, and cytoglobin, found in fibroblasts of all organs and in distinct neuronal cells, are recently discovered O2-binding respiratory proteins of vertebrates. Their physiological function is discussed related to O2 supply, ROS scavenging, NO detoxification and other possibly neuroprotective mechanisms. Here we report expression patterns of these globins by comparing *Spalax* to normal laboratory rats. Quantitative RT-PCR and Western blot analyses show that both globins are expressed at elevated levels in *Spalax* versus rat total

brain at normoxic conditions. This suggests that neuro- and cytoglobin contribute to the natural neuroprotection of *Spalax*, preadapting the animals to hypoxic stress. For neuroglobin, we also observed a shift in the cellular expression pattern: *Spalax* astroglia cells reveal a pronounced neuroglobin immunoreactivity, while rats (and mice) express neuroglobin almost exclusively in neurons. This expressional shift to glia cells may be a crucial neuroprotective feature of hypoxia-tolerant organisms, since it was recently also observed in deep-divings seals (Folkow et al., submitted).

Single-cell resolution mapping of metabolic alterations in focal cerebral ischemia – a thallium uptake study using thallium diethyldithiocarbamate as a tracer

Goldschmidt, J.[b,c], Baldauf, K.[d], Ziabreva, I.[d], Scheich, H.[b], Schröder, U. H.[a,d] and Reymann, K. G.[a,d]
[a]*Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Department Auditory Learning & Speech, Magdeburg, Germany*
[c]*Department Neurology II, Otto-von-Guericke University Magdeburg, Germany*
[d]*Research Institute for Applied Neuroscience* (*FAN*) *gGmbH, Magdeburg, Germany*

The lipophilic chelate complex thallium diethyldithiocarbamate, TlDDC, with the gamma-emitting thallium isotope 201Tl, has been introduced in the 1980s for single photon emission tomography (SPECT) imaging of cerebral blood flow in humans. We here analyzed the potential uses of TlDDC for delineation of ischemic injury in rats in hyperacute and late stages in a rat MCAO model of focal cerebral ischemia. We systemically injected TlDDC 15 minutes and 7 days after medial cerebral artery occlusion. We used a recently developed histochemical technique, thallium autometallography, for non-radioactive high-resolution mapping of the thallium distribution in the brain. Contrary to what is expected from a lipophilic compound we find, at the cellular level, complex patterns of the Tl+ distribution, which are incompatible with the assumption of passive diffusion of TlDDC into the brain. The distribution indicates instead that Tl+ is released from the complex into the brain extracellular space, from which neurons and glial cells take up the tracer in an activity-dependent manner. Since Tl+ is a well-established K+-probe the images we show here provide insight into brain potassium metabolism in hyperacute and late stages of cerebral ischemia. In the hyperacute phase we find a core region where Tl+-uptake is dramatically reduced in neurons while astrocytes still take up the tracer. In the region surrounding the core astrocytes are intensely stained while in neurons both decreases and increases in Tl+-content can be observed. In the late stage, glial uptake is markedly increased. As the TlDDC complex enables Tl+ to enter the brain tissue and thus also the gamma-emitting tracer 201Tl we suggest the use of TlDDC for imaging brain potassium metabolism and tissue viability in intact animals and in humans via single photon emission computed tomography.

Vesicular GABA transporter (VGAT) is cleaved by calpains under excitotoxic conditions, generating a non-synaptic protein

Gomes, J.[a], Melo, C.[a], Inacio, A.[b], Almeida, L.[a], Wieloch, T.[a,b] and Duarte, C.[a]
[a]*Center for Neuroscience and Cell Biology, University of Coimbra, Portugal*
[b]*Wallenberg Neuroscience Center, Lund University, Sweden*

GABA is the major inhibitory neurotransmitter in CNS, and may have a neuroprotective role under excitotoxic conditions. However, activation of GABAA receptors may also contribute to neuronal damage due to Chloride influx and consequent neuronal swelling. The extent of neurotransmitter release is regulated by the expression of vesicular transporters, which control the quantal efficacy of synaptic transmission. Therefore, putative changes in VGAT protein levels during ischemia may affect GABAergic transmission and cell death. In this work we investigated the fate of VGAT in cultured hippocampal neurons, under excitotoxic conditions. We found that VGAT (58 KDa) is cleaved upon glutamate stimulation of hippocampal neurons, in a biphasic manner, giving rise to a product of about 46 KDa (tVGAT). About 40% of VGAT is cleaved within the first hour after glutamate stimulation, and 35% of the protein is cleaved 2–7 h later. Truncated VGAT is stable for more than 24 h. Under the same conditions, VGAT mRNA is downregulated, by a process of active mRNA degradation. Calpain I inhibition with ALLN or MDL28170 prevented the cleavage of VGAT in hippocampal neurons subjected to excitotoxicity. Inhibition of Caspases with

Z-VAD-FMK did not affect glutamate-induced VGAT cleavage. VGAT cleavage was also observed in the cerebral cortex and striatum following transient Middle Cerebral Artery Occlusion (MCAO), a cerebral ischemia model. VGAT was also cleaved following intrahippocampal injection of kainate, a different model of *in vivo* excitotoxicity, but no effect was observed in transgenic mice overexpressing calpastatin, an endogenous calpain inhibitor. *In vitro* studies, using cerebrocortical synaptic vesicles and recombinant calpain I, also showed the cleavage of VGAT to the same product of about 46KDa. Immunoblot experiments using different antibodies against VGAT showed that calpain cleaves VGAT in the N-terminal region of the protein, giving rise to two similar truncated forms, at amino acid 52 and amino acid 60. Imunocytochemistry of GABAergic striatal neurons expressing GFP fusion proteins with VGAT or tVGAT proteins, showed that tVGAT immunoreactivity loses the synaptic localization, being homogenously distributed along the axons. This change in VGAT distribution was also observed with endogenous VGAT in glutamate stimulated hippocampal cultures. Taken together, our results show that glutamate toxicity causes a down-regulation of VGAT, with a concomitant generation of a truncated VGAT, which is likely to affect GABAergic neurotransmission and may influence cell death, during ischemia. (Supported by FCT and FEDER)

Activated protein C protects hippocampal neurons from thrombin-induced toxicity

Gorbacheva, L.[a], Strukova, S.[a], Pinelis, V.[b], Ishiwata, S.[c] and Reiser, G.[d]
[a]*The Lomonosov Moscow State University, Russia*
[b]*The Scientific Center for Children Health, Russian Academy Medical Science, Russia*
[c]*Waseda University, Tokyo, Japan*
[d]*Institute for Neurobiochemistry, Otto-von-Guericke University, Medical Faculty, Magdeburg, Germany*

Thrombin is produced immediately from blood prothrombin after primary or secondary brain trauma and its sufficiently high concentrations may be found within CNS. Activated protein C (APC) anti-inflammatory effects were believed to be linked to down-regulation of the generation of thrombin, which is known for its pro-inflammatory properties. It is well accepted that inflammation, coagulation and apoptosis occur concomitantly in sepsis and are intimately linked. We investigated the role of APC in cell survival and NFkB activation in cultured rat hippocampal neurons treated with the high concentration of thrombin. The level of NF-kBp65 was determined using two approaches, by the anti-NFkBp65 antibodies and NF-kBp65 total kit ELISA (Biosource). Cell death was determined using biochemical approach, which was based on the measurement of lactate dehydrogenase level (LDH) in the medium. We observed that thrombin in high concentrations (more then 10 nM) induces death in cultured hippocampal neurons. For example, 100 nM thrombin induced cell death in 32% of neurons, which is comparable with glutamate-induced death. The 15-minute pretreatment of neurons with APC protects cells from thrombin-induced toxicity. The investigations of NFkBp65 translocation in nucleus indicate that 10 nM thrombin did not change the activity of NFkBp65 but the increase of thrombin concentration until 50 nM resulted in almost two-fold growth the nuclear fraction of NFkBp65. The 15-minute pretreatment of cells with APC abolished the effect of high concentration of thrombin and the nuclear level of NFkBp65 did not differ from the control data. These results show that the high concentrations of thrombin may play the role of degeneration and pro-inflammatory factor and APC is able to protect neurons from thrombin by reduce activation of NFkB. Thus the neuroprotective effects of APC were realized by NFkB-depended pathway to like its' anti-inflammatory effect.

Ablation of proliferating microglia does not affect motor neuron degeneration in ALS caused by SOD1 mutations

Gowing, G.[a], Philips, T.[b], Robberecht, W.[b] and Julien, J. P.[a]
[a]*Research Centre of CHUQ, Laval University, Canada*
[b]*Laboratory for Neurobiology, University of Leuven, Belgium*

Microglial activation is a hallmark of all neurodegenerative diseases including amyotrophic lateral sclerosis. A detailed characterisation of the microglial cell population within the spinal cord of a mouse model of familial ALS was performed. Using flow cytometry we observed the presence of three distinct microglial populations within the spinal cord of mice overexpressing mutant superoxide dismutase (SOD1). Characterisation of cell proliferation within the CNS of SOD1G93A determined that the expansion in microglial cell population

is mainly due to the local proliferation of early myeloid (CD11b+, CD45int) cells. To assess the contribution of proliferating microglia in motor neuron degeneration, we generated CD11b-TKmut-30; SOD1G93A doubly transgenic mice that allow the elimination of proliferating microglia upon administration of the nucleoside analogue ganciclovir (GCV). Surprisingly, a 50% reduction in reactive microglia specifically in the lumbar spinal cord of CD11b-TKmut-30; SOD1G93A doubly transgenic mice had no effect on motor neuron degeneration.

FK506 reduces pro-inflammatory and cytotoxic activation of cytokine- stimulated rat astrocytes

Gozdz, A. and Kaminska, B.
Nencki Institute, Department of Cell Biology, Warsaw, Poland

Reactive astrogliosis is implicated in many acute and chronic neuropathological conditions and involves astrocyte proliferation, activation and hypertrophy accompanied by production of cytokines, growth factors and metabolic alterations. Astrocyte activation may exert both beneficial and detrimental effects on nervous system cells; therefore, its modulation is an attractive target for neuroprotective therapies. We have demonstrated that a widely used immunosuppressant and calcineurin inhibitior FK506 potently reduced gliosis *in vivo* and improved recovery in a rat stroke model (Zawadzka and Kaminska *Glia, 49:36-51, 2004*). To dissect the mechanism of FK506 action on activated astrocytes, we employed a model of "reactive astrogliosis *in vitro*" based on primary rat astrocyte cultures stimulated with the mixture of pro-inflammatory cytokines: IL1-b, IFN-γ and TNF-α. Cytokine cocktail induced activation of NFkB, p38 MAPK and JNK signaling pathways followed by cellular hypertrophy, rearrangement of astrocyte cytoskeleton, nitric oxide production and expression of mRNA for *il-6* and *trail*. FK506, as well as another calcineurin inhibitor cyclosporin A, reduced the astrocyte hypertrophy. FK506 decreased the level of activated p38 MAPK, as well as down-regulated *trail* mRNA. Interestingly, FK506 was also able to reduce the activation of p38 MAPK in astrocytes exposed to hydrogen peroxide implicating potential of this drug in counteracting some effects of oxidative stress observed during ischemic reperfusion or neuroinflammation. Our data suggest that FK506 may exert its neuroprotective effect partially via inhibition of the pro-inflammatory astroglia activation and implicate a calcineurin as a new candidate for triggering of astrogliosis. Supported by PBZ/MEiN/01/2006/32 (AG).

Progenitors with a neurogenic potential in the neocortex and striatum of the adult brain

Grande, A.[a,c], Sumiyoshi, K.[c,e], Nagao, M.[b,c], Cancelliere, A.[b,c], Sakthivel, B.[b,d], Waclaw, R. [b,c], Aronow, B.[b,d], Campbell, K.[b,c] and Nakafuku, M. [a,b,c,f]
[a]*Department of Neurosurgery, University of Cincinnati College of Medicine, Cincinnati, USA*
[b]*Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, USA*
[c]*Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, USA*
[d]*Division of Biomedical Informatics, Cincinnati Children's Hospital Research Foundation, Cincinnati, USA*
[e]*Department of Neurosurgery, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan*
[f]*Solution Oriented Research for Science and Technology* (*SORST*)*, Japan Science and Technology Agency, Chuo-ku, Tokyo, Japan*

In the adult mammalian brain, continuous production of new neurons and glia occurs only in a few restricted regions, namely the subventricular zone (SVZ) lining the lateral ventricle and subgranular zone of the hippocampal dentate gyrus. Thus, the occurrence of neural stem cells (NSCs) and other neurogenic progenitor cells [herein called neural progenitor cells (NPCs)] are thought to be confined to these so-called neurogenic regions. Previous cell culture studies from many laboratories, however, have reported that cells with the characteristics of NPCs also exist outside the neurogenic regions. The identity of such cells remains currently unknown. It could be that these cells are normally non-NPCs but transform into NPC-like cells in culture. Alternatively, they could represent cells that remain as dormant NPCs in the intact brain. These NPC-like cells may be a source of neuronal cell replacement under certain pathological conditions. In this study, we have identified NPC-like cells in the neocortex and striatum of the adult rodent brain. Our data show that these cells and NPCs derived from the SVZ exhibit overlapping, but distinct molecular properties. Our results further suggest that these cells can be mobilized in situ by exogenous growth factors and genetic manipulations to generate neurons in the ischemic brain.

Genomic response of the rat brain to global cerebral ischemia and reperfusion

Heimann, A.[b], Büttner, F.[a], Cordes, C.[b], Gerlach, F.[c], Alessandri, B.[b], Türeci, Ö.[d], Hankeln, T.[e], Kempski, O.[b] and Burmester, T.[c]
[a]*Institute of Zoology, Johannes-Gutenberg University Mainz, Germany*
[b]*Institute for Neurosurgical Pathophysiology, Johannes-Gutenberg University Mainz, Germany*
[c]*Institute of Zoology and Zoological Museum, University of Hamburg, Hamburg, Germany*
[d]*Department of Internal Medicine III, Division Johannes-Gutenberg University Mainz, Germany*
[e]*Institute of Molecular Genetics, University of Mainz, Germany*

To identify genes that are involved in stroke response, we have evaluated changes of gene expression in rat cerebrum after 15 min of complete global cerebral ischemia, followed by reperfusion for 1 h, 6 h or 24 h. Therefore thirty-four animals were randomized to sham ($n = 16$) and to 15 min ischemia ($n = 18$). Sham-operated animals underwent the same experimental procedures, omitting brain ischemia. Transient cerebral ischemia was induced by permanent occlusion of the left and temporary occlusion of the right common carotid artery combined with simultaneous hypobaric hypotension (reduction of the mean arterial blood pressure (MABP) to 35 mmHg by vacuum-induced venous blood pooling). Physiologic variables (cerebral blood flow, MABP, temperatures) were monitored during baseline, ischemia and during 30 min of reperfusion. Arterial blood gases were measured under baseline conditions and at the end of reperfusion. Animals (sham/ischemia) were allowed to survive 1 h ($n = 6/n = 6$), 6 h ($n = 5/n = 6$) or 24 h ($n = 5/n = 6$) before sacrifice. The left hemisphere was used to analyze gene expression. The transcript levels of seven genes by means of qPCR (std $< 10\%$ within each group) served to select three experimental and three sham controls per group for microarray studies. About 20,000 transcripts were detectable in brains from sham-operated rats. The levels of 576 transcripts ($\sim$2.9%) were significantly altered in response to experimental ischemia. 419 transcripts were up- and 157 downregulated; 39 transcripts changed after 1 h reperfusion, 174 after 6 h and 462 after 24 h. Results from quantitative real-time reverse transcription PCR of 18 selected genes showed excellent agreement with the microarray data. There is little overlap between gene regulation patterns at different reperfusion times (only nine genes displayed significant changes in transcript levels at all times). Several genes have been characterized that had not been identified before to be involved in ischemia-response. Analyses of gene ontology patterns and the most strongly regulated transcripts showed that the immediate response to an ischemia/reperfusion is mediated by the induction of specific transcription factors and stress genes. Inflammation and immune-related genes characterize later gene expression. These results confirm the notion that the brain's response to transient global cerebral ischemia is an active, specific and coordinated process.

Migration and differentiation of endogenous neural stem cells after venous stroke and spreading depression

Heimann, A.[a], Tamaki, R.[a,b], Itugbu, S.[a], Alessandri, B.[a] and Kempski, O.[a]
[a]*Institute for Neurosurgical Pathophysiology, Johannes-Gutenberg-University Mainz, Germany*
[b]*Nara Medical University, Department of Neurosurgery, Nara, Japan*

This study was designed to investigate neurogenesis after venous stroke in the adult rat. In order to examine whether hypoxia/ischemia or cortical spreading depression (SD) triggers migration and differentiation of neural stem cells we investigated 57 Wistar rats, randomized into four experimental groups at two different survival times (9 and 28 days). In order to label proliferating cells, bromodesoxyuridine (BrdU) was injected daily in all animals for seven days, starting after the acute experiment. ANaive@ rats received only BrdU without any operation or intervention. Sham operated animals were subjected to all operative procedures without induction of SD or ischemia (ANo SD@ group). In the ASD@ group spreading depression was induced 10 times every 7 minutes by intracortical micro-injection (2:l Kcl, 150 mmMol). In the ischemia group rats were subjected to a permanent focal cortical venous ischemia which was caused by photochemical occlusion of two adjacent bridging veins, followed by ten induced SDs (ASD plus 2-VO@). The two-vein-occlusion model (2-VO) is characterized by a reproducible infarction within the cerebral cortex and a rather large penumbra-to-core ratio. Physiological parameters were monitored in all operated animals. After 9 or 28 days the brains were harvested and dentate

gyrus (DG) and cortex were stained with doublecortin (DCX) for labeling of immature neurons on day 9, and with NeuN as marker for new neural cells on day 28, respectively. At day 9 there was a significant increase of BrdU positive cells and double stained DCX- and BrdU positive cells in the ipsilateral DG in the ASD@ and ASD plus 2-VO@ group. The cortex of both hemispheres, however, showed increased DCX- and BrdU positive cells only after SD induction and combination with ischemia (SD plus 2-VO). Again, at day 28 BrdU positive cells and NeuN- and BrdU positive cells were augmented in the DG of the ipsilateral hemisphere in the ASD@ and ASD plus 2-VO@ group. In the ipsilateral cortex double labeled NeuN- and BrdU positive cells increased only when SD was combined with ischemia. In conclusion, cortical spreading depression triggers proliferation and maturation of neuroblasts in the DG but not in the cortex. Fully matured neurons can only be found in the cortex after combination of spreading depression with venous ischemia. This suggests that stem cells only migrate and differentiate into the cortex when neuronal damage is present.

Mechanism of neurodegeneration induced by very long chain fatty acids, determined in glial cells and neurons from rat hippocampus

Hein, S.[a], Schönfeld, P.[b], Kahlert, S.[a] and Reiser, G.[a]
[a]*Institute of Neurobiochemistry, Otto-von-Guericke-University, Medical Faculty, Magdeburg, Germany*
[b]*Institute of Biochemistry, Otto-von-Guericke-University, Medical Faculty, Magdeburg, Germany*

Neurodegeneration is caused by multiple insults which are variable in its nature and severity. Moreover, cell death occurs through different mechanisms. Interestingly, free fatty acids play a decisive role in many steps of cell death and inflammation. The toxicity of fatty acids to cells depends on the length and saturation. In our previous work we found that phytanic acid, a saturated branched chain fatty acid, caused rapid cell death of cultured astrocytes (Kahlert et al. 2005). Here we analyzed the toxic activity of saturated very long chain fatty acids (VLCFA; $\geqslant$C22:0). The accumulation of VLCFA is a main characteristic of diseases with peroxisomal impairment, like X-linked adrenoleukodystrophy (X-ALD), a severe hereditary neurodegenerative disease. The toxic effect of VLCFA leading to severe symptoms with progressive and multifocal demyelination, adrenal insufficiency and inflammation remains still obscure. Additionally, the role of the accumulation of VLCFA in blood and tissue is also still unknown. Therefore, we exposed neural cells to VLCFA and analyzed the cellular consequences to understand the mechanism of neurodegeneration occurring in the brain. Our studies revealed a massive cell death of oligodendrocytes and astrocytes challenged with docosanoic- (C22:0), tetracosanoic- (C24:0) and hexacosanoic acids (C26:0) for 24 h. To elucidate the mechanism underlying the toxicity of VLCFA, we analyzed cell physiological parameters in oligodendrocytes, astrocytes and neurons. We found that VLCFA induced depolarization of mitochondria in situ and increased intracellular Ca2+ level in all three brain cell types. In isolated mitochondria, VLCFAs exert a detrimental effect by affecting the inner mitochondrial membrane and promoting the permeability transition. These data provide indications about the mechanism of toxicity of VLCFA. Our findings suggest that there is a potent toxic activity of VLCFA due to dramatic cell physiological effects. This, provides the first evidence for mitochondrially-based cell death mechanisms in neurodegenerative disease with peroxisomal defects and subsequent VLCFA accumulation. Kahlert S, Schonfeld P, Reiser G. 2005. The Refsum disease marker phytanic acid, a branched chain fatty acid, affects Ca2+ homeostasis and mitochondria, and reduces cell viability in rat hippocampal astrocytes. Neurobiol Dis 18(1):110-118.

Effect of transient focal ischemia on the infarct-size and on microglia activation in young and aged rats

Henrich-Noack, P., Miller, A. M. and Lynch, M. A.
Trinity College Institute of Neuroscience Dublin, Ireland

Stroke is predominantly a disease of the elderly population; however, pre-clinical experimental studies have been performed in young animals in the majority of cases so far. The aim of the current study was to compare the effects of transient focal ischemia in young and aged rodents. For this purpose we quantified the infarct areas and the microglia activation as determined by staining with the OX6 antibody. Transient focal ischemia was induced by injecting endothelin-1 under isoflurane anaesthesia bilaterally into the brain of young (3 month old) and aged (22–24 month old) Wistar rats. Seven days after the insult the rats were decapitated and the brains quickly frozen via isopen-

tane and stored at −80 °C. Sequential sections were taken through the brain and immunohistochemistry was performed (ABC-method with DAB as a substrate) on every 10^{th} slice; slices were counterstained with toluidine blue. The data show that there were a significantly greater number of OX6-positive cells in the brain tissue of the aged, compared with the young, animals indicating the presence of activated microglia. There was no difference in post-ischemic mortality in young and aged animals and similarly, infarct area was similar in the brains of young and aged rats. This finding suggests that it is important to use aged rodents for studying stroke, as the post-ischemic pathophysiology may differ between young and aged species.

Time-dependent reduction of infarct volume after focal ischemia in mice

Henrich-Noack, P.[a,c], Baldauf, K.[a,c], Reiser, G.[b] and Reymann, K. G.[a,c]
[a]*Research Institute for Applied Neurosciences* (*FAN*), *Magdeburg, Germany*
[b]*Institute for Neurobiochemistry, Otto-von-Guericke University, Medical Faculty, Magdeburg, Germany*
[c]*Leibniz Institute for Neurobiology, Magdeburg, Germany*

The mouse model of transcranial permanent occlusion of the middle cerebral artery (tpMCAO) is widely-used in stroke research. Here we quantified infarct size using a conventional histological method at several post-ischemic times, going beyond the commonly analysed period of up to 2 days, following artery occlusion. Two different mouse strains, which are widely used for pharmacological studies of neuroprotection and for genetic engineering, were used. For induction of ischemia a drill hole was made into the skull of anaesthetised mice and the middle cerebral artery was occluded by electrocoagulation. For evaluation of the infarct volume, systematic random sampled sections were Nissl-stained and the infarct areas measured on a microscope with an image analysing system. In both mouse strains tested (C57Black/6 and NMRI), the infarct volumes decreased significantly during the first days after tpMCAO. Notably, 13 days after surgery, ischemic and sham-operated animals had indistinguishably small lesions, which where in the range of only 5% of the infarct size on day 2 post ischemia. The standard method of calculating oedema and shrinkage correction provided no sufficient explanation for this significant decrease in infarct volume. There was, however, evidence reporting that structural changes in the residual ipsilateral hemisphere may compromise the significance of results arising from the method of calculating edema and shrinkage correction. In conclusion, our study indicates that the pronounced and fast, time-dependent decrease in histologically defined infarct volume can compromise results when studying the lasting neuroprotective effects of potential drugs.

Altered psychosocial behavior following "minor stroke" in the rat

Hewlett, K., Kelly, M. and Corbett, D.
Division of BioMedical Sciences, Faculty of Medicine Memorial, University Memorial University, St. John's, Canada

Stroke severity is given clinical prognosis based on consequent impairments, such that a stroke is considered 'major' if it results in lasting motor problems, aphasias or paralysis. Thus, patients that regain a near-normal ability to walk and speak are designated as having a 'minor' stroke and discharged without continuing care, despite enduring mental fatigue, disruptions in memory, emotional labiality, or compromised capacity to cope with stress (Jones and King, 2007) which can impede psychosocial health and quality of life. Our laboratory has established rodent stroke models of motor disability and rehabilitation; in the present study we examine the effects of a non-motor ('minor') stroke on social, cognitive and emotional behavior. Groups of male Sprague-Dawley rats were acclimatized to housing facilities in pairs and handled regularly for one week prior to sham surgery or focal ischemia of the left cingulate cortex. After one week of recovery subjects were randomized to chronic stress (isolation in a confinement cage for 6 h/day, 5 days/week for 3 weeks) or control handling. Emotional, cognitive and social behaviors were measured using the Morris water maze (an aversively motivated spatial learning task), elevated plus maze, open field test and observation of home cage social interactions. Testing began one month following surgery and concluded three months later. In a subset of animals cingulate cortex stroke did not cause motor deficits in the staircase test of fine motor function or the cylinder test of forelimb asymmetry. The elevated plus maze was used to determine if cingulate lesions or stress treatment caused pathological fear (anxiety-like behavior). There were no differences in rearing activ-

ity, total arm entries or the ratio of open and closed arm entries. However, there was a significant stroke-stress interaction for fecal boli counts. Stress did not affect the defecation rate in sham animals but caused a five-fold increase in rats with cingulate lesions. Minor stroke, but not stress, had a main effect on activity in the open field. All animals habituated to the apparatus over repeated exposure but animals that received cingulate lesions and stress remained hyperactive compared to other treatments. A similar pattern emerged from rearing activity during open field habituation, whereby ischemic injury augmented vertical exploration whereas stress did not affect rearing behavior. Cingulate lesions significantly slowed task acquisition in the water maze. By the last day of maze testing all animals were able to find the platform as efficiently as sham controls, but cingulate animals ranked higher on qualitative measures of emotionality during testing such as audible distress vocalizations. Interestingly there were no differences in long-term memory in the 48 hr probe test, suggesting that cingulate lesions alone or combined with chronic stress do not impair learning and memory, but rather alter subjective perception of stress. Preliminary results of adrenal mass indicate that both stress and cingulate stroke cause hypertrophy of the adrenal glands, a common result of over-stimulation of the hypothalamic-pituitary-adrenal axis. The findings will be discussed with relation to social behaviors (dominance, submissiveness), steroid hormone levels, and implications for modeling psychosocial symptoms of stroke in the rat.

An experimental model for the study of cerebral cell death after perinatal asphyxia

Hilario, E.[a], Alvarez, F. J.[b], Go ni-de-Cerio, F.[a], Rey-Santano, M.[b], Mielgo, V. E.[b], Caballero, A. [c], Alonso-Alconada, D.[a], Valls-i-Soler, A.[b,d] and Alvarez, A.[a]

[a]*Department Cell Biology & Histology, School of Medicine and Dentistry, University of the Basque Country, Leioa, Spain*

[b]*Research Unit on Pediatrics, School of Medicine and Dentistry, University of the Basque Country, Leioa, Spain*

[c]*Department Neurosciences, School of Medicine and Dentistry, University of the Basque Country, Leioa, Spain*

[d]*Department Pediatrics, School of Medicine and Dentistry, University of the Basque Country, Leioa, Spain*

Aim: Hypoxic-ischemic injury gives rise to brain damage in preterm neonates. To study early damage of hypoxic-ischemic injury in the brain, we set forth the utilization of an experimental model of preterm fetal lambs. *Material and Methods:* Fetal lambs at 90% of gestation (133 days of developmental age; term: 145 days), in which umbilical blood flow was partially occluded during 60 minutes, were randomly assigned to three different experimental groups: Healthy group: without asphyxia and with mechanical ventilation for 3 hours ($n = 5$); 0 hours post-Partial Cord Occlusion Group (0h-PCO group): sacrificed after 60 minutes of asphyxia. ($n = 5$); and 3 hours post-Partial Cord Occlusion Group (3h-PCO group): sacrificed after 60 minutes of asphyxia and mechanical ventilation for 3 hours ($n = 5$). Brain was fixed by perfusion with paraformaldehyde 4%. Serial gross sections were performed and multiple blocks of different brain territories selected. The damage was studied in cerebral cortex (frontal, parietal, temporal, occipital), basal nuclei, hypothalamus, thalamus, hippocampus, mesencephalon, pons, medulla oblongata, cerebellum (cortex and intracerebellar nuclei) and white matter. Samples were embedded in paraffin wax for hematoxylin-eosin (HE) studies and apoptosis detection by TUNEL. 1 mm^3 samples were also included for transmission electron microscopy studies. One-factor ANOVA was performed ($p < 0.05$). *Results:* In the morphological study, the most affected zones of the brain in both PCO groups were basal nuclei, mesencephalon, pons and intracerebellar nuclei (corresponding to subcortical areas). In these regions scattered great-size damaged cells were observed, whose cytoplasm had lost detail and acquired a homogeneous, eosinophilic appearance. The number of necrotic cells presented at 0 hours after HI injury did not increase after 3 hours from injury. The number of TUNEL positive cells was increased at 3 hours post-injury (3h-PCO group) with respect to both control (healthy group) and 0h-PCO group, in the cerebral cortex, cerebellum, mesencephalon and pons (mainly in cortical areas), as well as in the white matter. *Conclusion:* These results suggest selective cell damage in perinatal asphyxia. Cell death by necrosis involves elements, which participate in the extrapiramidal pathway whereas apoptosis was implicated in the cortical pathway. *Grants:* from the University of Basque Country (EHU06/99), Basque Government (IT-287-07) and from Ministry of Health (FIS PI06/0908, FIS PI06/0839).

Ischemic preconditioning alters the protein profile of CA1 pyramidal neurons to induce tolerance to global cerebral ischemia

Hoyte, L. C.[a], Zhao, Z.[b], Zhang, S.[b], Nagel, S.[a], Papadakis, M.[a], Brooks, K. J.[a] and Buchan, A. M.[a]
[a]*Acute Stroke Programme, Nuffield Department of Clinical Medicine, University of Oxford, UK*
[b]*Calgary Stroke Program, Hotchkiss Brain Institute, University of Calgary, Canada*

Background: Ischemic preconditioning (IPC) is a method to induce tolerance to cerebral ischemia whereby a short, sub-lethal, ischemia primes the brain to a more severe, injurious ischemia. IPC has been shown to improve survival of the sensitive CA1 pyramidal neurons following global cerebral ischemia. The exact mechanism for the tolerance has not been successfully elucidated, but theories include facilitated recovery of protein synthesis, altered expression of heat shock proteins, decreased calcium influx during injurious ischemia, and differential regulation of the alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropoinic acid (AMPA) receptor GluR1 and GluR2 subunits. The current study uses proteomics to compare the protein profiles of CA1 and CA3 pyramidal neurons, in both preconditioned and non-preconditioned rat brains. *Methods:* 55 adult male Wistar rats (Charles River, ON, Canada) were used for this study. Ischemia was induced via the four-vessel occlusion (4VO) method of global ischemia. The rats were either used for proteomics ($n = 13$) or histology ($n = 42$). The histology cohort was further divided into short-term survival (24 hours) ($n = 23$) or long-term survival (7 to 28 days) ($n = 19$), while the proteomics cohort was 24 hours survival. Animal groups included: 1) sham-preconditioning ischemia plus 10 min 4VO at 72 hours reperfusion, 2) Preconditioning (2 min 4VO) alone, and 3) Preconditioning ischemia plus 10 min 4VO at 72 hours reperfusion. Samples used for proteomics were homogenized in hypotonic buffer and differential centrifugation was used to generate several subcellular fractions. This study investigated the cytosolic and membrane plus organelles fractions. The samples used for histology were formalin fixed, paraffin embedded, sectioned at 6 microns and affixed to slides for analysis. *Results:* Ischemic preconditioning improved survival of the sensitive CA1 neurons out to the 28 day survival timepoint. Preconditioned CA1 neurons showed 63% survival at 28 days as compared to the 40% survival of the sham-preconditioned cohort. Ischemic preconditioning was shown to alter the protein profile of the CA1 pyramidal neurons, making their profile more similar to the CA3. In particular, the membrane proteins myelin basic protein, 60 kDa heat shock protein, VAMP2, syntaxin binding protein, sodium potassium transporting ATPase, fructose dehydrogenase, and malate dehydrogenase expression profiles were altered in the CA1 following preconditioning to show similar levels to the more robust CA3 neurons. Other proteins with increased expression due to preconditioning include ubiquitin carboxyl terminal hydrolase isozyme L1, fructose bis-phosphate aldolase C, dihydropyrimidinase related protein 2, and heat shock protein 70. *Conclusion:* Ischemic preconditioning improves the survival of sensitive CA1 neurons out to 28 days. The protein profile of the CA1 neurons is altered due to IPC; chaperone proteins and metabolic proteins showed increased expression and 24 hours after injurious ischemia. When examining the membrane fractions, it was evident that the protein profile of the CA1 neurons was altered so that the expression of several proteins was similar to the CA3. This indicates that preconditioning might induce neuronal survival of the CA1 by increasing the protein expression of key survival proteins.

Autophagy is required for ischemic tolerance

Jang, J.-Y., Park, H.-K., Chu, K., Lee, S.-T., Jung, K.-H., Bahn, J.-J., Kim, M., Lee, S. K. and Roh, J.-K.
Stroke & Stem Cell Laboratory, Department of Neurology, Clinical Research Institute, Seoul National University Hospital, Seoul, South Korea
Program in Neuroscience, Neuroscience Research Institute of SNUMRC, Seoul National University, Seoul, South Korea

The molecular mechanisms associated with apoptosis, a major type of active cell death (type I cell death) have largely been studied in the last decades. Compared with apoptosis, type II cell death is known to be associated with autophagosomes / autophagolysosomes and appear in the developing nervous system. The role of autophagy is known to increase the resistance for deprivation of nutrients and good environment. In this study, we sought to find the existence of the autophagy in ischemic preconditioning (IPC) and to reveal whether the autophagy could increase or decrease the preconditioning effect, using *in vitro* model. A model of ischemic preconditioning in the PC12 cell line was used. To find the existence of the autophagy

in ischemic preconditioning, we measured light chain (LC) 3-I band and LC3-II band simultaneously, using the immunoblotting. To increase the sensitivity of LC3-II despite the rapid autophagic vacuole degeneration in neuronal cell line, we inhibited the vacuole degradation by E64d and pepstatin A (AVI) and measured LC3-II and LC3-I at various time points after 6 hour IPC. To find the role of autophagy, we blocked the autophagic activity by 3-methyladenine (3MA) and wortmannin, well known chemical blockers of autophagy initiation. We also applied bafilomycin A, the inhibitor of the complement of the autophagy by inhibiting the fusion of autophagic vacuole to lysosome. IPC combined with or without autophagic blocker, was followed by 15 hour OGD. Pre-exposure of PC12 cells to 6 hours of IPC significantly increased cell viability after 15 hours of OGD, compared to non-preconditioned cells. Four hours after IPC, immunoblotting showed that the reduction of LC3-I band compared with actin. The addition of AVI on the culture media disclosed the LC3-II bands, a marker for activity of the autophagy from 4 hours to 8 hours after brief IPC. 3MA and wortmannin could reduce the effect of IPC compared with IPC effect without autophagic blocker. Intriguingly, bafilomycin A could reduce the effect of IPC minimally (to 95%), which suggested that the initial phase of autophagy might be more important to the IPC than the terminal phase of autophagy. Our results supported that the existence of autophagy in the ischemic preconditioning and the autophagy can be enhancing the tolerance to severe hypoxia.

Pharmacologic vagus nerve stimulation reduces cerebral inflammation in a hemorrhagic stroke model: Brain can speak immune language

Jang, J.-Y., Park, H.-K., Chu, K., Lee, S.-T., Jung, K.-H., Kim, M., Lee, S. K. and Roh, J.-K.
Stroke and Neural stem cell laboratory, Clinical Research Institute, Department of Neurology, Seoul National University Hospital, Seoul, South Korea

Background: Stimulated vagus nerve excretes acetylcholine into peripheral immune organs, such as spleen, and secreted acetylcholine binds to alpha7-nicotinic receptor of macrophage to reduce innate inflammation. Cerebral muscarinic stimulation increases vagus nerve activity. We aimed to investigate whether this "cholinergic anti-inflammatory pathway" can be applied to reduce the cerebral inflammation in a hemorrhagic stroke model. *Methods:* Experimental intracerebral hemorrhages were induced by stereotaxic collagenase injection in rats. Concomitantly, muscarine, methoctramine (M2 antagonist), or saline (as a control group) was injected into lateral ventricle. We monitored heart rate variabliity during muscarine injection and used splenectomy to eliminate cholinergic anti-inflammatory pathway. We measured inflammation molecules in brain and spleen at day 1, and brain water content and hemorrhage volume at day 3. Neurologic deficits were monitored for 4 weeks using modified limb placing behavioral test (MLPT) after ICH. *Results:* Intraventricular muscarine injection increased heart rate variability in ICH model, suggesting an increased vagus nerve output. Both muscarine or methoctramine injection, reduced brain water content and decreased inflammatory mediators in brain and spleen. Splenectomy prior to muscarinic stimulation eliminated the effect of muscarine on brain inflammation, which suggests that the effect of muscarine is mediated through the vagus nerve-spleen pathway rather than through direct interaction with hemorrhagic brain. Muscraine-injected rats showed improved neurologic outcomes. *Conclusions:* Our results show that cholinergic anti-inflammatory pathway stimulation can reduce cerebral inflammation in a hemorrhagic stroke model, and in other words, that the brain has an antagonistic tool for the innate inflammatory response to the damage of itself.

Neurovascular protection by the lipoxygenase inhibitor baicalein

Jin, G.[a], Arai, K.[a], Murata, Y.[a], Wang, S.[a], Stins, M. F.[a], Lo, E. H.[a] and van Leyen, K.[a]
[a]*Neuroprotection Research Laboratory, Massachusetts General Hospital, Charlestown, USA*
[b]*Department of Neurology, Johns Hopkins School of Medicine, Baltimore, USA*

We have previously shown that 12/15-lipoxygenase (12/15-LOX) is upregulated in the peri-infarct area following middle cerebral artery occlusion (MCAO) in mice, and we and others found that 12/15-LOX contributes to brain damage following ischemia/reperfusion. Building on our previous finding that the lipoxygenase inhibitor baicalein reduces infarct sizes, the current study was designed to investigate neurovascular effects of 12/15-LOX and the potential of baicalein as a neuroprotective agent, both *in vivo* and *in*

vitro. The LOX inhibitor baicalein protected cultured brain endothelial cells against oxidative stress induced either by hydrogen peroxide or by hypoxia. Following transient focal ischemia, 12/15-LOX was increased in neurons and endothelial cells. Baicalein protected mice against MCAO when administered before ischemia or at reperfusion. The vascular tight junction protein claudin-5 underwent extensive degradation in the peri-infarct area, which was partially prevented by baicalein. Brain edema was significantly ameliorated in 12/15-LOX knockout mice as well as wild-type mice treated with baicalein. Likewise, extravasation of immunoglobulin G (as a measure of blood-brain barrier leakage), was reduced in both 12/15-LOX ko mice or baicalein-treated wild-type mice. 12/15-LOX may contribute to ischemic brain damage not just by causing neuronal cell death, but also by detrimental effects on the brain microvasculature. 12/15-LOX inhibitors may thus be effective as both neuroprotectants and vasculoprotectants. Baicalein has potential as a neuroprotective agent, but must be further investigated.

Simvastatin, Bcl-2 and neuroprotection

Johnson, L. N.[a], Eckert, G. P.[b], Butterick, T.[a], Igbavboa, U.[a], Muller, W. E.[b] and Wood, W. G.[b]
[a] *Geriatric Research, Education and Clinical Centre, VA Medical Center and Department of Pharmacology, University of Minnesota, School of Medicine, Minneapolis, USA*
[b] *Department of Pharmacology, Biocenter Niederursel, Johann-Wolfgang-Goethe-University Frankfurt/a.M., Germany*

Statins are most commonly prescribed to reduce hypercholesterolemia; however, recent studies have shown that statins have additional benefits, including neuroprotection. Until now, the mechanism underlying statin-induced neuroprotection has been poorly understood. Recent *in vivo* studies from our lab reported the novel finding that simvastatin increased expression levels of a gene encoding for a major cell survival protein, Bcl-2. The purpose of the present experiments was to determine if simvastatin could protect neurons from cytotoxicity by increasing Bcl-2 mRNA and protein levels and if such protection would be altered by Bcl-2 suppression. Neurons were pretreated with simvastatin and challenged with a compound known to reduce Bcl-2 levels and induce cell death. Simvastatin pretreatment resulted in a significant reduction in cytotoxicity (LDH release and caspase-3 activation) following challenge compared with unchallenged neurons. Chronic simvastatin treatment significantly increased Bcl-2 mRNA and protein levels while challenge resulted in a significant reduction in Bcl-2 protein abundance. G3139, an antisense oligonucleotide directed against Bcl-2, abolished the protective effects of simvastatin and eliminated simvastatin-induced upregulation of Bcl-2 protein. These findings suggest that neuroprotection by simvastatin is dependent on the drug's previously unexplored and important effect of upregulating Bcl-2. Discovering how statins are mediating pro- and anti-apoptotic genes will provide new insight into the potential use of other cholesterol lowering and lipid related drugs that might also be able to impart neuronal protection in numerous neurodegenerative diseases (ischemic stroke, multiple sclerosis, AIDS dementia and others) and conditions outside of brain in which programmed cell death has been implicated. This work was supported by grants from the National Institutes of Health AG-23524, AG-18357, NATO Collaborative Linkage Grant (980136) and resources/facilities of the Minneapolis VA Medical Center.

Beta-amyloid pathology and calcification in the thalamus following focal cerebral ischemia in rats

Jolkkonen, J., Hiltunen, M., Soininen, H. and Sivenius, J.
Department of Neurology, University of Kuopio, Finland

We recently showed atypical b-amyloid (Ab) and amyloid precursor protein (APP) staining in the thalamus following transient occlusion of the middle cerebral artery (MCAO). Interestingly, staining was diffuse acutely after the infarct, but accumulated, leading to dense, permanent plaque-like deposits particularly in the ventroposterior lateral and ventroposterior medial nuclei (VPL/VPM). In addition, these deposits were positive for Aβ and N-terminal APP, but not for C-terminal APP. The most intense staining was obtained with antibodies recognizing Aβ3–16 and Aβ1-x epitopes. APP processing and levels of Ab degrading enzymes IDE, BACE1 and NEP remained unchanged after 48 hours of MCAO in the thalamus. Interestingly, however, APP processing was significantly enhanced between by the end of 1 month follow-up in the ipsilateral thalamus. Simultaneously, BACE1 and IDE levels were increased an average 50% whereas the

NEP levels decreased steadily. These results suggest that APP processing is enhanced in the both cortical and thalamic regions leading to the increased maturation of APP as well as increased APP CTF production after focal cerebral ischemia. Furthermore, inverse correlation between IDE and NEP protein levels in the thalamus suggests a cross-regulation between these two Aβ degrading enzymes after focal cerebral ischemia. Surprisingly, Aβ deposits in the thalamus showed an overlapping distribution pattern with calcium in MCAO rats. Alizarin red staining 3 weeks after MCAO showed diffuse calcium accumulation covering most of the thalamic nuclei. Small granular deposits were also located in cortical areas adjacent to infarcts, but not in other brain areas. A completely different picture was seen 7 months after MCAO. Diffuse calcium staining in the thalamus transformed to small granules, and larger formations often organized as clusters, with variable locations. In these chronic MCAO rats, scanning electron microscopy showed large mineral deposits in the thalamus with coral-shaped protrusions. In addition to calcium, phosphorus was detected in all deposits with an overlapping distribution. The calcium-to-phosphorus ratio was 1.28 $\pm$ 0.15, which is characteristic to hydroxy-apatites. X-ray analysis showed no evidence of other minerals, or metals such as iron, zinc, or copper in deposits. β-amyloid pathology and calcification in the thalamus may have functional implications. Beam-walking test showed impaired performance in hAPP overexpressing rats following MCAO indicating that excessive load of Aβ in the thalamus may impair behavioural outcome. More importantly, our human post mortem study from 484 subjects showed that experimental data are to some extent seen in patients with cerebrovascular lesion. That is the subjects with cerebrovascular lesions had increased Aβ overload in the thalamus compared to subjects without evidence of previous ischemic event. We have characterized a unique experimental condition in the thalamus of stroke rats, which includes a continuous neurodegenerative process, microglia activation and Aβ deposition and calcification similar to the pathology in Alzheimer's disease. The model is expected to provide valuable information on pathological processes in various neurodegenerative diseases and help in development of novel drug treatments.

TGF-beta1 signaling in the healthy and diseased brain: A focus on neurogenic regions

Kandasamy, M., Rivera, F., Lehner, B., Couillard-Despres, S., Bogdahn, U., von Hoersten, S. and Aigner, L.
Department of Neurology, University of Regensburg, Germany

Transforming growth factor (TGF)-beta1 has multiple physiological functions in the adult central nervous system (CNS). It modulates inflammatory responses and controls proliferation of microglia and astrocytes. In the diseased brain, TGF-beta1 expression is upregulated and, depending on the cellular context, its activity can be beneficial or detrimental regarding regeneration. Recent data suggest that TGF-beta1 might be involved in the control of neural stem and progenitor cell proliferation during adult neurogenesis, in particular during neurodegeneration. Unfortunately, very little is known on TGF-beta signaling in neurogenic regions. Here, we provide a comprehensive and systematic analysis of the expression patterns of TGF-beta signaling components in the adult rodent CNS with focus on the neurogenic regions of healthy and transgenic Huntington's disease (HD) brains. We demonstrate the anatomical distribution of the TGF-beta receptors RI and RII and of the downstream signaling element phospho-Smad 2 using Western blot analysis and immunehistochemistry. Moreover, we analyzed TGF-beta1 signaling *in vitro* using neural progenitor cell cultures derived from adult hippocampus or from SVZ. In contrast to the healthy brain, where TGF-beta signaling seems to be confined to differentiating and mature neurons, high levels of TGF-beta signaling were detected in the neurogenic regions animal models of HD. This correlates with reduced rates of cell proliferation and neurogenesis in the HD brains. We conclude that TGF-beta1 signaling might be an important molecular feature involved in maintenance of neuronal integrity in the healthy brain, but limit neural progenitor proliferation and neurogenesis under disease conditions. Funding: Bayerische Forschungsstiftung (M.K.) and Bavarian State Ministry of Sciences, Research and the Arts, "Forneurocell grant" (L.A.).

Subcellular mitochondrial heterogeneity in hippocampal glial cells

Keil, V. C., Funke, F. and Müller, M.
Center of Physioloy and Pathophysiology, Georg-August-University, Göttingen, Germany

Mitochondria – even in a single cell – clearly differ in shape, subcellular localization, and functional determination. To analyze such diversity, we used the mitochondrial membrane potential marker JC-1. Mitochondria were visualized using either a wide-field microscope equipped with an optical image splitter device (Dual View) or a two-photon laser scanning microscope equipped with multiple photomultiplier detectors. Since JC-1 forms red aggregates in polarized mitochondria and less polarized mitochondria appear green, the activity of single mitochondria can be visualized in real time. Cultured hippocampal glial cells contained clearly separated mitochondria, and both highly and less polarized mitochondria coexisted in a single cell. A high density of polarized, tubular mitochondria was found in the perinuclear region. Single, roundish mitochondria often appeared green, i.e. less polarized. Challenging mitochondria by cyanide or glutamate caused a heterogenous shift from red to green fluorescence. Especially perinuclear mitochondria responded with strong but reversible depolarization. Rhythmic changes in the mitochondrial membrane potential appeared well synchronized in spatially confined mostly perinuclear mitochondrial clusters. They persisted in Ca^{2+}-free solutions, but were antagonized by dantrolene. Fluo-3 recordings revealed localized cytosolic Ca^{2+} sparks, suggesting Ca^{2+} release from the endoplasmic reticulum (ER). In conclusion, we obtained evidence for functional mitochondrial heterogeneity in glial cells, a less vulnerable mitochondrial population around the nucleus and spatially confined functional interactions of mitochondria and the ER. Supported by the Deutsche Forschungsgemeinschaft (CMPB, EXC171).

Effects of intrathecal application of brain-derived neurotrophic factor on the perilesional response after focal cortical infarcts

Keiner, S., Witte, O. W. and Redecker, C.
Department of Neurology, Friedrich-Schiller-University Jena, Germany

Brain-derived neurotrophic factor (BDNF) is known as a member of the neurotrophin family of growth factors, which plays an important role in the complex cellular and functional changes after ischemic infarcts. Up to now only little is known about the effects of exogenous BDNF on cellular proliferation and differentiation in the peri-infarct zone. Here we analyzed the effects of BDNF on the perilesional cellular response after focal infarcts induced in the sensorimotor fore- and hindlimb cortex using the photothrombosis model in rats. BDNF or vehicle was continuously infused into the ipsilateral ventricle directly after infarct induction for 2 weeks by osmotic minipumps. The proliferating cells were labelled with bromodeoxyuridine (BrdU) within the period of drug administration starting one day post surgery. At day 14 and 42 after infarct induction, BrdU-positive cells were immunocytochemically stained and quantified in the peri-infarct zone using semiautomatic stereology. To further analyze the phenotypes, triple-immunofluorescence with antibodies against the immature neuronal marker (DCX), mature makers (NeuN, Hu), astrocytic markers (GFAP, S100β) and microglia/macrophages marker (CD68) was performed. Sensorimotor function was assessed using the limb-use asymmetry test, the ladder rung walking test as well as the tapered beam walking test at day 1, 3, 14, and 42 post surgery. Our study demonstrates that intrathecal BDNF application significantly increased the number of DCX-positive neuroblasts in the perilesional area 2 weeks after infarct induction and provoked the generation of mature neurons 42 days post surgery. Furthermore, exogenous BDNF reduced the number of newly generated microglia/macrophages two weeks after the lesion, whereas S100β and GFAP expressing astrocytes were not significantly influenced. Intrathecal BDNF administration modified the cellular response in the perilesional area. However, functional recovery was not significantly improved.

Rehabilitative therapies differentially alter proliferation and survival of glial cell populations in the perilesional zone of cortical infarcts

Keiner, S., Wurm, F., Kunze, A., Witte, O. W. and Redecker, C.
Department of Neurology, Friedrich-Schiller-University Jena, Germany

The present study examined the effects of rehabilitative therapies on proliferation and survival of distinct

glial populations in the perilesional area of photochemically induced focal ischemic infarcts in the forelimb sensorimotor cortex in rats. Immediately after the infarct, one group of animals housed in standard cages received daily sessions of skilled reaching training of the impaired forelimb; a second group was transferred to an enriched environment, whereas a third control group remained in the standard cage without further treatment. Functional recovery was assessed in a sensorimotor walking task. To label proliferating cells, bromodeoxyuridine (BrdU) was administered from day 2 until day 6 postinfarct. Proliferation and survival of astrocytes, microglia/macrophages, immature and mature oligodendrocytes in the perilesional zone were immunocytochemically quantified at day 10 and 42. Using this approach, we demonstrate that enriched environment and reaching training both improve functional recovery and strongly reduce the proliferation of microglia/macrophages in the perilesional zone. Furthermore, daily training of the impaired forelimb significantly increased the survival of activated astrocytes. Our data therefore provide evidence that rehabilitative therapies not only modify the postlesional reorganisation on the neuronal level but also significantly influence the glial response in the perilesional zone.

Monitoring of metabolism and excitotoxicity after middle cerebral artery occlusion in mice

Kiewert, C.[a], Lang, D.[b], Mdzinarishvili, A.[a], Hartmann, J.[a] and Klein, J.[a,b]
[a]*Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Science Center, Amarillo, USA*
[b]*Department of Pharmacological Sciences, School of Pharmacy, Johann Wolfgang von Goethe University, Frankfurt/ a.M., Germany*

Microdialysis sampling during experimental stroke allows for monitoring of changes in the composition of the extracellular fluid in real time. The technique adds important data to quantitative endpoints of ischemic damage. In the present study, microdialysis probes were implanted into the striatum of adult CD-1 mice. On the next day, permanent middle cerebral artery occlusion (MCAO) was induced by thread insertion while monitoring bloodflow with laser-Doppler flowmetry. We continuously collected microdialysis samples from 2 hrs before until 3 hrs after MCAO, and again 22 to 24 hrs after MCAO. Infarct area and edema formation were determined by 2,3,5-triphenyltetrazolium chloride staining 24 hrs after MCAO. In the microdialysate, glucose, glutamate, and glycerol were determined photometrically with the CMA 600 Microanalyzer. Choline and acetylcholine were measured by HPLC with electrochemical detection. Potassium was determined with a potassium selective electrode. Immediately after MCAO, glucose decreased to $<5\%$ of baseline values. Glutamate rapidly increased 10 to 100-fold. Glycerol and choline, both markers of lipid membrane breakdown, increased to more than 500% of baseline. The levels of acetylcholine decreased to $<10\%$ of baseline values. Potassium concentration in the microdialysate increased from 3.5 mM to 7 mM. Twenty-four hours after occlusion, glutamate, choline and glycerol levels were still elevated at about 30-fold, 10-fold and 3-fold, respectively; glucose was $<5\%$ of baseline and acetylcholine was below detection limit ($<5\%$ of baseline values). We conclude that the combination of stroke with microdialysis is feasible in mice and will be helpful in future investigations, e.g. of transgenic mouse models, to gain a better understanding of stroke pathophysiology. It can also be used as an *in vivo* model to test for new stroke therapeutics. Measurements of extracellular stroke markers in animals treated with putative neuroprotective compounds are in progress.

Death-associated protein kinase is activated in oxygen-glucose-deprivation induced cell death in organotypic hippocampal slice culture

Klette, C.[b], Straßburger, M.[a], Schröder, U. H.[a], Schneider-Stock, R.[c] and Reymann, K. G.[a,b]
[a]*Research Institute for Applied Neuroscience* (*FAN*) *gGmbH, Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[c]*Department of Pathology, Otto-von-Guericke University Magdeburg, Medical Faculty, Germany*

The death-associated protein kinase (DAP-kinase) is a calcium calmodulin-regulated serine/threonine protein kinase consisting of several domains with various functions. Beside the kinase domain and the calmodulin regulatory domain, the protein contains of 8 ankyrin repeats, a cytoskeleton binding region, 2 potential P-loop motifs and a death domain. DAP-kinase, which is crucially involved in the induction of early apoptotic pathways, is negatively regulated by au-

tophosphorylation on serine 308 in the calcium calmodulin regulatory domain. In order to investigate the mechanism of neurodegeneration following ischemia we study the role of DAPK in organotypical slice culture model. Our study shows that after oxygen glucose deprivation (OGD) the DAP-kinase becomes rapidly dephosphorylated and activated. The neuroprotective NMDA receptor antagonist MK-801 inhibits the dephosphorylation of DAP-kinase after OGD. Our data indicate that the DAP-kinase is one of the mechanisms activated by the excessive glutamate release during ischemia via the NMDA receptor and that DAP-kinase activation maybe a major cause for the subsequent delayed neuronal death.

In-vivo imaging of the inflammatory receptor CD40 after cerebral ischemia using a fluorescent antibody

Klohs, J.[a], Grafe, M.[b], Graf, K.[b], Stiebenz, D.[b], Greger, K.[c], Stelzer, E.[c], Steinbrink, J.[d], Bourayou, R.[d], Kronenberg, G.[a], Endres, M.[a], Lindauer, U.[a], Dirnagl, U.[a] and Wunder, A.[a]
[a]*Experimental Neurology and Center for Stroke Research, Charité University medicine Berlin, Germany*
[b]*German Heart center Berlin, Germany*
[c]*European Molecular Biology Laboratory* (*EMBL*) *Heidelberg, Germany*
[d]*Berlin NeuroImaging Center* (*BNIC*), *Charité University medicine Berlin, Germany*

Introduction: Imaging technologies that enable the specific and sensitive detection of brain inflammation after stroke are highly desirable. In this study, we explored whether the inflammatory receptor CD40 can be non-invasively and specifically visualized in mice after cerebral ischemia using a fluorescent monoclonal antibody, labeled with the near-infrared fluorescence (NIRF) dye Cy5.5 (Cy5.5-CD40MAb). *Methods:* Wild type and CD40-deficient mice were subjected to transient middle cerebral artery occlusion. Mice were either intravenously injected with Cy5.5-CD40MAb or control Cy5.5-IgGMAb. Non-invasive and *ex-vivo* NIRF imaging was performed after injection of the compounds. Specificity of the antibody was investigated using microscopic techniques. *Results:* Significantly higher fluorescence intensities over the stroke-affected hemisphere, compared to the contralateral side, were only detected non-invasively in wild type mice that received Cy5.5-CD40MAb, but not in CD40-deficient mice injected with Cy5.5-CD40MAb or in wild type mice that were injected with Cy5.5-IgGMAb. *Ex-vivo* NIRF showed an intense fluorescence within the ischemic territory only in wild type mice injected with Cy5.5-CD40MAb. Confocal microscopy revealed a partial co-localization of parenchymal fluorescence from the injected Cy5.5-CD40MAb with activated microglia and blood-derived cells in the ischemic region. *Conclusions:* The study demonstrates that a CD40-targeted fluorescent antibody enables specific non-invasive detection of the inflammatory receptor CD40 after cerebral ischemia using optical techniques.

The proliferation potential, migration and the differentiation of neural stem cells derived from human umbilical cord blood (HUCB-NSC) after their transplantation into the brain of neonatal and adult rats

Kozlowska, H., Markiewicz, I., Janowski, M., Wanacka, E., Domanska-Janik, K. and Lukomska, B.
NeuroRepair Department, Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Transplantation of neural stem cells (NSC) is the key strategy of cell replacement therapy in the central nervous system (CNS). However, important factors that greatly affect graft cell fate include microenvironment, which consists of local cues and the host versus graft reaction. *The purpose of the study* was to compare survival, migration, and differentiation of HUCB-NSC after their transplantation into the brain of neonatal and adult Wistar rats. *Methods:* HUCB-NSC (2×10^5) labeled with CMFDA cell tracker were transplanted (tx) into SVZ of the postnatal day 0 (P0) rats or into intact brain of the CsA immunocompromized adult rats. After 1, 3, 7, 14 or 21 days brains were removed, frozen and cut into 20 μm coronary slices, then immunohistochemical studies were performed to visualize HUCB-NSCs fate in the brain. *Results:* In neonatal rats, 3 days after tx most of HUCB-NSC remained in the graft. During the first week HUCB-NSC started to disperse and migrate. HUCB-NSC situated at the periphery of the transplant or in migratory stream display proliferation marker (Ki67). After 7-21 days HUCB-NSC survived in the host brain with many cells expressing neuronal or astrocytic phenotypes. Few of HUCB-NSC presented the features of adult neurons ($MAP2^+$ with long protrusions). In adult rats, 3 days after tx, HUCB-NSC form

dense deposit with only single cells migrating into brain tissue. Most of the grafted HUCB-NSC stayed undifferentiated with few cells expressing neuronal (NF200) or astrocytic (GFAP) markers. After 7 days numerous HUCB-NSC situated inside the graft underwent successive degeneration and subsequent depletion. Transplanted HUCB-NSC induced heavy inflammatory response of the host detected by macrophage/microglia ($ED1^+$) accumulation and astrogliosis ($GFAP^+$). No viable HUCB-NSC were found after 14 days. *Conclusions:* Host environment dictates the fate of transplanted neural progenitors derived from human cord blood however immunological response in the brain of adult rats limits the time of observation due to short survival of transplanted HUCB-NSC.

Intravenous transplantation of human placenta-derived mesenchymal stem cells upon experimental stroke dose-dependently produced beneficial effects

Kranz, A.[a], Wagner, D.-C.[a], Schmidt, U. R.[a], Förschler, A.[b], Kamprad, M.[c], Aberman, Z.[d], Emmrich, F.[a,c,e] and Boltze, J.[a,e]
[a]*Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany*
[b]*Department of Neuroradiology, University Hospital rechts der Isa, Munich, Germany*
[c]*Institute of Clinical Immunology and Transfusion Medicine, University of Leipzig, Germany*
[d]*Pluristem Inc., Haifa, Israel*
[e]*Translational Centre for Regenerative Medicine, University of Leipzig, Germany*

Background: The beneficial effects of mesenchymal stem cells (MSC) treatment upon stroke have already been shown in murine models. The human placenta affords an easy accessible source for this kind of adult stem cells. In our study we investigated the therapeutic potency of MSC obtained from the fetal and maternal part of the human placenta. The MSC were provided by Pluristem Therapeutics, Inc. (PLC fetal and PLC maternal). *Methods:* Permanent MCAO was induced in 38 male spontaneously hypertensive rats, weighting 300–350 g. Animals were randomly distributed to the following experimental groups: (1) PLC fetal 1x10E6, $n = 8$; (2) PLC maternal 1x10E6, $n = 8$; (3) Vehicle solution, $n = 8$; (4) PLC fetal 2x10E6, $n = 7$; (5) PLC maternal 2x10E6, $n = 7$. Single injections took place 24 h upon stroke, administration of double-doses were initiated 8h and 24 h upon MCAO. Two behavioral tests (Beam-Walk and mNSS) were performed from Day 1 to Day 60 regularly. Infarct volumetry and brain atrophy was measured *in vivo*, using a clinic 1.5T MR scanner at Days 1, 8, 29 and 60. For investigations of glial reactivity we investigated a 750 μm broad area next to the infarct border for GFAP-positive cells semi-quantitatively. *Results:* Double injection of PLC with maternal origin significantly reduced behavioral defects compared to the control group as well as to the single injection group. Furthermore, the double injection of maternal PLC significantly decreased the MRI-infarct volume at Day 60 compared to control. Transplantation of 2x10E6 PLC fetal showed a significant improvement of functional recovery compared to the mono-injection group and a trend to therapeutic superiority compared to the control. The appraisal of glial reactivity is still ongoing. *Conclusions:* The sensorimotor results precisely displayed a dose-dependency of effective PLC treatment. While single injections are equal to the control group, increased doses effectuated a distinct improvement of functional recovery. Surprisingly, the superiority of increased cell-doses was not confirmed in MR investigations. A remarkable effect concerning the development of infarct volume was only observed between the control and the double PLC maternal administration. Potentially, the discriminatory power of the MR-depend infarct volumetry seems to be inadequate to detect marginal differences.

Development of mouse model for live imaging of innate immune response: A role of TLR2 in the brain injuries

Lalancette-Hebert, M., Phaneuf, D., Soucy, G., Weng, Y. C. and Kriz, J.
Department of Anatomy and Physiology, University Laval, QC, Canada

Microglial cells are the main effectors of the innate response following CNS injuries, including ischemia. However, whether microglial activation has beneficial or detrimental effects on adjacent ischemic neurons remains controversial. Microglial activation following brain ischemia is associated with the induction of Toll-like receptor 2 (TLR2). In the mouse brain, TLR2 expression is very low or undetectable but it is strongly induced in microglial cells in response to inflammatory stimuli or brain injury. In cerebral ischemia, the spatial and temporal dynamics of microglial responses is not yet fully characterized. The big advantage will

be to study these responses in real-time. Therefore, in order to investigate and analyze microglial/innate immune responses from the brains of live animals, we generated transgenic mice bearing the dual reporter system, firefly-luciferase and GFP, under transcriptional control of the murine TLR2 gene promoter. Using live imaging approach, we pursued the characterization of immune response in two different models such as cerebral ischemia and endotoxin (LPS) cerebral injection. Our live imaging results followed by double immunofluorescence and *in situ* hybridization analysis revealed that our reporter transgenes were properly induced. Moreover, the expression pattern of luciferase and GFP in our transgenic model was following the expression of endogenous TLR2 at protein and mRNA levels. Using biophotonic/bioluminescent imaging and transgenic reporter mouse models will help us to better understand real-time pathological changes associated with the brain inflammatory responses and allow us to better select for therapeutic targets and disease markers.

Microglia protects neurons against ischemia by synthesis of tumor necrosis factor

Lambertsen, K. L.[a], Hjelm Clausen, B.[a], Babcock, A.[a], Gregersen, R.[a], Nielsen, M.[b], Wirenfeldt, M.[a], Haugaard, L. S.[a], Færgeman, N. J. K.[c], Dagnaes-Hansen, F.[d], Bluethmann, H.[e], Meldgaard, M.[a] and Finsen, B.[a]
[a]*Medical Biotechnology Center, University of Southern Denmark, Odense, Denmark*
[b]*Department of Anatomy and Neurobiology, University of Southern Denmark, Odense, Denmark*
[c]*Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark*
[d]*Department of Medical Microbiology and Immunology, University of Aarhus, Denmark*
[e]*Roche Center for Medical Genomics, F. Hoffmann-La Roche, Basel, Switzerland*

Acute cerebrovascular disease and traumatic brain injury are accompanied by glial activation and microglial-macrophage synthesis of proinflammatory cytokines such as tumor necrosis factor (TNF) within the first critical hours. Although intensively studied, the beneficial or detrimental contributions of microglial-derived and inflammatory macrophage-derived TNF to the neuronal damage remains to be clarified. We investigated the neuroprotective function of microglial- and macrophage-derived TNF using a murine model of stroke and different combinations of genetically modified bone marrow (BM)-chimeric mice. We report that infarct volumes were greatly exacerbated in TNF-knock out (KO) mice compared with wild-type (WT) mice 24 hours after arterial occlusion. At this time, the infarct and the penumbra were equally infiltrated by TNF-producing microglia and BM-derived macrophages. The detection of larger infarcts in BM-chimeric TNF-KO mice grafted with WT BM cells, than in BM-chimeric WT mice grafted with TNF-KO BM cells, pointed to a neuroprotective effect of microglial-derived TNF. Observations of increased neurodegeneration in TNF-p55 receptor (R)-KO mice compared to TNF-p55R-KO,TNF-p55p75R-KO and WT mice were suggestive of a neuroprotective effect of microglial-derived TNF acting through the TNF-p55R. The observation of a neuroprotective effect of microglial-derived TNF in the acute phase after focal cerebral ischemia in mice point to parenchymal microglia as key regulators of neuronal sensitivity to acute cerebrovascular insults.

SPECT imaging shows accumulation of stem cells into internal organs after systemic administration in middle cerebral artery occlusion rats

Lappalainen, R. S.[a], Narkilahti, S.[a], Huhtala, T.[b], Liimatainen, T.[c], Suuronen, T.[d], Närvänen, A.[b], Suuronen, R.[a], Hovatta, O.[e] and Jolkkonen, J.[d]
[a]*Regea – Institute for Regenerative Medicine, University of Tampere and Tampere University Hospital, Finland*
[b]*Department of Biosciences, University of Kuopio, Finland*
[c]*A.I.Virtanen Institute for Molecular Sciences, University of Kuopio, Finland*
[d]*Department of Neurology, University of Kuopio, Finland*
[e]*Karolinska Institutet, CLINTEC, Karolinska University Hospital, Huddinge, Sweden*

The neuroregenerative therapeutic potential of human embryonic stem cell (hESC)-derived neural cells has been under intense investigation during the past few years. Before clinical trials, hESC-derived neural cell grafts are preferably tested in experimental models of neurodegenerative diseases. To end up with a satisfactory therapeutic outcome it is crucial to get the transplanted cells to the site of the damage. Here, we tested the definite accumulation of 111In-oxine labeled hESC-derived neural progenitors and rat hippocampal

progenitors after intravenous administration (femoral vein vs. carotid artery) in sham-operated and middle cerebral artery occlusion (MCAO) rats. Cells were detected *in vivo* using SPECT/CT device designed for rodents. In comparison, a given number of 111In-oxine labeled cells were injected stereotactically to the brain parenchyma to determine the sensitivity of the SPECT/CT device. Our results showed that after the intravenous injections, despite of the injection site, both cell types accumulated primarily into the internal organs instead of into the brain. Additional studies showed that detection sensitivity of SPECT/CT device was approximately 1000 111In-oxine labeled cells *in vitro*, and labeling of cells with 111In-oxine did not affect the cell viability or did not explain the lack of cell migration to the ischemic brain. In conclusion, our results indicate that intravenous administration is not an optimal route to deliver cell transplants to the brain after MCAO.

In vitro effect of isovaleric acid on oxidative stress parameters in rat brain mitochondria

Latini, A.[a], Bortoli, G.[a], Amaral, A. U.[b], Leipnitz, G.[b], Solano, A. F.[b], Glaser, V.[a] and Wajner, M.[b,c]
[a]*Laboratório de Bioenergética e Estresse Oxidativo, Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, Brazil*
[b]*Laboratório de Erros Inatos do Metabolismo, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil*
[c]*Universidade Luterana de Brasil, Canoas, Brazil*

Isovaleric acid (IVA) accumulates in patients affected by isovaleric acidemia (IVAcidemia), an autosomal recessive inborn error of leucine catabolism caused by the deficiency of the mitochondrial enzyme isovaleryl-CoA. Patients affected by this disorder suffer from acute episodes of encephalopathy; severe neurological symptoms and increased levels of ammonia generally accompany the accumulation of IVA. Considering that the neurotoxic mechanisms in IVAacidemia are virtually unknown, the objective of the present study was to investigate the *in vitro* effect of IVA (0.01–10 mM) on various parameters of oxidative stress in rat brain mitochondria. Thiobarbituric acid-reactive substances (TBA-RS), protein carbonyl formation (PCF), total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), and the activity of the antioxidant enzyme glutathione peroxidase (GPx) were assessed. In some experiments it was also tested the combine effect of IVA plus ammonia (50–200 μM). Significant increased TBA-RS levels were observed when rat brain mitochondria were exposed to IVA plus ammonia. IVA also provoked increased PCF in the presence or absence of ammonia. TRAP and TAR values as well as the activity GPx were not altered by the acid. The data indicate that IVA provokes oxidation of lipids and proteins, mainly in the presence of ammonia, possibly by inducing free radicals. Thus, in case these findings could be extrapolated to the human condition, it may be presumed that oxidative stress is involved in the IVAcidemia neurotoxicity. Financial support: CNPq, FAPESC, FINEP research grant – Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00.

Effects of post-ischemic hypoxia on the functional consequences of focal cerebral ischemia in the mouse

Leconte-Duval, C.[a,b], Tixier, E.[a], Bouët, V.[b], Freret, T.[b], Toutain, J.[a], Fauchon, C.[b], Roussel, S.[a], Boulouard, M.[b], Bernaudin, M.[a] and Schumann-Bard, P.[b]
[a]*CERVOxy team "Hypoxia and cerebrovascular pathology" UMR CI-NAPS Cyceron, Caen, France*
[b]*Memory and behavoiural plasticity group, Departement of Pharmaceutical sciences, University of Caen, France*

Experimental application of slight hypoxia before cerebral ischemia is known to induce cerebral adaptive processes leading to an increased brain's resistance to further ischemia, a phenomenon so called "ischemic tolerance" (Gidday et al., 1994 ; Bernaudin et al., 2002). The mechanisms of hypoxic preconditioning may involve of the transcription factor "Hypoxia Inducible Factor1" (HIF1) and its potentially beneficial target genes. The effects of hypoxia, applied after focal cerebral ischemia on the ischemia-induced functional deficits have not been explored although the ischemic postconditioning of the brain has been recently reported in the Rat (Pignataro et al., 2007). Therefore, the objective of this study was to expose mice after focal cerebral ischemia to chronic intermittent hypoxia in order to stimulate brain's adaptive responses, such as neuroprotection, neurogenesis. In addition, we have examined whether it is possible to induce a postconditioning on an *in vitro* model of oxygen glucose deprivation (OGD) on primary cortical neurons. Mice were

submitted to hypoxia (8% O2, 1 h, 3 times per week) (Hpx group, $n = 10$) initiated 5 days after transient (1 h) intraluminal middle cerebral artery occlusion and compared to a mice undergoing ischemia only (Nmx group, $n = 10$). We evaluated the effects of hypoxia on ischemic infarction measured by T2weighted MRI at 48 h and 43 days after ischemia, and on sensorimotor as well as mnesic functional recovery. Post ischemic hypoxia did not modify ischemiainduced behavioural deficits, but improved the non spatial recognition memory performances of shamoperated animals. Moreover, hypoxia reduced thalamic atrophy, a delayed histological consequence of focal ischemia. Futhermore, hypoxia (0.1% O2, 1 h), applied 14 h post OGD is neuroprotective on primary neurons *in vitro*. Our study is in favour of a protective effect of delayed postischemic chronic intermittent hypoxia against delayed thalamic neurodegeneration. The mechanisms underlying these beneficial effects have now to be investigated. We will particularly focus on the possible involvement of one of HIF1 target genes, such as EPO, and the potential contribution of neurogenesis.

Erythropoietin enhances functional recovery after focal cerebral ischemia through increase of synaptogenesis

Lee, J.[a], Jeon, J. Y.[b] and Kim, Y. J.[c]
[a]*Konkuk University School of Medicine, Seoul, South Korea*
[b]*Dankook University College of Medicine, Seoul, South Korea*
[c]*Kyunghee University College of Nursing Science, Seoul, South Korea*

Background and Purpose: Erythropoietin (EPO) has been shown to promote neural repair in cerebral ischemia through enhanced angiogenesis and neurogenesis. However, the role of EPO on synaptogenesis and synaptic plasticity has not been clearly defined. In this study, we investigated to determine the effect of EPO on synaptic plasticity following focal ischemic stroke with environment enrichment (EE) exposure. *Methods:* Male Sprague-Dawley rats (10–18 weeks, 275 $\sim$ 325 g) were subjected to middle cerebral artery occlusion (MCAO) for 90 min and were divided into 4 groups; Control, EPO, EE and EPO+EE. EPO was administered subcutaneously at a dose of 5,000 units/kg daily for 5 days starting at 24 hours after MCAO. Environment enrichment exposure was provided with the cage filled with a variety of objects such as toys, wooden blocks, and running wheel. On day 9 and 16, all rats were performed the behavioral tests for motor function recovery. Infract volumes were measured, and Immunohistochemistry and western blotting for NeuN, synaptophysin and GAP43 were performed to determine the synaptogenesis and synaptic plasticity. *Results:* The average infarct volume was 112.4 $\pm$ 17.3 mm^3 in control, 85.7 $\pm$ 13.5 mm^3 in EPO treated group, 92.8 $\pm$ 19.4 mm^3 in EE treated group, and 59.4 $\pm$ 11.6 mm^3 in EPO +EE treated group, respectively. EE and EPO+EE group showed significant improvement of motor function in behavioral test when compared with control group. Immunoreactivity of synaptophysin and GAP43 were significantly more increased in EPO+EE treated group. *Conclusions:* Our data suggest that EPO administered after focal cerebral ischemia may enhance functional recovery associated with environment enrichment through synaptogenesis and synaptic plasticity.

The influence of chronic arterial hypertension on the evolution of the ischemic penumbra: A sequential study with MRI in the rat

Letourneur, A., Roussel, S., Toutain, J., Bernaudin, M. and Touzani, O.
CERVOxy team "Hypoxia and cerebrovascular pathophysiology", Centre d'Imagerie-Neurosciences et Applications aux Pathologies (*CI-NAPS*),*Université de Caen Basse-Normandie, Université Paris Descartes, Caen, France*

Chronic arterial hypertension (CAH) and stroke are two major pathologies that affect the worldwide population. CAH increases the risk of stroke as well as the severity of the resultant lesion. In spite of this fact, arterial hypertension is rarely taken into consideration during preclinical investigations. In order to better define the therapeutic window in hypertensive subjects, the aim of our study was the analysis of the impact of CAH on the spatio-temporal evolution of the ischemic lesion in the acute phase. Special attention was paid to the ischemic penumbra. The evolution of the ischemic lesion was analysed using sequential magnetic resonance imaging (MRI) examinations from 30 minutes up to 4 hours after permanent middle cerebral artery occlusion in spontaneously hypertensive rats (SHR) and their normotensive control rats (WKY). Our results show that the ischemic lesion was bigger in hypertensive rats than

in normotensive ones at all time points (e.g. 155 ± 62; 91 ± 52 mm^2 at 30 minutes). Interestingly, and in contrast to normotensive rats, there was no penumbra in the SHR defined as the mismatch between perfusion- and diffusion-weighted imaging at all the time points analysed. This suggests that even if a treatment is precociously administered, the ischemic lesion will remain bigger in hypertensive subjects. The fact that arterial hypertension is not considered in pre-clinical studies could explain the failures of the pharmacological treatment of stroke in human.

Fluoxetine affords robust neuroprotection in the postischemic brain with a wide therapeutic window

Lim, C.-M.[a], Kim, S.-W.[a], Jung, J.-Y.[a], Kim, K.-S.[b], Han, P.-L.[b], Lee and J.-K.[a]
[a]*Department of Anatomy and Center for Advanced Medical Education, Inha University School of Medicine, Inchon, Korea*
[b]*Division of Nano Sciences and Brain Disease Research Institute, Ewha Womans University, Seoul, Korea*

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that is widely used in the treatment of major depression. Fluoxetine is also effective at alleviating post-stroke depression and induces motor recovery in stroke patients and facilitates cognition after traumatic brain injury. Although one mechanism underlying these diverse effects may be ascribed to its anti-depressant action, it remains to be investigated whether fluoxetine confers any effects other than anti-depression. In this study, we tested whether fluoxetine protects neuronal death in a rat cerebral ischemia model of middle cerebral artery occlusion (MCAO). The administration of fluoxetine intravenously (10 mg/kg) at 30 min, 3 hrs, or 6 hrs after MCAO reduced infarct volumes to 22.3 $\pm$ 7.3%, 20.5 $\pm$ 4.2%, and 25.1 $\pm$ 3.6%, respectively, of that of the untreated control. Moreover, the neuroprotective effect of fluoxetine was evident when it was administered as late as 9 hrs after MCAO/reperfusion. These neuroprotective effects were accompanied by improvement of motor impairment and neurological deficits. The fluoxetine-treated brain was found to show marked repressions of infiltration of inflammatory cells and proinflammatory marker expressions. Consistently, fluoxetine suppressed the LPS-induced activation of primary neutrophil cultures, as evidenced by a reduction in ROS production and the accompanying induction of proinflammatory cytokines. Moreover, fluoxetine suppressed NF-kB activity dose-dependently in the postischemic brain, suggesting that NF-kB activity inhibition explains in part its anti-inflammatory effect. These results suggest that curative treatment of fluoxetine affords strong protection against delayed cerebral ischemic injury, and that these neuroprotective effects might be associated with its anti-inflammatory effects.

Prevention of neuronal damage by calcium channel blockers with antioxidative effects after transient focal ischemia in rats

Lukic-Panin, V., Kamiya, T., Zhang, H., Hayashi, T., Tsuchiya, A., Sehara, Y., Deguchi, K., Yamashita, T. and Abe, K.
Department of Neurology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan

Background: Cerebral ischemia is a major leading cause of death and at first place cause of disability all over the world. Many drugs are in experimental stage for treatment of stroke. Among them are calcium channel blockers (CCBs) that have, in animal models, different effectiveness in healing of ischemic damage in brain. Mechanism of CCB action in cerebral ischemia is still unclear, but antioxidative property is supposed to be implicated. In the present study, we investigated antioxidative and neuroprotective properties of two CCBs, azelnidipine and amlodipine. *Methods:* Male Wistar Kyoto rats were subjected to 90 minutes of transient middle cerebral artery occlusion (MCAO) by a nylon thread. Animals were divided into 3 groups, vehicle, azelnidipine and amlodipine group. In azelnidipine and amlodipine groups, rats were treated with azelnidipine (1 mg/kg) and amlodipine (1 mg/kg) by gastric gavage for two weeks before MCAO. Vehicle group was treated by solution of methyl cellulose for two weeks. Rats were killed 24 hs after MCAO. Clinical parameters (mean arterial pressure, heart rate, body weight), infarct volume, brain edema index, cerebral blood flow (CBF), oxidative stress markers that are HEL, 4-HNE, AGE and 8-OHdG, and evidence of apoptosis by TUNEL, were investigated. *Results:* There were no significant differences among groups in mean arterial pressure, heart rate and body weight. Treatment with azelnidipine and amlodipine reduced infarct volume and brain edema. Azelnidipine treated group showed more marked reduction of infarct volume

and cerebral edema than amlodipine group. There was no attenuation of CBF in CCB groups. The number of HEL, 4-HNE, AGE and 8-OHdG positive cells were significantly decreased in CCB treated groups. These molecules were again fewer in azelnidipine group than in amlodipine group. In TUNEL staining, the number of positive cells was smaller in the CCB treated groups, especially in azelnidipine group. *Conclusions:* Pretreatment of azelnidipine and amlodipine had a neuroprotective effect in ischemic brain. Antioxidative property is one of the important profiles of CCBs that is implicated in brain protection. *Keywords:* cerebral ischemia, calcium channel blockers, azelnidipine, amlodipine, oxidative stress.

Transduction of injury signals between retinae after unilateral crush of the rat optic nerve

Macharadze, T.[a], Marunde, M.[a], Wanger, T.[b], Scheich, H.[b], Goldschmidt, J.[b], Zuschratter, W.[c], Gundelfinger, E. D.[d] and Kreutz, M. R.[a]
[a]*Project Group Neuroplasticity, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[b]*Department of Acoustics, Learning and Memory, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[c]*Special Lab Confocal Lasercan Microscopy and Electronmicroscopy, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[d]*Department of Neurochemistry / Molecular Biology, Leibniz Institute for Neurobiology, Magdeburg, Germany*

In this study we report that partial unilateral optic nerve crush (ONC) affects the number of retinal ganglion cells of the contralateral eye still in continuity with the ipsilateral superior colliculus. The reduction in cell number of the uncrossed retinal projection was accompanied by a microglia response largely restricted to their region of origin, i.e. the ventro-temporal retina. The cell loss could be prevented by the local intravitreal application of the anti-inflammatory agent dexamethasone. Axotomy had the same effect on the number of retinal ganglion cells of the uncrossed projection as incomplete ONC. Moreover, the level of neuronal activity after ONC as evidenced by thallium autometallography was much less altered in the termination area of the uncrossed projection, the rostro-medial superior colliculus, as compared to other areas of this region. We propose that injury signals from the damaged optic nerve and retina are transduced directly to the unaffected eye. These signals do not require a direct interaction between the contralateral and the ipsilateral pathways and they will induce an inflammatory response restricted to the uncrossed projection.

Rehabilitation augments stem cell transplantation after focal ischemia in rats

MacLellan, C. L., Cumby, N., Granter-Button, S., Hewlett, K., McKay, K., Chernenko, G. and Corbett, D.
Division of BioMedical Sciences, Faculty of Medicin Memorial, University Memorial University, St. John's, Canada

The potential for using stem cells to treat a number of neurological disorders, including stroke, has garnered much interest, but the ability of stem cells to promote functional recovery must be rigorously assessed in animal models before transplantation studies progress to clinical trials. Our laboratory showed that enriched housing and exercise enhanced transplanted subventricular zone (SVZ) stem cell migration and improved functional benefit following stroke in rats, but that the majority of cells died within one month of transplantation. We tested whether motor and cognitive rehabilitation augments stem cell transplantation after stroke. Prior to surgery, 62 male Sprague Dawley rats were trained to reach for food reward pellets in the staircase task. Performance was also assessed in the horizontal ladder-walking task, cylinder task, and elevated plus maze. Focal ischemia was induced by injecting the vasoconstrictive peptide endothelin-1 into the forelimb motor cortex and lateral striatum. Six days later, green fluorescent protein-expressing adult neural stem cells isolated from transgenic mouse SVZ (800,000 cells or vehicle) were injected into the ipsilateral sensorymotor cortex and striatum. Rats were then assigned to the control (standard housing; 2 rats per cage) or enriched rehabilitation condition that consisted of living in an enriched environment (8 rats per large cage with various objects used to stimulate exploratory behaviour) plus motor (skilled reaching) and cognitive (Hebb-Williams maze) rehabilitation 6 d/week. Functional recovery was assessed repeatedly over 2 months following transplantation, at which time rats were euthanized. Survival and migration of SVZ cells will be quantified using stereology. Preliminary analyses suggest that a significant proportion of stem cells sur-

vive out to 2 months and are located in the tissue surrounding the cortical injury and throughout the striatum. Immunohistochemistry (e.g., NeuN, GFAP, NG-2 and DCX) and confocal microscopy will be used to identify transplanted cell phenotype. In the staircase task, rehabilitation and stem cells each provide modest functional improvement, but greatest recovery occurred with the combination of therapies. Our results suggest that rehabilitation augments stem cell transplantation, possibly by enhancing neuronal plasticity required to support long-term stem cell survival.

Minocycline protects LHON-cybrids against thapsigargin induced cell death

Mansfeld, S.[a], Haroon, F.[a], Kreutzmann, P.[a], Mawrin, C.[a] and Kirches, E.[a]
[a]*Neuropathology and Medical Neurobiology of the Otto-von-Guericke University, Medical Faculty, Magdeburg, Germany*
[b]*Neuropathology of the Friedrich-Schiller University, Jena, Germany*

Leber's hereditary optic neuropathy (LHON) is a maternally inherited mitochondrial disorder, characterized by acute or subacute loss of retinal ganglion cells, leading to severe visual impairment or even blindness. The primary causes are mitochondrial DNA point mutations with amino acid exchanges in respiratory chain complex I, which can elicit ATP decline and enhanced ROS production. We recently reported, that 100 μM of the antibiotic minocycline can protect mitochondrial functions and inhibit apoptosis and cell death in a LHON-cybrid line under conditions of thapsigargin induced calcium overload (Haroon et al., Neurobiol Dis 28, 2007). In terms of cell survival, protection against 1 μM thapsigargin (over night) by a 30 min preincubation of cells with 100 μM minocycline, occurred selectively in a LHON clone, but not in a wt clone. We now demonstrate that a siginificant, dose-dependent cytoprotective effect occurred in two LHON and two wt clones in experiments with longer minocycline preincubation (6 h) and a weaker stimulus (0.1 μM thapsigargin). In this assay, even the more relevant concentration of 10 μM minocycline was protective, suggesting that the drug may generally be active against a moderate calcium stress. The results were reproduced with various cell densities, but experiments with some batches of the frozen cybrids failed to show any protective effect, suggesting unknown confounding variables.

Interactions of LPS or TMT – activated glia and neural stem cells derived from cord blood: Insights into the regulation of neurogenesis

Markiewicz, I., Winiarska, H., Domanska-Janik, K. and Lukomska, B.
Department of NeuroRepair, Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Brain inflammation contributes to the propagation of neuropathological events that involves activation of astrocytes and microglia. It remains obscure how activated glial cells affect the survival and differentiation of neural stem cells (NSC). *The aim of the study* was to analyze neuronal commitment of Human Umbilical Cord Blood derived Neural Stem Cells (HUCB-NSC) cultured in the presence of normal and LPS- or TMT-activated glial cells. *Methods:* HUCB-NSC (5×10^4/cm^2) were co-cultured with normal or LPS (0.1 μg/ml) and TMT (1 μM) – stimulated astrocytes and microglial cells isolated from neonatal rat brain for proliferation and cell phenotype assessment. Pro-inflammatory cytokines were estimated (ELISA). *Results:* Normal rat astrocytes induce HUCB-NSC to differentiate mostly into neurones (75% TUJ1+; 65% MAP-2+) but microglia stimulate HUCB-NSC to differentiate into neurons (45% TUJ1+) as well as into astrocytes (56% S100ß+). LPS – and TMT – induced astrocytes diminish neurogenesis of HUCB-NSC (29% and 33%, respectively vs 75% TUJ1+) and increase astrocyte differentiation (52% and 53%, respectively vs 1% S100ß+) in comparison to non-stimulated astrocytes. Microglia activation by LPS and TMT decreases HUCB-NSC differentiation into neurons (27% and 26%, respectively vs 45% TUJ1+) but enhances oligodendrogenesis (9% and 7%, respectively vs 1% O4+) compared to normal microglia. Stimulation of astrocytes and microglia by LPS and TMT declines HUCB-NSC proliferation (2% and 1% vs 24% co-cultured with astrocytes or 4% and 6% vs 16% co-cultured with microglia). The presence of IL-1β, IL-6 and TNF-α was observed in glia cell culture supernatants after LPS and TMT implementation. *Conclusions:* Activation of astrocytes and microglia induced by LPS and TMT attenuate pro-neural effect of non-stimulated (resting) glia and suppress proliferation of HUCB-NSC *in vitro*. The release of pro-inflammatory cytokines might be partly responsible for this effect. Supported by grants No: 2P05/A177/29; 1309/P01/2006/31.

Pro- and anti-regenerative processes characterized in the neonatal mouse brain

Maxwell, K., Toole, C., Butt, R., Coorssen, J. and Dyck, R.
Department of Physiology and Biophysics, Hotchkiss Brain Institute, Faculty of Medicine, University of Calgary, Canada

The murine medial frontal cortex (MFC) regenerates when removed by aspiration during the early postnatal period. However, a stroke injury induced in the same region, at the same age, does not. As such, these injury models provide a means to identify and differentiate pro- and anti-regenerative processes following cortical injury. We have now undertaken proteomic and genomic comparisons of the brain after stroke- and aspiration-induced injuries. Here we report the constellation of proteins and genes that change following each particular mode of cortical injury. In addition, we compare these expression data to that seen when the stroke-damaged tissue is removed by aspiration, 3 h after injury. Mouse pups (postnatal day 7; C57BL/6) were randomly assigned to have the MFC removed by aspiration, damaged by photothrombosis or damaged by photothrombosis followed by aspiration (stroke+aspiration). The inflammatory response was also characterized in each injury model using the microglial marker (Iba-1). Protein changes were assessed using 2-D gel electrophoresis coupled with mass spectrometric analyses, while changes in gene expression were assessed by RNA microarray. We identified specific proteins and genes that were uniquely or commonly regulated by mode of injury. Functionally, they are involved in physiological pathways including: cytoskeletal organization, stress response, redox regulation, cell death/survival, protein transport, growth factor signaling, and immune response. Remarkably, we found the stroke-damaged MFC will regenerate following removal of the injured tissue 3 h later. The microglial response in the cortical grey matter after stroke+aspiration was most similar to a stroke injury however the response in the white matter was most similar to that seen following aspiration injury. As for activation of biological pathways, the signature of the stroke+aspiration injured brain contained components that were seen in both stroke and aspiration injury models. These studies will help us to identify the specific cellular and molecular pathways that promote regeneration from those that inhibit it.

Cellular and behavioral neuroprotective effects of IGF-I following kainic acid induced degeneration of the hippocampus in rat and mice

Miltiadous P., Stamatakis A. and Stylianopoulou F.
Laboratory of Biology- Biochemistry, Faculty of Nursing, University of Athens, Greece

Insulin-like Growth Factor-I (IGF-I) has been used as a therapeutic agent in a number of models of neurodegenerative diseases. It has been shown that IGF-I plays a role in the repair processes following brain trauma exhibiting mitogenic and trophic actions and that it can improve the clinical outcome in animal models of ALS. In the present study, we investigated the neuroprotective action of IGF-I on kainic acid (KA)-induced neurodegeneration, since KA has been used in animal models of various human neurodegenerative diseases. KA or KA with IGF-I were stereotactically injected into the CA3 hippocampal area of adult rodents (rats or mice) and cell trauma and death markers were studied histologically. Following KA administration extended degeneration of the hippocampus was observed both ipsilaterally and contralaterally to the injection site as shown by cresyl violet staining. In addition, cell death was clearly evident in the ipsilateral hemisphere using FluoroJade B staining. Furthermore, activated astrocytes with typical astroglial processes, were detected in the ipsilateral hippocampus. Finally, Hsp70, an index of cellular stress, was induced in both the ipsilateral and the contralateral hemisphere. In the brain of rodents receiving both KA and IGF-I all the above markers of neurodegeneration were not observed in the contralateral hemisphere, while they were significantly reduced in the ipsilateral. These results suggest that IGF-I has neuroprotective properties, decreasing both neuronal death and astrogliosis. We also determined the effects of KA or KA with IGF-I administration on spatial learning and memory using the Morris water maze: KA injected mice had learning and memory impairments compared to animals injected with KA and IGF-I. Thus, IGF-I emerges as a potent neuroprotective factor both at the cellular and the behavioral level. This research project (PENED) is co-financed by E.U.-European Social Fund (75%) and the Greek Ministry of Development-GSRT (25%).

Potential effects of cell therapy on local inflammatory processes after MCAO

Möller, K.[a], Wagner, D.-C.[b], Kamprad, M.[c], Boltze, J.[b,d], Seeger, J.[a] and Stahl, T.[a]
[a]*Department of Anatomy, Histology and Embryology; University of Leipzig, Germany*
[b]*Fraunhofer Institute of Cell Therapy and Immunology, Leipzig, Germany*
[c]*Institute of Clinical Immunology and Transfusion Medicine, University of Leipzig, Germany*
[d]*Translational Centre for Regenerative Medicine, University of Leipzig, Germany*

Administration of adult hematopoetic stem cells is well known to reduce infarct size and to improve clinical outcome after cerebral ischemia in rodents and other animal models. First thought to promote recovery via replacement of damaged neurons and neuroglia, it is now assumed that these cells modulate the immune status through systemic and local effects and therefore influence the inflammatory response and the regenerative process in the brain. However, the exact nature of those alterations remains to be elucidated. There is rising evidence that T-cell subpopulations trigger resident microglia to shift from cytotoxic to neuroprotective profile as well as stimulate endogenous neurogenesis. On the other hand cell therapy is supposed to lead to a general decrease of inflammatory cell activation and infiltration.To characterize the inflammatory response we are currently using flow cytometric methods to quantify inflammatory cell populations in nervous tissue of rats after permanent occlusion of the middle cerebral artery. Thereby, we put the focus on the discrimination of resident and infiltrating macrophages by using markers for CD45, CD11b and MHC II as well as on the identification of T-cell subpopulations. Finally, we aim to specify potential differences between animals treated with human umbilical cord blood cells and untreated control groups. Besides, to obtain spatial information, a descriptive evaluation through histological and immunhistochemical methods is going to take place.

Cannabinoid CB2 receptor (CB2) activation in cerebral ischemia

Murikinati, S. R.[a], Jüttler, E.[a], Keinert, T.[a], Waibler, Z.[a], Sommer, C.[a], Kalinke, U.[a] and Schwaninger, M.[a]
[a]*Institute for Pharmacology, University Heidelberg, Germany*
[b]*Paul Ehrlich Institute, Langen, Germany*
[c]*Institute for Pathology, Johannes-Gutenberg-University, Mainz, Germany*

The incidence of stroke is increasing around the world. Endogenous cannabinoids (CBs) and their receptors acquired a great interest in stroke. The endogenous cannabinoids bind to and activate two Gi/o protein coupled receptors, central cannabinoid receptor type 1 (CB1) and the peripheral cannabinoid receptor type 2 (CB2). The latter is particularly abundant in the immune system. Here, we have investigated the role of the CB2 receptor and its selective agonist JWH-133 at the cellular and molecular level in focal cerebral ischemia. Mice were subjected to middle cerebral artery occlusion and infarct volumes were measured after 48 h. Using CB1- and CB2-deficient mice we found that JWH-133 has neuroprotective effects through CB2 receptors. Using bone marrow transplantations between wild-type (WT) and CB2-/- mice, we obtained evidence that the CB2 agonist JWH-133 reduces infarct size through bone marrow-derived cells. Immunohistochemical detection of Iba1-positive cells showed that microglia is significantly reduced by the CB2 agonist JWH-133. In conclusion, our data show that activation of the CB2 receptor is neuroprotective and suggest that the neuroprotection is mediated by bone marrow-derived cells, possibly microglia.

Is the PHD-inhibitor dimethyloxalylglycine neuroprotective in focal cerebral ischemia?

Nagel, S.[a], Brooks, K. J.[a], Hoyte, L.[a], Papadakis, M.[a], Sibson, N. R.[b] and Buchan, A. M.[a]
[a]*Acute Stroke Programme, Nuffield Department of Clinical Medicine, University of Oxford, UK*
[b]*Experimental Neuroimaging, Department of Physiology, Anatomy & Genetics, University of Oxford, UK*

Background: Recent studies in focal cerebral ischemia, in contrast to global forebrain ischemia, have proposed that an increase in the Hypoxia Inducing Factor (HIF)-1alpha protein levels may result in better outcome after ischemia. Moreover, neuron-specific inactivation of HIF-1alpha seems to increase brain injury in transient focal ischemia. Dimethyloxalylglycine (DMOG) is a cell penetrant oxoglutarate analogue that stabilizes HIF-1alpha *in vitro* in cell cultures and *in vivo* in ischemic muscle models by inhibiting the Prolyl-4-Hydroxylases (PHDs). We are investigating the ef-

fect of DMOG on ischemic damage, cerebral perfusion and neurological outcome using a permanent focal cerebral ischemia rat model. *Material and Methods:* Male wistar rats (225 g $+/-$ 10%) are randomly treated for two days twice daily with 40 mg/kg BW DMOG in saline (cumulative dose: 160 mg/kg BW) or saline alone. Permanent focal cerebral ischemia is induced with the filament model at day 2. One, three and twenty four hours after ischemia MRI is performed using diffusion-weighted imaging (DWI) and arterial spin labeling (ASL) perfusion-weighted imaging. At twenty-four hours, a neuroscore is obtained and brains are either perfusion fixed in 4% formaldehyde or snapfrozen following the MRI investigation. Immunohistochemistry (IHC) and western blotting (WB) are performed to assess expression levels of HIF-1 alpha as well as downstream proteins, such as vascular endothelial growth factor (VEGF). *Results:* Preliminary results show an approximately 40% reduction of infarct sizes on DW images, and improved neuroscores at 24 hours in the DMOG treated animals compared to saline treated rats. The combination of DMOG and ischemia produces a significant increase in VEGF protein levels in the ipsilateral cortex relative to ischemia alone or DMOG treatment without ischemia. The full analysis of MRI parameters, including perfusion imaging, as well as IHC and WB results of HIF-1alpha and downstream proteins will be presented. *Conclusions:* Pre-treatment with DMOG appears to attenuate brain injury after permanent focal cerebral ischemia.

Microglia protect neurons after ischemia in different ways

Neumann, J.[a], Dinkel, K.[a], Gunzer, M.[b] and Reymann, K. G.[a,c]
[a]*Leibniz Institute for Neurobiology, Magdeburg, Germany*
[b]*Institute for Molecular and Clinical Immunology, Otto von Guericke University, Medical Faculty, Magdeburg, Germany*
[c]*Institute for Applied Neurosciences* (*FAN*), *Magdeburg, Germany*

Cerebral ischemia is accompanied by an acute inflammation, involving the activation of microglia and the infiltration of neutrophils (PMN) and monocytes into the brain. Whether and how microglia can protect neurons from ischemic death is still debated. Using a model of application of innate immune cells onto hippcampal slices (OHC), we investigated their effects on neuronal death after ischemia. We show that applied PMN exacerbated neuronal damage, whereas microglia protected neurons from oxygen glucose deprivation (OGD). Applying an approach of transgenic OHC from mice (eYFP expression in neurons) and fluorescently labeled microglia, we observed that microglia engaged in close physical contact with neurons after OGD. Blockage of microglia migration to the neurons after OGD abolished the microglia mediated protection. Moreover, the application of microglia and PMN simultaneously indicated that microglia counteracted the PMN neurotoxicity. Time-lapse imaging revealed the phagocytosis of PMN by microglia. Interference with the phagocytosis using RGDS and N-Acetyl-Glucosamine abrogated the microglial ability to reduce the PMN neurotoxicity. Taken both observations together: microglia might protect neurons after ischemia in two ways: (i) direct by physical interaction with neurons and (ii) indirect by clearance the inflamed tissue from toxic PMN. Thus, non-selective anti-inflammatory treatment after ischemia would be counterintuitive.

Aquaporin-4 mediated waterfluxes are compromised by histamine in astrocytes *in vitro* and in rat brain *in vivo*

Nuydens, R.[a], Buist, A.[a], De Jongh, R.[b], Smets, A.,[a,c] and Meert, T.[a]
[a]*CNS department, Johnson & Johnson Pharmaceutical Research & Development, a division of Janssen Pharmaceutica N.V, Beerse, Belgium*
[b]*Department of Anaesthesia, Intensive and Emergency Care, Ziekenhuis Oost-Limburg, Genk, Belgium*
[c]*University Hasselt, Diepenbeek, Belgium*

Aquaporins (AQP) determine bi-directional, transmembraneous water transport and regulate cell volume and tissue water homeostasis. AQP-4, the channel with the highest water permeability, is most abundantly expressed in the brain and predominantly localized on astrocyte endfeet. Its expression rate and polarization changes during various central nervous system diseases accompanied by cerebral edema, including stroke, trauma, infections and brain tumors. AQP-4 is thought to contribute to brain edema formation and clearance, however, little is known about gating, shedding, internalization and degradation of AQP-4. Histaminergic neurons project throughout the entire brain

and are activated during brain diseases accompanied by edema. Interfering with histamine signaling influences the outcome after neurological insults in animals. In addition, histamine challenge to an AQP-4 transfected gastric cell line decreased the fraction of functional membrane-associated AQP-4 and attenuated hypotonic cell swelling.The present study shows, in primary astrocyte cultures and in AQP-4 transfected 1321-N1 cells, that AQP-4 expression is mainly restricted to the cell membrane with a small fraction confined to a vesicular compartment in the cytoplasm. Challenges with various degrees of hypotonicity, results in a fast, reversible cell swelling as measured by quantitative microscopy. AQP-4 overexpression in 1321-N1 cells results in an increased swelling upon hypotonicity. Exposure of primary astrocytes or AQP-4 transfected 1321-N1 cells to histamine induces a rapid decrease of membrane-associated AQP-4 with an accumulation of AQP-4 containing vesicles inside the cell. Histamine pretreatment dose-dependently reduces the cell swelling upon hypotonic stress. *In vivo*, ICV application of histamine dose dependently impairs water homeostasis in the adult rat brain thereby increasing the ICP. The ICP changes over time show a biphasic pattern that ultimately reached pathological levels and leads to death of the injected rats.

Dissecting the molecular pathways responsible for the selective vulnerability of CA1 hippocampal cells following transient global forebrain ischemia by proteomic analysis

Papadakis, M.[a], Hoyte, L.[a], Nagel, S.[a], Batycka, M.[a], Zhang, S.[b], Kessler, B.[a] and Buchan, A. M.[a]
[a]*Nuffield Department of Clinical Medicine, University of Oxford, UK*
[b]*Department of Clinical Neurosciences, University of Calgary, Alberta, Canada*

Background: Global and transient forebrain ischemia results in a differential response in particular areas of the hippocampus. CA1 pyramidal neurons exhibit selective and delayed neuronal death, whereas, the CA3 area is resistant to ischemia. Therefore, the global model of transient forebrain ischemia provides a promising platform to study the differential susceptibility of distinct neuronal populations to cerebral ischemia. *Methods:* Adult rats were subjected to either transient, but severe forebrain ischemia, using the four-vessel occlusion model, or to sham-forebrain ischaemia. Animals were sacrificed 24 hours, following reperfusion, CA1 and CA3 area were microdissected, cytoplasmic and membrane/organelle fractions were generated, and analysed by 2D electrophoresis. Isoelectric focusing was carried out using a 3–10 pH gradient and proteins were separated according to their molecular mass using 12% SDS-PAGE gels. Quantitative analysis was performed to identify proteins that were upregulated or downregulated due to ischemia, spots of interest were excised and their identity was determined by mass spectrometry. *Results:* Analysis of the membrane/organelle proteins of the CA1 area of ischemic brains, revealed a similar profile to sham-ischemic CA1 areas. However, particular chaperone proteins were significantly upregulated in the vulnerable CA1 area, compared to sham-ischemic CA1 cells, indicating the cellular response of ischemic CA1 cells to compensate for the accumulation of misfolded proteins. Further investigation of cytoplasmic proteins present in ischemic and sham-ischemic CA1 areas demonstrate that the proteomic analysis of subcellular fractions from CA1 and CA3 areas can provide novel pathways to confer neuroprotection.

Single-cell resolution mapping of potassium metabolism in the hippocampus of naive rats and after transient global cerebral ischaemia

Pforte, C.[b], Henrich-Noack, P.[a], Schroeder, U. H.[b], Hecht, T.[b], Scheich, H.[b], Goldschmidt, J.[b] and Reymann, K. G.[a,b]
[a]*Research Institute Applied Neurosciences* (*FAN gGmbH*), *ZENIT, Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Magdeburg, Germany*

With the hitherto available imaging techniques it is hardly possible to investigate hippocampal activity patterns at the level of individual cells *in vivo.* Using the newly developed method of thallium autometallography with the lipophilic chelate complex thallium diethyldithiocarbamate it is possible to trace the cellular potassium metabolism. Using this technique we were able to perform for the first time single cell mapping of potassium metabolism and neuronal activity in the hippocampus from naive rats and after transient two-vessel occlusion with hypotension.In naive animals the neuronal bands were clearly labelled and individual cells could be distinguished by a differentiated staining intensity. In the hilus single, strongly thallium-positive

neurons became visible. Two weeks after global ischaemia, we could show a regularly alternating vertical laminar periodicity of the selective vulnerability in the CA1/*stratum radiatum* in animals, which developed a partial damage in CA1. The surviving CA1 neurons were intensively stained. In animals with complete CA1 cell death, the *stratum moleculare* and the granular neurons of the DG had changed from a slight staining (controls) to a very intensive labeling. This is the first morphological evidence of functional impairments in the granular neurons after global ischemia and it suggests that a pronounced hyperpolarization may be the underlying mechanism of the loss of function in the DG post ischemia. These results have significant implications for the interpretation of neuroprotective effects and for the use of regenerative strategies and they reveal extraordinary new insights of post-ischemic brain function by imaging neuronal activity at the cellular level.

Pigment epithelium derived factor during postnatal development, ageing and disease of the nervous system

Pina, A. L., Kubitza, M., Van Wagenen, S., Ertl, M. and Brawanski, A.
Department of Neurosurgery, Regensburg University Clinic, Regensburg, Germany

Pigment Epithelium Derived Factor (PEDF) is a secreted glycoprotein, which possesses multiple and varied biological properties, it is neurotrophic, neuroprotective, antitumorigenic and has a potent antiangiogenic activity. We have focused our attention on exploring the specific localization, function, and effects of PEDF in the nervous system during normal development, aging, and disease. The following is a brief account of some of our results. We have demonstrated that PEDF protein is naturally down-regulated with age in the roedent eye and brain, leading to an age-related increase of the VEGF/PEDF ratio. This suggests a potential higher risk for neovascularization and partly the cause of some degenerative diseases at older stages of life in these organs. With Immunohistochemistry and cell specific double labeling, we have shown the specific expression of PEDF in endothelial cells and neurons in various regions of the adult brain. This defined localization of PEDF open the possibility to search for other relevant effects of this factor in the brain. Our *in vivo* results show that intraventricular infusion of PEDF has a stimulatory influence on subventricular zone neural stem cells while reducing the proliferation of microglia cells in the traumatic injured brain. These results support the importance of this molecule in the control of neurogenesis and inflammation in the lesioned brain and indicate the significant therapeutic potential that PEDF might have for the diseased nervous system.

Efficient immunosuppressive treatment promotes graft survival and allows tumour development from xenografted h-ESC in models of cerebrovascular diseases

Polentes, J.[a], Tine, S.[a], Seminatore, C.[a], Ellman, D.[b], Jendelova, P.[c], Lambertsen, K.L.[b], Kozubenko, N.[c], Blondeau, J.[a], Sykova, E.[c], Finsen, B.[b], Onteniente, B.[a] and the STEMS consortium
[a]*INSERM UMR 894, Centre de Recherche en Psychiatrie et Neurosciences, Paris and INSERM UMR 861/I-STEM/AFM/UEVE, Evry, France*
[b]*Medical Biotechnology Center, University of Southern Denmark, Odense, Denmark*
[c]*Institute of Experimental Medicine, Prague, Czech Republic*

Xenografting for experimental transplantation studies or allografting in humans requires an immunosuppressive treatment (IT) to avoid graft rejection. Evidence is accumulating in support of a role for innate immune and adaptive immune responses in cerebrovascular diseases. In particular, T lymphocyte depletion has been described after brain infarction. In addition, cerebrovascular diseases involve the rupture of the blood-brain barrier, with a strong inflammatory reaction that might impair the survival of transplanted cells. We have compared the efficacy of two IT and one anti-inflammatory treatment on the survival of h-ESC-derived progenitors transplanted into the rat brain after an ischemic insult. Sprague-Dawley rats received a 90 min occlusion of the middle cerebral artery (tMCAO) and were transplanted with neural progenitors derived from the SA-001 h-ESC line (Cellartis AB). Rats were randomly assigned to 3 groups: IT#1: cyclosporine A (CsA) 4 mg/kg, azathioprin (Aza) 4 mg/kg, and methylprednisolone (MP) 2 mg/kg every other day; IT#2: CsA 10 mg/kg, Aza 5 mg/kg, MP 2 mg/kg daily; and IT#3: 4 mg/kg of ketoprofen, a non-steroidal anti-inflammatory drug, daily for 3 weeks followed by IT#2. Rats were sacrificed after 1 month. Graft survival was 65% with IT#1, 100% with IT#2 and 60%

with IT#3. After 1 month, graft size in IT#3 was smaller than in IT#2. However, tumor formation was observed in 100% of the animals that had received IT#2. Tumor development was confirmed before sacrifice of the animals by the deterioration of behavioural scores.

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Identification of ischemic regions in a rat model of stroke

Popp, A., Jaenisch, N., Witte, O. W. and Frahm, C.
Department of Neurology, Friedrich-Schiller-University, Jena, Germany

Background and Purpose: Investigations following stroke first of all require information about the spatiotemporal dimension of the ischemic core as well as of perilesional and remote affected tissue. Here we immunohistochemically evaluated regions differently impaired by focal ischemia. *Methods:* Wistar rats underwent a transient (30min) suture-occlusion of the middle cerebral artery (MCAO) followed by reperfusion times of 2 h, 1 d, 7 d, and 30 d. Ischemic samples were processed for western blotting concerning their expression level of two inducible heat shock proteins, HSP27 and HSP70. Adjacent brain slices were stained with TTC and subsequently processed for two distinctive Nissl methods and for immunohistochemistry using MAP2, HSP27, and HSP70. *Results:* For identification of the infarct core conventional TTC staining is reliably applicable at 1 d of reperfusion whereas Nissl histology works well from 1 d of reperfusion on. HSP70 reacts in a limited post-ischemic time (1d) but precisely defines the ischemic penumbra. Though MAP2 is an excellent marker at all investigated times, its precedence lies in a reliable recognition of injured tissue as early as 2 h after stroke. HSP27 can be visualized from 1 d of reperfusion on and is sensitive enough to detect perilesional and even remote impaired tissue. HSP27 expression strength in western blots correlates well with the infarct dimension whereas HSP70 intensity displays the dimension of penumbral tissue. *Conclusions:* A reliable identification of the infarct core as well as of perilesional and remote affected ischemic tissue requires different approaches at variable times after focal stroke.

Functional properties of D6/GFP-neural stem/progenitor cells during *in vitro* differentiation and after transplantation into the injured rat brain

Prajerova, I.[a,b], Anderova, M.[a,c], Honsa, P.[a], Machon, O.[d] and Chvatal, A.[a,b,c]
[a]*Laboratory of Neurobiology, Department of Cellular Neurophysiology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[b]*Department of Neuroscience, 2nd Med. Faculty, Charles Universit, Prague, Czech Republic*
[c]*Center for Cell Therapy and Tissue Repair, Charles University, Prague, Czech Republic*
[d]*Department of Transcriptional Regulation, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic; Prague, Czech Republic*

D6 is a promoter/enhancer of mDach1 gene, which is involved in the development of neocortex including ventricular zone and hippocampus, and is expressed in the proliferating neural stem/progenitor cells in the cortex. The differentiation potential of embryonic neural stem/progenitor cells, isolated from E12 mouse embryos, in which the expression of the GFP is driven by D6 promoter/enhancer, has been studied *in vitro*, and after transplantation into intact adult rat brain as well as into the site of photochemical lesion. The electrophysiological properties of D6/GFP cells were studied using the whole-cell patch clamp technique, and immunohistochemical analyses were carried out. Six days after the onset of *in vitro* differentiation two cell populations were identified. Large flat cells forming an underlying layer expressed GFAP and/or nestin. Smaller cells with multiple long processes expressed neuronal markers βIII tubulin, MAP-2 or DCX. These cells with an average membrane potential of -61.2 mV, a membrane resistance of 909.5 MOhms and a membrane capacitance of 11.2 pF displayed voltage-dependent A-type K+-channels, delayed outwardly rectifying K+-channels and Na+-channel, which was blocked by 1 μM TTX. One week after transplantation into the intact tissue and 4 weeks after the transplantation into the site of photochemical lesion the D6/GFP-cells survived and expressed markers characteristic of mature neurons, such as NeuN, NF68, βIII tubulin and MAP2. Based on these results D6/GFP-cells could provide a suitable tool for studying cell survival, migration and differentiation under pathological conditions.

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Neuroprotective potential of human umbilical cord blood MNC after OGD insult in organotypic hippocampal slice cultures

Reich, D. M.[a], Hau, S.[b], Straßburger, M.[c], Naumann, W.[d], Emmrich, F.[a,e,f], Reymann, K. G.[c] and Boltze, J.[a]
[a]*Fraunhofer Institute of Cell Therapy and Immunology, Leipzig, Germany*
[b]*Vita 34 AG, Leipzig, Germany*
[c]*Research Institute for Applied Neurosciences* (*FAN gGmbH*), *Magdeburg, Germany*
[d]*Faculty of Bioscience, Pharmacy and Psychology, Institute of Zoology II, University of Leipzig, Germany*
[e]*Institute of Clinical Immunology and Transfusion Medicine, University of Leipzig, Germany*
[f]*Translational Centre for Regenerative Medicine, University of Leipzig, Germany*

The potential of cell therapy after stroke has been investigated in several experimental paradigms using *in vivo* models of global and focal ischemia. However, due to complexity of the situation *in vivo* causal relationships between observed improvements of behavioral skills and impact of grafts have not been revealed up to now. Complex three-dimensional organization of nervous tissue retaining organotypic hippocampal slice cultures (OHC) provide an adequate and at the same time simplified experimental basis to determine effects of therapeutic cells. In this study, we investigated the ability of human umbilical cord blood mononuclear cells (HUCB-MNC) and HUCB derived stem cells (CD34+), (i) to influence neuronal cell death and (ii) to migrate towards injured neuronal tissue using OHC that were exposed to oxygen-glucose deprivation (OGD).Over an observation period of three days HUCB-MNC were able to reduce neuronal damage measured by PI uptake to 77% $\pm$ 10% when they were placed onto OGD OHC. In addition, HUCB-MNC exhibited an elongated migration promoting morphology and in fact migrated into the slices. In indirect cocultures HUCB-MNC required a five-fold elevation in cell numbers to accomplish efficient cell death reducing effects. Direct application of CD 34+ stem cells (enriched to 94% $\pm$ 3%) could only provide remarkable protection during the first two days (47% $\pm$ 11%).

Gene expression in the cortex after exposure to low frequency (60 kHz) ultrasound

Reuter, P.[a,b,c], Nedelmann, M.[d,e], Schülke, S.[b], Nürnberg, S.[c], Walberer, M.[d,e], Ritschel, N. [d,e], Alessandri, B.[c], Kempski, O.[c], Marx, J.[a], Kaps, M.[d], Gerriets, T.[d,e] and Hankeln, T.[b]
[a]*Department of Neurology, Johannes Gutenberg-University Mainz, Germany*
[b]*Institute of Molecular Genetics, Johannes Gutenberg University Mainz, Germany*
[c]*Institute for Neurosurgical Pathophysiology, Johannes Gutenberg University Mainz, Germany*
[d]*Department of Neurology, Justus Liebig-University Giessen, Germany*
[e]*Department of Radiology - Experimental Neurology Research Group, Kerkhoff Clinic, Bad Nauheim, Germany*

Currently the most effective therapy for thrombotic stroke is the application of rt-PA. Therapeutic ultrasound can support the thrombolytic effect of rt-PA. However previous histological and MRT studies revealed detrimental effects of low frequency ultrasound on the brain. An enlarged ischemic volume and intracerebral bleeding were observed. Until now there are no data concerning the molecular response to transcranial ultrasound treatment. Our study was therefore designed to evaluate the molecular effects of 60 kHz ultrasound on the brain. We compared gene expression levels in native rats to ultrasound treated rats and rats, which underwent experimental middle cerebral artery occlusion (MCAO). The results are hoped to contribute to preventing future problems using ultrasound thrombolysis. Wistar rats were anaesthetized with isoflurane and treated with transcranial ultrasound (60 kHz) for one hour. After 4 or 24 hours brains were extracted and frozen in liquid nitrogen. Gene expression levels in the cortex were measured by qRT-PCR. We investigated a selection of genes coding for transcriptional factors, apoptosis markers, angiogenesis factors, heat shock proteins and respiratory molecules to obtain an initial overview of the molecular responses of brain tissue. Our results indicate that most of the genes are down-regulated in the ultrasound treated groups compared to the native animals or ischemic animals. Additionally, it appears that isoflurane has a repressive effect on gene expression. Possibly this down-regulation of many genes inhibits protective processes and therefore causes the observed detrimental effects.

Microglia is modulated by protease-activated receptor 4

Riek-Burchardt, M.[a], Dökert, M.[a], Neumann, J. [a], Reiser, G.[c] and Reymann, K. G.[a,b]
[a]*Leibniz Institute for Neurobiology, project group Neuropharmacology, Magdeburg, Germany*
[b]*Institute for Applied Neurosciences* (*FAN gGmbH*), *Magdeburg, Germany*
[c]*Institute for Neurochemistry, Otto-von-Guericke-University Magdeburg, Medical Faculty, Germany*

Cerebral ischemia is among other immunological events characterised by an early response of microglia cells within the endangered region and microglia from surrounding parenchyma. Migration of surrounding microglia to the site of injury is a key event in exerting the response in the respective region. Whether microglia cells then exhibit beneficial or detrimental effects is still highly debated. It has been shown that the serine protease thrombin is an inflammatory mediator that acts via protease-activated receptors (PAR1, PAR3 and PAR4) and modulates microglia function (e.g. cytokine production). We have been focusing on the role of PAR4 on microglia after ischemia. Using an *in vitro* co-culture of primary hippocampal neurons and the microglia cell line BV-2, we show that microglia migration activity is dramatically enhanced after ischemia. Similar migration enhancement of microglia in the co-culture has been seen after selective PAR4 activation by an activating peptide without ischemic exposure. Moreover, we investigated the PAR4 dependent regulation of ED-1 expression that shows the lysosomal activity of phagocytotic cells. We found a down regulation of ED-1 expression after selective activation of PAR-4 on microglia cells. Thus, our results implicate that microglia activation by PAR4 might regulate microglia function after cerebral ischemia.

Higher subventricular zone activation and neuroblast migration when the adherent fraction of the bone marrow is transplanted into the brain of ischemic rats

Romaguera-Ros, M.[a], Borreda Gascó, I.[a], Pérez-García, A.[a], Sirerol-Piquer, M. S.[a], Gómez-Pinedo, U.[a], García-Verdugo and J. M.[a,b]
[a]*Centro de Investigación Príncipe Felipe, Valencia, Spain*
[b]*Universitat de València, Valencia, Spain*

España Stroke remains a leading cause of death and disability worldwide. Over the last decade, stem cell therapy has been introduced as an experimental treatment for stroke. In this study, the permanent intraluminal middle cerebral artery occlusion was performed and the adherent fraction of the bone marrow transplanted into the penumbra site, due to their homing and plasticity ability in injured tissues. These transplanted cells are supposed to provide trophic support to tissue at risk in the penumbra surrounding the infarct area, enhance vasculogenesis, and help promote survival, migration, and differentiation of the endogenous precursor cells after stroke. A comparison between the SVZ activation in stroke vs stroke with cell transplantation revealed a higher increase in the SVZ activation with the cell transplantation than stroke per se. Electron microscopy revealed an increase in the cell layer number at the SVZ. This result was confirmed with immunohistological staining, where not only an increase in BrdU positive cells were observed with cell transplantation, but also an increase in progenitors and migrating neuroblast towards the damaged tissue as can be observed by TUJ-1, PSA-NCAM and GFAP immunostaining. Future perspectives in our work consist in providing an adequate environment for these new generated cells at the damaged area.

Aβ mediated diminution of MTT reduction – an artefact of single cell culture?

Rönicke, R.[a], Klemm, A.[a], Meinhardt, J.[c], Schröder, U. H.[a], Fändrich, M.[c] and Reymann, K. G.[a,b]
[a]*Project Group Neuropharmacology, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[b]*Research Institute for Applied Neurosciences* (*FAN gGmbH*), *Magdeburg, Germany*
[c]*Leibniz Institute for Age Research* (*Fritz Lipmann*), *Jena, Germany*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) reduction assay is a frequently used and easily reproducible method to measure beta-amyloid (Aβ) toxicity in different types of single cell culture. To our knowledge, the influence of Aβ on MTT reduction has never been tested in more complex tissue. Initially, we reproduced the disturbed MTT reduction in neuron and astroglia primary cell cultures from rats as well as in the BV2 microglia cell line, utilizing three different Aβ species, namely freshly dissolved Aβ (25–35), fibrillar Aβ (1–40) and oligomeric Aβ (1–

42). In contrast to the findings in single cell cultures, none of these Aβ species altered MTT reduction in rat organotypic hippocampal slice cultures (OHC). Moreover, application of Aβ to acutely isolated hippocampal slices from adult rats and *in vivo* intracerebroventricular injection of Aβ also did not influence the MTT reduction in the respective tissue. Failure of Aβ penetration into the tissue cannot explain the differences between single cells and the more complex brain tissue. Thus electrophysiological investigations disclosed an impairment of long-term potentiation (LTP) in the CA1 region of hippocampal slices from rat by application of oligomeric Aβ (1–40), but not by freshly dissolved Aβ (25–35) or fibrillar Aβ (1–40). In conclusion, the experiments revealed a glaring discrepancy between single cell cultures and complex brain tissue regarding the effect of different Aβ species on MTT reduction. Particularly, the differential effect of oligomeric versus other Aβ forms on LTP was not reflected in the MTT reduction assay. This may indicate that the Aβ oligomer effect on synaptic function reflected by LTP impairment precedes changes in formazane formation rate or that cells embedded in a more natural environment in the tissue are less susceptible to damage by Aβ, raising cautions against the consideration of single cell MTT reduction activity as a reliable assay in Alzheimer's drug discovery studies.

The chemokine SDF-1 mediates 'homing' of human umbilical cord blood cells to hypoxic-ischemic brain lesions

Rosenkranz, K.[a], Kumbruch, S.[a], Lebermann, K.[a], Marschner, K.[b], Jensen, A.[c], Dermietzel, R. [a] and Meier, C.[a]
[a]*Department of Neuroanatomy and Molecular Brain Research, Ruhr-University Bochum, Germany*
[b]*Department of Obstetrics and Gynecology, St. Elisabeth-Hospital, Bochum, Germany*
[c]*Department of Obstetrics and Gynecology, Knappschaftskrankenhaus, Ruhr-University Bochum, Germany*

Previous studies have shown that intraperitoneal transplantation of human umbilical cord blood (hUCB)-derived mononuclear cells led to the specific 'homing' of these cells to a hypoxic-ischemic brain lesion in perinatal rats. Motor deficits resulting from the lesion were alleviated upon transplantation. Thus, the presence of hUCB cells at the lesion site seems to be a major prerequisite for their potential beneficial effect. In this study, we focused on elucidating mechanisms underlying the specific migration of hUCB cells to the brain lesion. The presence of chemotactic signals at the lesion site is one possibility to induce cell 'homing'. The chemokine stromal derived factor-1 (SDF-1/CXCL12), which was previously shown to be a potent chemoattractant for directed migration of other stem and progenitor cells, is a putative candidate of chemotactic factors. Therefore we investigated the spatial and temporal expression of SDF-1 in brain hemispheres with or without hypoxic-ischemic lesion. SDF-1 expression was substantially increased at the lesion site during the investigated period of fourteen days after the insult. Furthermore, HLA-positive hUCB cells were mainly detected in SDF-1 expressing brain regions and we were able to show that these cells express the SDF-1 receptor CXCR4 on their surface. The functional implication of SDF-1 in directing hUCB cell migration was determined by application of neutralizing SDF-1 antibodies *in vivo*, resulting in a reduced number of hUCB-derived mononuclear cells residing at the lesion site. With these functional effects, together with the observed timing and location of its expression, the involvement of the chemokine SDF-1 in hUCB cell 'homing' seems conceivable.

Mobilizing the brain's endogenous stem cell niche *in vivo*

Rueger, M. A., Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoeppner, D. J., Ravin, R., Poser, S. W., Bae, S.-K., Kittappa, R. and McKay, R. D. G.
Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, USA

Stem cells provide a critical component of regenerative medicine. Neural progenitor cells reside in the adult brain with the ability to self-renew and differentiate into tissue appropriate, functional cell types. However, this inherent regenerative potential is insufficient to facilitate substantial recovery of the adult brain following severe insults or neurodegeneration. Furthermore stem cell transplantation therapies are limited by inefficient stem cell growth and immunological incompatibilities with the host. Therefore, it is desirable to boost the generation and survival of endogenous stem/progenitor cells *in vivo* to achieve maximum functional recovery following injury. We identified a general signal transduction pathway that is initiated by

activation of the Notch receptor and controls both the growth and survival of stem cells. Notch receptor activation induced the expression of the specific target genes hairy and enhancer of split 3 (Hes3) and Sonic hedgehog (Shh) through rapid activation of cytoplasmic signals, including the serine/threonine kinase Akt and the mammalian target of rapamycin (mTOR) along with activation of the transcription factor STAT3. This resulted in improved survival of neural progenitor cells in culture. Intracerebroventricular infusion of Notch ligands into the brain of adult rats significantly increased the number of newly generated progenitor cells *in vivo*, and produced significant motor recovery in rats following the middle cerebral artery occlusion (MCAO) stroke model. These data indicate that two central goals of regenerative medicine, enhanced stem cell expansion *in vitro* and *in vivo*, can be achieved through modulation of fundamental signal transduction cascades by Notch ligands.

Erythropoietin impedes morphological consequences of chronic neurodegeneration

Sargin, D., Hassouna, I., Sperling, S., Sirén, A.-L. and Ehrenreich, H.
Max-Planck-Institute of Experimental Medicine, Göttingen, Germany

Erythropoietin (EPO) is a hematopoietic growth factor with a wide range of neuroprotective properties. Using our model of chronic progressive neurodegeneration, induced by a standardized cryo-lesion of the right parietal cortex of 4-week-old mice, we found global brain atrophy and distinct cognitive impairment many months after lesion, which could both be completely prevented by early EPO treatment. This ideal situation of a subtraction approach allows us to study processes of neurodegeneration and mechanisms of neuroprotective EPO action. The present work focuses on understanding the morphological basis of the observed atrophy and the EPO effect on numbers of glial cells and neurons including neuronal subpopulations and synaptic density. We show that unilateral parietal lesion at early age causes persistent bilateral microglial activation in the hippocampus, consistent with a chronic inflammatory response. Although total number of neurons and glial cells remain unaltered, a subgroup of neurons, the parvalbumin expressing GABAergic interneurons, is significantly increased bilaterally in the hippocampus upon lesion. Importantly, oligodendrocytes are reduced upon lesion, explaining the observed brain atrophy. These late bilateral consequences of the unilateral lesion are entirely abolished by early EPO application. Understanding these profound effects of EPO in our model of global brain atrophy will open new avenues for treatment of neurological and psychiatric diseases.

Bone marrow stem cells do not need cell contact with hypoxic injured tissue to evoke neuroprotective effect

Sarnowska, A.[a,c], Braun, H.[b], Sauerzweig, S.[b] and Reymann, K. G.[a,b]
[a]*Research Institute for Applied Neuroscience,* (*FAN gGmBH*), *Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Magdeburg, Germany*
[c]*NeuroRepair Department, Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland*

Bone marrow stem cells (*BMSCs*) are able to confer beneficial effects after transplantation into animals with ischemic brain injuries. This effect is probably mainly caused by the release of trophic factors, although the replacement of dead neural cells by *BMSCs* cannot be excluded. The aim of this study was to answer the question, whether the neuroprotective effects depend on direct cell-cell contacts between *BMSCs* and injured tissue. We investigated that interplay in an *in vitro* model of organotypic slice cultures in order to avoid the interference of immunological rejection processes after transplantation *in vivo*. To perform ischemic injury *in vitro* organotypic hippocampal slice cultures (OHC) were exposed to oxygen-glucose deprivation (OGD). Possible direct or indirect neuroprotective effect evoked by *BMSCs* was evaluated in two experimental paradigms using ischemic injured hippocampal slices: (i) cell transplantation on the top of OGD-treated OHC (ii) co-cultivation of cell culture with OHC space separated for 24 h. In both paradigms, *BMSCs* treatment evoked significant neuroprotection in OGD-injured tissue. This effect increased after treatment with *BMSCs* serum deprived, enriched with cells expressing nestin and GFAP. Comparing cell transplantation and cell co-cultivation with injured tissue we concluded that the neuroprotective effect of *BMSCs* does not depend on cell-cell contacts. Supported by Marie Curie development host fellowship program (MCFH-2001-00639).

Identification of a subset of bone marrow cells expressing embryonic and early neural markers after proliferation under serum-free culture conditions

Sauerzweig, S.[a,b], Reymann, K. G.[a,b] and Braun, H.[a,b]
[a]*Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[b]*Research Institute Applied Neurosciences* (*FAN gGmbH*), *Magdeburg, Germany*

The stem cell debate concerning the ability of bone marrow stromal cells (BMSC) to differentiate into neural cell types, were incremental affected by indications for the existence of a pluripotent subpopulation responsible for the observed effects. In our study, we initially analyzed the role of fetal bovine serum (FBS) regarding the neural differentiability of rat BMSC *in vitro*. By performing immunocytochemistry and various microscopy techniques including time-lapse imaging, we were able to verify a subset of cells, which are able to proliferate during a period of serum-free cultivation. The progenies possess a round respectively bipolar morphology and co-express the neuroglial marker nestin, GFAP and S100ß. Moreover, these cells are immunopositive for the embryonic stem cell marker Oct-4 and Sox2. The present data support the arising concept of a pluripotent subpopulation within a BMSC culture.

Late detection problems of cells due to removal of incorporated BrdU and indicated by fragmented labeled nuclei

Sauerzweig, S., Baldauf, K., Braun, H. and Reymann, K. G.
Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany

Bromodeoxyuridine (BrdU) is currently one of the most popular markers for cell division and has also been used for labeling of transplanted cells. Structurally being a thymidine analog BrdU is incorporated into DNA of dividing cells during the S-phase of the cell cycle. However, over the last years critics arose concerning the reliability of cell-labeling by BrdU. Since BrdU is a marker of DNA synthesis one has to distinguish between BrdU-incorporation into mitotic active cells during S-phase and BrdU-labeling of cells undergoing certain metabolic events such as DNA repair, abortive cell cycle entry and gene duplications, leading to an overestimation of cell proliferation. While this problem has been recently addressed, the stability of BrdU incorporation into nuclear DNA over defined time periods has not been sufficiently investigated. We labeled mesenchymal stem cells with different concentrations of BrdU and observed these cells *in vitro* over 4 weeks. Within this period we detected a shift of the BrdU signal from the nucleus to the cytosol. Similar changes were observed *in vivo*, where we injected BrdU intraperitoneally in rats and detected BrdU-positive cells in the hippocampus after 7 and 28 days. We found an increasing fragmentation of the BrdU signal pattern over time. These results lead us to the conclusion that the BrdU-signal in labeled cells is gradually degraded over time. Consequently, new generated cells, which were originally labeled by BrdU, were no longer detectable after defined time points, although these cells are still alive.

Neuroprotective effects of the survival promoting peptide Y-P30

Schneeberg, J.[a], Riek-Burchardt, M.[a], Braun, H. [a], Landgraf, P.[b], Kreutz, M. R.[b] and Reymann, K. G.[a,c,]
[a]*Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Project Group Neuroplasticity, Magdeburg, Germany*
[c]*Institute for Applied Neurosciences* (*FAN gGmbH*), *Magdeburg, Germany*

Y-P30 is a polypeptide produced by peripheral blood mononuclear cells (PBMC) of the maternal immune system during pregnancy. The peptide passes the blood-placenta barrier and accumulates in neurons of the developing infant brain, where it enhances survival of thalamic neurons and displays neuritogenic activities. Interestingly, expression of the peptide factor can be induced after optic nerve crush in adult rats. To further address its potential role in brain injury we followed up its expression after brain ischemia (filament model in rats) and tested its neuroprotective potential in the oxygen-glucose-deprivation (OGD) model with hippocampal slices as an assay for ischemia. Y-P30 gene expression in PBMC's was surprisingly low after experimental brain ischemia as compared to optic nerve crush. However, Y-P30 conferred significant neuroprotection when the peptide was added at concentrations of 200 nM and 2 μM to the medium of hippocampal slice cultures two hours before starting the

deprivation of oxygen and glucose. A modest but still significant neuroprotective effect was found when the peptide was applied two hours after injury to the medium. Y-P30 has been shown to built up larger oligomers which might hinder passage through the culture membranes. To further enhance the peptides neuroprotective potential we therefore applied Y-P30 directly on top of the hippocampal slice. This administration regime let to the most robust neuroprotection even at very low doses.Supported by the Leibniz Society-Pakt für Forschung and the LSA (N2/TP5).

Inhibition of Na^+/H^+ exchangers protects hippocampal slices from oxygen-glucose-deprivation induced injury

Schröder, U. H.[a,b], Straßburger, M.[a], Breder, J.[b], Sabelhaus, C. F.[b], Kahlert, S.[c], Reiser, G.[c] and Reymann, K. G.[a,b]
[a]*Research Institute for Applied Neuroscience* (*FAN gGmbH*), *Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[c]*Institute of Neurobiochemistry, Medical Faculty, Otto-von-Guericke University Magdeburg, Germany*

Intra- and extracellular acidosis are ubiquitous events in cerebral ischemia. In neurons intracellular pH is mainly regulated by the Na+/H+ exchange system. Inhibition of Na+/H+ exchangers has been intensely studied in models of myocardial ischemia, but there are not many reports available on cerebral ischemia. In the present study, we investigated the effects of inhibition of Na+/H+ exchangers on ischemic damage in organotypic hippocampal slice cultures (OSCs) from 10-day-old rats and acute hippocampal slices from adult animals. The broad-spectrum Na+/H+ exchange inhibitor harmaline reduced neuronal cell death in slice cultures both when present during the insult and the recovery period as well as when applied only during the recovery period. The protective effect of harmaline was mimicked by the more specific inhibitors EIPA and S3226, but not by typical inhibitors for the Na+/H+ exchanger isoform 1, indicating that the protective effect is not mediated by this isoform. Harmaline also protected organotypic cultures from neonate rats but, contrasting EIPA, not acute hippocampal slices from adult animals against functional neuronal damage. This may indicate that the protective effects involve different Na+/H+ exchangers. Also supporting the notion that regulation of intracellular pH is developmentally regulated, EIPA did not affect the recovery from an acid load in isolated neurons from neonate rats, although EIPA sensitive Na+/H+ exchangers were expressed. Our data show that Na+/H+ exchange inhibition can protect hippocampal neurons from oxygen-glucose-deprivation induced injury. We speculate that the protective effects are either due to the suppression of pH-sensitive injury mechanisms or to a restriction of Na+ entry, which would limit subsequent Ca2+ accumulation via the Na+/Ca2+ exchanger.

Strain-specific differences in secondary thalamic degeneration after focal cerebral infarction

Schroeter, M.[a,b] and Jander, S.[a,b]
[a]*Department of Neurology, University of Cologne, Germany*
[b]*Department of Neurology, Heinrich Heine University, Düsseldorf, Germany*

After cerebral ischemia, tissue damage is not restricted to the ischemic focus. Cortical damage leads to secondary degeneration of connected thalamic nuclei. After spinal cord injury, differences between mouse strains has been reported with respect to secondary growth, cavitation, and scarring of the lesion. We therefore asked for strain-specific differences of thalamic damage in C57BL/6 vs. SV 129 mice that may impact on functional recovery. Focal cortical infarction was induced by photothrombosis. At day 14 and 28, secondary thalamic damage was assessed by neuron counts, immunohistochemical analysis of MHC upregulation, and microglia activation. Photothrombosis induced circumscribed, cone-shaped, reproducible cortical infarctions sparing subcortical structures in both strains. C57Bl/6 mice showed rapidly occurring and extensive thalamic degeneration in ipsilateral thalamic nuclei connected to the infarcted sensorimotor cortex. Degeneration was accompanied by MHC upregulation, microglia activation, and phagocytosis. In SV 129 mice, thalamic degeneration as well as microglia activation was mild. We show substantial differences in the extent of secondary thalamic degeneration and microglia activation after focal cerebral infarction. Similar to the findings in spinal cord injury, C57Bl/6 mice succumb to increased neuronal damage and secondary tissue damage after focal cortical infarction. Our findings have implications for the impact of genetic factors on the evolution brain damage following ischemia and

should be taken into account when designing knock-out studies in these frequently employed mouse strains.

The induction of HIF-1α under anoxic conditions in SH–SY5Y neuroblastoma cells is strongly modulated by the availability of glucose

Serra, A., Planas, A. M. and Santalucia, T.
Department of Brain Ischemia and Neurodegeneration, Institut d'Investigacions Biomèdiques de Barcelona (*IIBB*)-*Consejo Superior de Investigaciones Científicas* (*CSIC*), *IDIBAPS, Barcelona, Spain*

The hypoxia-inducible transcription factor (HIF-1) mediates the cellular response against diminishing oxygen concentrations by activating among others a number of genes involved in the production of ATP by glycolysis. HIF-1 is a heterodimeric factor composed of an oxygen-regulated subunit, HIF-1α, and a constitutive one, HIF-1β. Under normal oxygen concentrations, HIF-1α expression is very low as a result of proteasomal degradation induced by the oxygen-dependent hydroxylation of certain proline residues in HIF-1α catalysed by enzymes called prolylhydroxylases. Nevertheless, a decrease in oxygen concentration results in reduced degradation and accumulation of the protein. The regulation of HIF-1α expression and transcriptional activity by low oxygen concentration on its own has been the subject of intensive research. However, little attention has been paid to the fact that low oxygen concentrations are often associated to diminished glucose supply under ischemic conditions, and whether the induction of HIF-1α would be regulated by the availability of glucose besides the absence of oxygen. We have investigated whether glucose may exert a modulating effect on the induction of HIF-1α by anoxia in SH-SY5Y neuroblastoma cells. Despite anoxia alone was able to induce the expression of HIF-1α, the additional suppression of glucose caused a larger induction of HIF-1α. This seemed to be due to a larger degradation of HIF-1α in the presence of glucose, since the levels of expression were greatly increased by addition of a proteasome inhibitor, while Inhibition of the prolylhydroxylases caused a comparatively smaller increase. We found no differences in the oxygen consumption rate or in the extent of hypoxia achieved under both incubation conditions that could account for our results. Analysis of the proteasome activity showed that it was not altered by either of the incubation conditions. This suggests that there may be an additional activity targeting HIF-1α for degradation, which is induced by the presence of glucose in cells incubated under anoxic conditions.

Inter-age variability of bona fide unvaried transcripts - Normalization of quantitative PCR data in ischemic stroke

Sieber, M.[a,b], Guenther, M.[a], Kohl, M.[c], Witte, O. W.[a], Claus, R.[b] and Frahm, C.[a]
[a]*Departments of Neurologyy, University Hospital, Friedrich-Schiller-University, Jena, Germany*
[b]*Departments of Anaesthesiology and Intensive Care Medicine, University Hospital, Friedrich-Schiller-University, Jena, Germany*
[c]*Mathematical Statistics, University of Bayreuth, Germany*

Background: Aging is a major risk factor for a variety of neurobiological diseases leading to variations of transcriptional expression in affected tissues. Reverse transcription of RNA followed by quantitative PCR is a powerful technique for detection and quantification of specific transcripts differentially expressed. An essential prerequisite for accurate interpretation of quantitative PCR data obtained from expression studies is an appropriate normalization process. Therefore, we validated the expression of the most frequently used reference genes consisting of *Gapdh* and *Actb* as well as *Hmbs*, *Hprt1* and *Gusb* in an animal model of mice in respect to two major influence factors, aging and ischemia. In the experimental settings, we intended to reflect variations in both, the local and systemic immune response. *Results:* The consistency in gene expression of the tested transcripts were quantified based on standard deviation, correlation analysis and two algorithms available as VBA applets termed *GeNorm* and *Normfinder*. Overall, the results of the study proofed the suitability of *Actb* in combination with *Gapdh* and with tissue-specific limitations *Hmbs* in brain and *Gusb* in white blood cells as the most stable transcripts for accurate normalization. We clearly demonstrated that both, the aging process *per se* and aging in combination with ischemia are confounding factors with respect to the expression stability of *Hprt1*. *Conclusions:* The present study confirms the need to analyze the stability of *bona fide* unvaried transcripts in detail according to the specific conditions of interest. Based on the expression stability, the use of *Gapdh* and *Actb* as highly abundant transcripts for normalization of qPCR data under

conditions of aging and ischemia in a mouse model was evaluated. However, for low abundant genes the use of *Hmbs* in brain and *Gusb* in white blood cells is recommended.

Targeted blockade of individual surface arterioles and voltage sensitive dye imaging reveal a capacity for rapid re-weighting of cortical circuit function in mice after stroke

Sigler, A., Mohajerani, M. H. and Murphy, T. H.
Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, Canada

Recovery from brain damage following ischemic stroke is proposed to result from the production of new synaptic pathways within surviving brain regions. This process is thought to take place over a period of days to weeks and parallel recovery of sensory and motor function. Here we have addressed whether more rapid redistributions of brain function might occur following loss of blood flow to select regions of somatosensory cortex in mice by using voltage sensitive dye imaging to monitor widespread patterns of neuronal activity in response contralateral limb stimulation in somatosensory cortex. We report rapid signaling between related areas of cortex such as the fore and hindlimb cortices over a millisecond time scale that were not observed with lower temporal resolution imaging techniques such as intrinsic signal imaging. Using targeted photothrombotic stroke directed at individual surface arteries we have examined how the pattern of activity within the somatosensory cortex changes following ischemia directed at a subset of the forelimb map. As expected, somatosensory function was lost in areas that were ischemic as confirmed by laser speckle imaging. The loss of function as assessed by voltage sensitive dye imaging extended 0.3 mm into areas with relatively normal blood flow indicating a potentially salvageable area containing adequate perfusion but little function. If an ischemic stroke led to partial loss of forelimb function (anterolateral response lost), the remaining forelimb-stimulated voltage sensitive dye responses became unexpectedly centered within the adjacent hindlimb areas in the majority of mice. This re-weighting of circuit activity could underlie some forms of rapid behavioral compensation or direct future synaptically mediated stroke recovery at later time points.

Resveratrol prevents the inhibition of Na^+,K^+-ATPase activity after global cerebral ischemia

Simão, F., Matté, C., Wyse, A. T. S., Netto, C. A. and Salbego, C.
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, Brazil

The effects of resveratrol (RSV) treatment on the Na+,K+-ATPase activity were examined in hippocampus and cerebral cortex of the rats exposed to global cerebral ischemia. Adults Wistar rats were submitted to 10 min of ischemia by the four-vessel occlusion method. RSV (30 mg/Kg) was injected i.p for 7 consecutive days before ischemia. The effects of RSV on Na+,K+-ATPase was determined at 1 h, 24 h, and 7 days after global ischemia. It was found that global cerebral ischemia induced a statistically significant decrease in the Na+,K+-ATPase activity of the hippocampus and cortex, from 1 h to 7 days of reperfusion. Maximal enzymatic inhibition was registered 24 h after the ischemic damage. The decline in the Na+, K+-ATPase activity was prevented in the animals exposed to RSV treatment within the first 24 h of reperfusion. Our results indicate that global cerebral ischemia induced a significant alterations in the Na+,K+-ATPase activity in the hippocampus and cortex during different periods of reperfusion. RSV treatment prevented ischemia-induced changes in the Na+,K+-ATPase activity. We suggest that the maintenance of Na+,K+-ATPase activity afforded by RSV be related to cellular neuroprotection.

Synaptic plasticity in hippocampus after ischemia-reperfusion injury

Skibo G. and Nikonenko A.
O. O. Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kiev, Ukraine

Cerebral ischemia followed by reperfusion activates numerous events leading to brain cell death. The disruption of cerebral blood flow produces a central core of dead neurons surrounded by a penumbra of damaged but still functional neurons, which gradually die if reperfusion is not renewed. Intimate cellular mechanisms of this process remain obscure. This study used *in vivo* and *in vitro* rat models of cerebral ischemia to

analyze early post-ischemic events of synaptic plasticity in hippocampal CA1 stratum radiatum. *In vivo* transient cerebral ischemia was modeled by permanent vertebral artery occlusion and transient common carotid artery occlusion. *In vitro* oxigen-glucose paradigm was used to model ischemia in long-term hippocampal organotypic slice cultures. Electron microscopy of post-ischemic asymmetric synapses in hippocampal CA1 area provided an evidence for rapid changes both in pre- and postsynaptic structures. *In vivo* the frequency of perforated synapses, defined as having a perforated postsynaptic density (PSD), experienced increase by 70% at 15 min and nearly 2.5-fold – at 24 hours after the ischemic episode. The frequency of multiple spine boutons was 60% higher at 15 min and 2.5-fold higher – at 2 hours gradually returning to the level of control afterwards. At the same time significant increase in PSD thickness was observed being equal to 24% at 15 min and 2.7-fold – at 7 days after the ischemic episode. Less pronounced changes in PSD length followed the same trend. Post-ischemic synaptic contacts were surrounded by protrusions of ramified activated astrocytes. *In vitro* changes in PSD dimensions assessed 15 min and 2 h after the OGD episode were similar to those observed *in vivo*. In presynaptic terminals *in vivo* and *in vitro*, both the ischemic and OGD episodes induced changes in spatial arrangement of synaptic vesicles as well as depletion in the readily releasable pool of these organelles. Synaptic vesicles became more distant from active zones and had larger inter-vesicle spacing with respect to controls. Taken together, these data suggest that adaptive plasticity in the hippocampus takes place early after ischemia-reperfusion injury and may contribute to the delayed neuronal death in the ischemic penumbra.

Neuroprotective effect of VEGF overexpression on mice cognition after unilateral common carotid artery ligation

Staub, J.[a], Ernst, E.[b], Marti, H. H.[b] and Plaschke, K.[a]
[a]*Department of Anesthesiology, University of Heidelberg, Germany*
[b]*Department of Physiology, University of Heidelberg, Germany*

Objective: Neurons are susceptible to hypoxia and ischemia. However, endogenous adaptive responses aim at protecting the tissue from hypoxic-ischemic injury. Angiogenesis and neurogenesis are two of such adaptive responses, and both seem to be governed by the same hypoxia-induced growth factor, vascular endothelial growth factor (VEGF). VEGF is upregulated during hypoxia and ischemia and improves outcome after stroke. The aim of the present study was to investigate the influence of VEGF overexpression on behavior and cognition under permanent moderate cerebral oligemia. Furthermore, cellular changes as detectable by immunohistochemistry were analyzed. *Methods:* The left common carotid artery was permanently occluded (CCAO) in wild-type C57BL/6 and VEGF transgenic mice (V1) (1) ($n = 24$) for 12 days (subchronic group). Skin incision and anesthesia without artery occlusion was performed in a sham group ($n = 25$). Mice behavior was then tested for 12 consecutive days: Spontaneous locomotor activity was measured in a mice-adapted holeboard box (2) and time-to-platform, distance-to-platform, and swimming-velocity were measured using a water maze with video controlled analysis. A second group ($n = 19$) was sacrificed after 48 h to compare acute occlusion effects with subchronic changes. Differences in Pecam-1 (CD 31 (angiogenesis marker) and nestin (neurogenesis marker) expression were then detected and quantified by standard immunohistochemistry. *Results:* VEGF overexpression resulted in significant behavioral changes after unilateral CCAO. V1 mice spent more time in the target quadrant of the water maze as compared to wild-type (wt) animals. Times and distances to a hidden platform were shorter in V1 animals. No significant changes were found in locomotor activity and swimming speed. Improved cognitive performance in behavioral testing corresponded with increased nestin cell count in the V1 group. *Conclusion*: VEGF overexpression led to a protective effect on cognition under conditions of cerebral oligemia.

MAPK signalling pathways as target for anti-inflammatory treatment – neuroprotection, microglia and neurogenesis

Strassburger, M., Braun, H. and Reymann, K. G.
Leibniz Institute for Neurobiology, Magdeburg, Germany

The mitogen-activated protein kinase (MAPK) regulates a wide variety of cellular signal transduction processes and plays an important role in inflammation after cerebral ischemia. We investigated the interplay between neuronal damage (propidium iodide uptake), mi-

croglia activation (Ox-42 immunohistochemistry) and neurogenesis (double labeling of bromodeoxyuridine with doublecortin) after ischemia in organotypic hippocampal slice cultures treated with the p38-MAPK inhibitor SB239063. Our study shows that after oxygen glucose deprivation (OGD) the p38-MAPK and the extracellular-signal-regulated kinase 1/2 (ERK1/2) are strongly activated. The p38-MAPK phosphorylation returned to basal levels within 1 h after OGD, whereas the ERK1/2 phosphorylation reached the original level only after 24 h. Treatment with 20 μM and 100 μM SB239063 strikingly reduced cell death after OGD and significantly diminished microglia activation in the cornu ammonis (CA-region), but not in the area dentata (AD). The pro-inflammatory cytokine IL-1β was reduced by 84% after treatment with SB239063 whereas the cytokines IL-6 and TNF-α were not affected. After 6 days, neurogenesis was significantly increased in the posterior periventricle (pPV). Based on these findings our study shows that an anti-inflammatory treatment with SB239063 reduces cell death, inflammation, and microglia activation and at high concentration enhances the OGD-induced neurogenesis in the pPV.

Prostaglandin synthesis in rat brain astrocytes is under the control of the n-3 docosahexaenoic acid, released by group VIB calcium independent phospholipase A_2

Strokin, M.[a], Sergeeva, M.[b] and Reiser G.[a]
[a] *Institute of Neurobiochemistry, Medical Faculty, Otto-von-Guericke University Magdeburg,, Germany*
[b]*Belozersky Institute of Physico-Chemical Biology, Moscow State University, Russia*

In the current study we reveal that in astrocytes the VIB Ca2+-independent phospholipase A2 is the enzyme responsible for release of docosahexaenoic acid (22:6n-3). After pharmacological inhibition and siRNA silencing of VIB Ca^{2+}-independent phospholipase A2, docosahexaenoic acid release was strongly suppressed in astrocytes, which were acutely stimulated (30 min) with ATP and glutamate or after prolonged (6 h) stimulation with the endotoxin lipopolysaccharide. Docosahexaenoic acid release proceeds simultaneously with arachidonic acid (20:4n-6) release and prostaglandin liberation from astrocytes. We found that prostaglandin production is negatively controlled by endogenous docosahexaenoic acid, since pharmacological inhibition and siRNA silencing of VIB Ca^{2+}-independent phospholipase A2 significantly amplified the prostaglandin release by astrocytes stimulated with ATP, glutamate, and lipopolysaccharide. Addition of exogenous docosahexaenoic acid inhibited prostaglandin synthesis, which suggests that the negative control of prostaglandin synthesis observed here is likely due to competitive inhibition of cyclooxygenase-1/2 by free docosahexaenoic acid. Additionally, treatment of astrocytes with docosahexaenoic acid lead to reduction in cyclooxygenase-1 expression, which also contributes to reduced prostaglandin production observed in lipopolysaccharide-stimulated cells. Thus, we identify a regulatory mechanism important for the brain, in which docosahexaenoic acid released from astrocytes by VIB Ca^{2+}-independent phospholipase A2 negatively controls prostaglandin production.

Tin protoporphyrin provides neuroprotection following hypoxia-ischemia by modification of nitric oxide synthase, cyclooxygenase and mitochondrial complex 1

Sutherland, B. A., Shaw, O. M. and Clarkson, A.
Department of Pharmacology and Toxicology, University of Otago, Dunedin, New Zealand

The aim of this study was to determine whether heme oxygenase (HO) modulation affected hypoxia-ischemia (HI)-induced brain damage and to identify the possible mechanisms involved. HI was induced in 26 day old male Wistar rats by left common carotid artery ligation and exposure to 8% oxygen for 1 hour. 30 μmol/kg tin protoporphyrin (SnPP – HO inhibitor), 30 μmol/kg ferriprotoporphyrin (FePP – HO inducer) or the equivalent volume of the vehicle 0.9% saline were administered intraperitoneally daily from 1 day prior to HI until sacrifice 3 days post-HI. Infarct volume was quantified in 1mm thick brain slices using triphenyltetrazolium chloride staining. SnPP administration significantly ($P < 0.05$) reduced infarct volume (62.33 ± 12.32 mm^3) compared to saline-treated animals (125.52 ± 13.43 mm^3) while FePP had no effect (126.04 ± 18.05 mm^3). SnPP did not inhibit HO activity significantly at 3 days post-HI so other mechanisms must have contributed to the neuroprotection by SnPP. In the ipsilateral hemisphere, HI + saline significantly ($P < 0.05$) increased total nitric oxide synthase (NOS) activity, compared to non-intervention controls, which was further augmented ($P < 0.001$) by HI + SnPP. Inducible NOS (iNOS) activity in the ipsilater-

al hemisphere was raised by HI + saline compared to non-intervention controls, but was significantly ($P < 0.05$) inhibited by HI + SnPP. Cyclooxygenase (COX) activity in the ipsilateral hemisphere was increased by HI + saline compared to non-intervention controls but was significantly ($P < 0.05$) reduced by HI + SnPP. Mitochondrial complex I activity (part of the electron transport chain) was raised significantly ($P < 0.05$) by HI + SnPP compared to HI + saline animals in the ipsilateral hemisphere. Therefore, SnPP acts on many mechanisms to produce neuroprotection 72 hours post-HI including total NOS, iNOS, COX and mitochondrial respiratory function, and may provide an attractive multimodal neuroprotective strategy.

New neurons generated from neonatal glial progenitors

Sypecka, J., Sarnowska, A. and Domanska-Janik, K.
NeuroRepair Department, Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

The fate choice of neural progenitors could be dictated by local cellular environment of adult CNS. The aim of our study was to investigate the effect of hippocampal slice culture on the differentiation and maturation of the oligodendrocyte NG2 precursors. *Methods:* The hippocampal slice culture was established from the brains of 7-days old rats. The NG2 precursors, obtained from old mixed primary culture of neonatal rat hemispheres, were labeled with CMFDA and seeded on hippocampal slices. After 7–14 days in co-culture, the cells were stained with neural markers. *Results:* The NG2 cells differentiated predominantly into oligodendrocytes, presenting various stages of maturation: progenitors (NG2), pre-oligodendrocytes (O4) and finally mature GalC-positive cells. However, a considerable number of the cells differentiated into neurons: TUJ+ and even MAP-2+ cells were frequently observed. Moreover, a certain population gathered the proliferative properties, as revealed by Ki67 expression. *Conclusions:* Neuronal microenvironment provided by the culture of hippocampal slices is potent to induce neurogenesis from oligodendrocyte progenitors and promotes their differentiation not only into OLS but also into neurons. It also supports their proliferative capacity. The results indicate the crucial role of local cellular environment in fate-decision of neural progenitor cells and thus may affect their differentiation after transplantation into CNS.

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Pro-apoptotic role of Erk in glutamate-dependent death of cultured cortical astrocytes and after transient focal ischemia

Szydlowska, K., Gozdz, A. and Kaminska, B.
The Nencki Institute of Experimental Biology, Warsaw, Poland

Astrocytes participate in detection, propagation, and modulation of excitatory synaptic signals, and provide metabolic support to neurons. Astrocytes can modulate glutamate (GLU) homeostasis by its uptake, preventing GLU elevations. However, an excess of GLU under pathological conditions can induce death of astrocytes by unknown mechanisms. Impairments of astrocyte function may play an important role in cerebral ischemia.We have demonstrated that GLU (>50 mM) affects survival of cultured cortical astrocytes, induces mitochondrial potential disruption followed by caspase-9 and -3 activation, DNA fragmentation and cell death. This apoptotic cell death was inhibited by an addition of 1 μM FK506, a calcineurin inhibitor and neuroprotective drug. Studying molecular mechanisms of astrocyte death, we found that extracellular signal-regulated kinase (Erk1/2) and JNK are strongly activated. Application of JNK and MEK inhibitors (SP600125 and UO126 - respectively) inhibited GLU induced death of astrocytes suggesting that Erk1/2 activation contributes to cell death of glutamate-treated astrocytes. FK506 treatment reduced glutamate-induced Erk1/2 activation and subsequent cell death. To evaluate an *in vivo* relevance of this finding, brain slices of rats subjected to transient MCAo were stained for active, phospho-Erk, cell type specific markers and DNA fragmentation (TUNEL staining), and analyzed by confocal microscopy. A rapid increase of phosphorylated Erk1/2 levels was detected in the injured hemisphere, both in neurons and astrocytes, and was decreasing with time. In similar brain regions, we observed numerous GFAP/TUNEL positive astrocytes. FK506 treatment reduced an elevation of phosphorylated Erk1/2 level in the injured hemisphere and decreased TUNEL staining in the brain areas where it blocked Erk activation. Concomitant ischemia-induced changes in the levels of phospho-Erk were observed in protein extracts from cortex and striatum. FK506 reduced ischemia-induced elevation of phospho-Erk levels in extracts only from the cortex. Our findings suggest that a sustained Erk activation can contribute to glutamate-induced astrocyte death *in vitro* and *in vivo*. Furthermore, we demonstrate that a neuroprotectant FK506 potently inhibits

Erk activation and subsequent cell death. Erk signaling have been implicated in neuronal survival pathways, however a growing evidence incriminates an acute Erk activation in initiation of cell death. Thus, we hypothesize that modulation of glutamate-induced Erk activation early after reperfusion and blocking of astrocyte death might be a novel target for neuroprotectant in ischemia.

Taurine attenuates mitochondrial toxicity induced by tamoxifen

Tabassum, H.[a], Parvez, S.[b], Banerjee, B. D.[a], Siemen, D.[b] and Raisuddin, S.[c]
[a]*Environmental Biochemistry and Immunology Laboratory, Department of Biochemistry, University College of Medical Sciences and G.T.B. Hospital* (*University of Delhi*), *Delhi, India*
[b]*Institute of Medical Neurobiology, Medical Faculty, Otto-von-Guericke University Magdeburg, Germany*
[c]*Immunotoxicology Laboratory, Department of Medical Elementology and Toxicology, Jamia Hamdard* (*Hamdard University*), *New Delhi, India*

One of the most attractive approaches to disease prevention involves the use of specific nutrients to protect tissue against toxic and carcinogenic injury and degenerative diseases. Tamoxifen (TAM) is a selective estrogen receptor modulator widely used in the treatment of breast cancer. TAM potentially affects mitochondrial functions as it acts as an uncoupling agent and a powerful inhibitor of mitochondrial electron transport chain. Several naturally occurring antioxidants influence the antioxidant enzymes and provide protection against free radical induced damage. Taurine, which is an important intracellular free beta-amino acid, is known to be an antioxidant and a membrane-stabilizing agent. Taurine has been reported to attenuate anticancer drug induced toxicity. We studied of taurine pretreatment on the toxicity of TAM in mouse liver mitochondria focusing specifically on the redox cycle biomarkers. TAM caused a significant rise in the mitochondrial lipid peroxidation (LPO) and protein carbonyl (PC) content and superoxide radical generation. There was a significant change in the mitochondrial thiol profile in the TAM-treated animals. Pretreatment of mice with taurine (100 mg/kg) markedly lowered mitochondrial LPO, PC content, and superoxide radical generation. The present findings demonstrate that the antioxidative potential of taurine could be attributed to its modulatory effect on the xenobiotic bioactivation and detoxification processes. It is also suggested that the protection afforded by taurine is either by reversing the decline of antioxidants or by the direct free radical scavenging activity.

Adrenomedullin protects neurons against oxygen-glucose deprivation stress by an autocrine and a paracrine manner

Tixier, E., Leconte-Duval, C., Roussel, S., Petit, E. and Bernaudin, M.
CI-NAPS UMR 6232, Université de Caen Basse-Normandie, Université Paris Descartes, CNRS, CEA, INSERM. CERVOxy group "Hypoxia and cerebrovascular pathophysiology", Centre Cyceron, Caen, France

The understanding of mechanisms involved in ischemic brain tolerance may provide new therapeutical targets for stroke. *In vivo* genomic studies revealed an upregulation of adrenomedullin (AM) expression by hypoxic preconditioning. Furthermore, AM reduced ischemia-induced brain damages in rodents. However, whether AM is involved in hypoxia-induced brain tolerance and whether AM, produced by neurons themselves, endothelial cells or microglia, protects neurons against ischemic stress remain unknown. Using a neuronal model of hypoxia-induced tolerance against oxygen glucose deprivation (OGD), we showed that hypoxic preconditioning (0.1% or 0.5% of oxygen) reduced OGD-induced neuronal death, whereas hypoxic treatment with 1% or 2% of oxygen is not neuroprotective. AM expression (mRNA and protein) was not only increased following the hypoxic preconditioning but also further increased following OGD in preconditioned neurons (0.1% or 0.5% of oxygen) and not in neurons pre-treated with 1% of oxygen. Furthermore, AM antagonism abolished hypoxia-induced tolerance and recombinant AM reduced OGD-induced neuronal death. Finally, we found that AM is also expressed by endothelial cells, microglia, and further increased by hypoxia. Supernatants of OGD-exposed endothelial cells or microglia reduced OGD-induced neuronal death through AM-dependent mechanism. Altogether, our results suggest that AM is a potent autocrine and paracrine neuroprotective factor during cerebral ischemia.

Pro-urokinase mutant (M5) vs recombinant tissutal plasminogen activator (rt-PA) in the acute treatment of stroke in the rat: Preliminary data

Tomasi, S.[a], Gurewich, V.[b], Sarmientos, P.[c] and Vercelli, A.[a]
[a]*Department of Anatomy, Pharmacology and Forensic Medicine, University of Torino, Italy*
[b]*Vascular Research Laboratory, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, USA*
[c]*Laboratorio di Neuroscienze "R. Levi-Montalcini" and Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Milan, Italy*

Prourokinase (proUK) is a zymogenic plasminogen activator that induces fibrin-specific clot lysis without binding to fibrin; its unusual high intrinsic activity, however, causes important side effects like major bleeding, limiting its therapeutic exploitation. A single site mutant (M5) of proUK was developed, in order to reduce spontaneous activation. C1-inhibitor was shown to inhibit both recombinant tissutal plasminogen activator (rt-PA) and M5 activation. M5- vs rt-PA-mediated toxicity was tested in a rat model of ischemia. Animals were infused four hours after ischemia onset with different drugs associations: group 1 ($n = 7$) received rt-PA; group 2 ($n = 7$) received rt-PA and C1-inhibitor; group 3 ($n = 5$) was infused with M5 and C1-inhibitor; group 4 ($n = 5$) received vehicle only; group 5 ($n = 5$) C1-inhibitor only. Animals were sacrified 24 hours later; mortality rate and histological appearance of brain ischemic sections were compared. Rats infused with rt-PA only showed tipically important bleeding soon after infusion with highest mortality rate (57%) and important haemorrhagic infiltration of ischemic brain. Group 2 showed reduced mortality rate (25%) and short lasting bleeding with a thin blood epidural infiltration. Only one animal died in group 3 (12.5%); important epidural hematome was observed in one rat. Rt-PA infusion seems to cause important bleeding with a higher frequency than M5; this risk of hemorrhage is higher when rt-PA is given alone. This variation in mortality probably reflects different kind of bleeding observed: intense, long lasting, often lethal in group 1, very less intense in groups 2–3. Bleeding after M5 infusion was particularly weak and short lasting. The different clinical behavior did not correlate clearly with histopathological appearance, but seems to be more related to different timings and liability of M5 and rt-PA respectively to cause bleeding. C1-inhibitor protective role seems to be stronger with M5 than rt-PA.

Fluorescence-activated cell sorting for the transplantation of human embryonic stem cell-derived neural precursors after MCAO in rats

Turnovcová, K.[a,b], Kozubenko, N.[a,b], Kapcalová, M.[a,b], Jendelová, P.[a,b]., Syková, E.[a,b]
[a]*Institute of Experimental Medicine, Prague, Czech Republic*
[b]*Department of Neuroscience, 2nd Medical Faculty, Charles University, Prague, Czech Republic*

Stroke is a medical emergency and can cause permanent neurological damage, complications, and death if not promptly diagnosed and treated. Human embryonic stem cell-derived neural precursors (hESC-NPs) can be used for effective therapies to restore function following neurological deficit. Neural precursors derived *in vitro*, however, show broad heterogeneity depending on cell lineage and developmental stage. Such heterogeneity can often lead to tumor formation after transplantation in experimental models of stroke. Here, we describe the cell surface markers characterizing our CCTL14 hESC line in subsequent sub-cultures and used to exclude pluripotent precursors by means of fluorescence-activated cell sorting (FACS). We focused on undifferentiated embryonic stem cell markers such as SSEA4 and nanog as well as neural stem cell markers (NCAM [CD56], CD24, CD133, SSEA1 [CD15]). Our study revealed that NCAM-positive cells appear first during neural differentiation with the strong coexpression of SSEA4. During the next several passages of hESC-NPs, the expression of SSEA4 was downregulated, the expression of NCAM remained the same, and CD133 appeared. As an animal model of stroke, we used male Sprague-Dawley rats (300–350 g) that underwent transient (90 min) intraluminal occlusion of the middle cerebral artery (MCAO). The rats were examined using magnetic resonance imaging during the subsequent 1–3 months and histological analysis was performed at the end of the experiment. We found that the transplantation of SSEA4-positive hESC-NPs led to tumor formation, while the transplantation of SSEA4-negative hESC-NPs did not cause tumor formation.

Prothymosin α plays a key role in cell death mode-switch, a new concept for europrotective mechanisms in stroke

Ueda, H.
Division of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Science, Japan

Following stroke or traumatic damages, both necrotic and apoptotic neuronal death cause a loss of functions including memory, sensory perception and motor skills. From the fact that necrosis has a nature to expand, while apoptosis to cease the cell death cascade in the brain, it is considered that the promising target for the rapid treatment for stroke is the necrosis. Here I introduce the discovery of prothymosin α (ProTα), which inhibits neuronal necrosis, and propose its potentiality of clinical use for stroke. First of all, it should be noted that ProTα inhibits the neuronal necrosis induced by serum-free starvation or ischemia-reperfusion stress, which causes a rapid internalization of GLUT1/4, leading a decrease in glucose uptake and cellular ATP levels. Underlying mechanisms are determined to be through an activation of Gi/o, phospholipase C and PKCβII. ProTα also causes apoptosis later through a similar mechanism. However, the concomitant treatment with neurotrophins completely inhibits the ProTα-induced apoptosis. Of most importance is the finding that the systemic injection of ProTα completely inhibits the brain damages, motor dysfunction and learning memory defect induced by cerebral ischemia-reperfusion stress, which up-regulates neurotrophins. As ProTα almost entirely prevents the focal ischemia-induced motor dysfunction 4 h after the start of ischemia, this protein seems to have a promising potentiality for clinical use.

Cortical spreading depressions increase cell proliferation and gliogenesis in the entorhinal cortex of rats

Urbach, A., Brueckner, J. and Witte, O. W.
Department of Neurology, Friedrich-Schiller-University, Jena, Germany

Recently we showed that cortical spreading depression (CSD), an epiphenomenon of diseases like stroke or traumatic brain injury induces neurogenesis in the adult dentate gyrus of rats. Here, we evaluated the effect of CSD on cell proliferation and differentiation in the entorhinal cortex, which provides the major afferent projections to the dentate gyrus. CSD were induced by epidural application of 3M KCl to the cerebral cortex of rats, controls received 3M NaCl. Cell birth and fate were analyzed at different time points thereafter by means of intraperitoneal bromodeoxyuridine (BrdU) injections and immunocytochemistry with antibodies against BrdU, nestin, astrocyte (GFAP, S100β), microglial (lectin), synantocyte (NG2), and immature and mature neuronal (DCX, NeuN) markers. Repetitive CSD resulted in a significant increase in BrdU-positive cell numbers in the ipsilateral entorhinal cortex. Almost all of these newborn cells displayed a glial phenotype. We show that CSD continuously increase the number of newborn astrocytes, microglia and synantocytes, though these cell populations were differently affected by CSD as reflected by a shift in their relative abundance. Furthermore, we found a successive increase in BrdU/DCX co-expressing cells, which became significant 6 weeks following CSD. There was no evidence of neurogenesis in this brain region. Our data demonstrate that CSD significantly induce proliferation of cortical precursor cells with their progeny differentiating into various types of glia cells.

Monocytes as a cellular vehicle for the therapeutic of stroke

Valable, S.[a,b], Tixier, E.[a,b], Divoux, D.[a,b], Touzani, O.[a,b], Roussel, S.[a,b], Bernaudin, M.[a,b] and Petit, E.[a,b]
[a]*CI-NAPS, UMR 6232, Université de Caen Basse-Normandie, Université Paris Descartes, France*
[b]*CNRS, CEA, INSERM. CERVOxy group "Hypoxia and cerebrovascular pathophysiology. Centre CYCERON, Caen, France*

Introduction: To date, stroke therapies remain limited despite a great number of neuroprotectants discovered on *in vitro* and *in vivo* models. The failure of most drugs is often mainly inherent to their poor capacity to cross the blood-brain barrier and cell-based vectorization of therapeutic agents could be a very promising approach to circumvent these difficulties. Focal cerebral ischemia is caused, at least initially, by a reduction of blood flow consecutively to a vascular obstruction leading to oxygen (hypoxia) and metabolic substrates depletion. Ischemic neurodegeneration depends on cellular reactions such as gliosis, inflammation, and angiogenesis that modify survival and repair. The contribution

of the inflammatory reaction to ischemic tissue damage remains controversial (Hendrix and Nitsch, 2007). Besides microglial cells, circulating monocytes and macrophages could contribute to reparation processes. Indeed, these cells rapidly migrate into pathological hypoxic area as we previously described in a glioma model (Valable et al., 2007). Thus, we hypothesized that monocytes genetically modified to overexpress neuroprotective genes may represent a potential strategy for the treatment of stroke. Here, we studied on a preclinical model whether cerebral ischemic tissue could exert chemoattractive properties for circulating monocytes. *Methods:* mice were subjected to permanent focal cerebral ischemia by occluding the middle cerebral artery (MCAo). Monocyte/macrophage cells (P388-D1) transfected with a pc-DNA3.1-eGFP plasmid were injected in the tail vein (2.106cells/100 μl) at the same time as the cerebral artery occlusion. After different times of MCAo, histochemical studies were realized on coronal free-floating brain sections. *Results:* The histochemical study with the isolectin BS-I-B4 revealed that activated microglia/macrophages cells migrate to the ischemic core as a function of the duration of ischemia (from 12 hours to 7 days). The systemic injection of GFP-transfected monocytes showed that, on this permanent ischemia model, similarly to resident microglial cells, circulating monocytes are specifically detected at the border of the ischemic area suggesting that hypoxia-ischemia tissues exert a tropism for these circulating cells, according to Stroh et al., (2006). In addition, by analyzing the ischemic volume, we established that exogenous monocytes displayed no deleterious effect on the ischemic area. These results were also confirmed by *in vitro* studies.These observations, suggest that monocytes participate in the inflammatory response induced by cerebral ischemia and may be used as a safely cellular strategy to deliver specifically molecular therapeutics to the cerebral injured tissue.
Hendrix and Nitsch, J Neuroimmunol. 2007, 184:100-12. *Valable et al., Neuroimage. 2007,* 37 Suppl 1:S47-58. *Stroh et al., Neuroimage. 2006, 33:* 886-97.

INFγ exacerbates oxygen/glucose deprivation-induced cell death in cortical neurons and this effect is prevented by IL10

Verdaguer, E. and Planas, A.
Department of Brain Ischemia and Neurodegeneration, Institut d'Investigacions Biomèdiques de Barcelona (*IIBB*)*-Consejo Superior de Investigaciones Científicas* (*CSIC*)*, IDIBAPS, Barcelona, Spain*

Interferon gamma (INFγ) is a pro-inflammatory cytokine that in the CNS is mainly responsible for the activation of microglial cells and the upregulation of MHC class II antigens. INFγ-inducible responses are attributed to STAT1-dependent mechanisms, but recent reports demonstrated a number of STAT1-independent responses.In primary cultures of rat cortical neurons, INFγ (50 ng/μl) upon oxygen glucose deprivation (OGD) for 1 h plus reoxygenation, significantly increases OGD-induced neuronal death, as assessed by LDH release measured after 4 h and 24 h of reoxygenation. The negative effect of INFγ on OGD is prevented by the anti-inflammatory cytokine IL10 (10 ng/μl). Phosphorylation of pSTAT1 (Tyr701) and pSTAT3 (Tyr 705) was examined at 1 h of reoxygenation after OGD. OGD increased pSTAT3 but not pSTAT1. INFγ increased the phosphorylation of STAT1, whereas IL10 enhanced pSTAT3, as expected. However, treatment with INFγ after OGD increased pSTAT3, also. Combination of INFγ plus IL-10 under OGD conditions did not prevent INFγ-induced pSTAT1 and reduced INFγ-induced pSTAT3. The complex signal transduction after cytokine treatment and its relation to cell death and survival is under investigation.

Neuronal apoptosis prevention by carbon monoxide (CO): Preconditioning-like effect

Vieira, H. L. A.[a,b], Queiroga, C. S. F.[a] and Alves, P. M.[a,b]
[a]*Instituto de Biologia Experimental e Tecnológica* (*IBET*), *Oeiras, Portugal*
[b]*Instituto de Tecnologia Química e Biológica* (*ITQB*), *Universidade Nova de Lisboa, Oeiras, Portugal*

Carbon monoxide (CO), an endogenous product of mammalian cells, generated by heme-oxygenase (HO), has been claimed to present anti-apoptotic properties in several tissues. The present work demonstrates the effect of CO in preventing neuronal apoptosis induced by excitotoxicity and oxidative stress, in mice primary culture of cerebellar granule cells. In addition, endogenous CO appears to be an important product of HO activity in order to confer neuroprotection against apoptosis. Despite being neuroprotective, CO also induces ROS generation in neurons. These two phenomena suggest a CO preconditioning mechanism to prevent cell death. The role of several players in the preconditioning event, namely soluble guanylyl cyclase (sGC), nitric oxide synthase (NOS) and ATP dependent mito-

chondrial K channel (mitoKATP) was addressed. Inhibition of sGC or NOS activity, or closing of mitoKATP reverses the protective effect of CO. In addition, CO treatment triggers cGMP and NO production in neurons. Moreover, ROS generation seems to be necessary for CO to confer preconditioning and neuroprotection. Opening of mitoKATP, which appears to be critical for CO prevention of apoptosis, might be a downstream event in the process of preconditioning. In conclusion, CO induces preconditioning, prevents neuronal apoptosis and might constitute a novel and strong candidate for neuroprotective therapies.

Differential post-lesional response of distinct populations of hippocampal precursors in the young and aged brain

Walter, J., Witte, O. W. and Redecker, C.
Department of Neurology, Friedrich-Schiller-University, Jena, Germany

During hippocampal neurogenesis slowly dividing precursor cells with astrocytic, radial glia-like properties could be distinguished from highly dividing neuronal precursors in the subgranular zone (SGZ). Several studies indicate that these distinct subtypes of precursors are differentially stimulated under pathophysiological conditions. Even the relatively quiescent radial glia-like precursors (type 1 cells) increase their proliferative activity within hours after cortical infarcts. It is the aim of the present study, to analyze whether this proliferative response is also present in the aged brain. To this purpose, we used the photothrombosis model to induce focal infarcts in the forelimb cortex of 3 and 16 months old transgenic mice expressing green-fluorescent protein (GFP) under control of the stem cell marker nestin. Sham-operated mice served as controls. To label proliferating cells all mice received three single injections of bromodeoxyuridine (BrdU, 50 mg/kg i.p. every 2 h) at day 4 after the infarct. Two hours after the last injection the animals were transcardially perfused and processed for immunocytochemistry using antibodies against BrdU, Nestin-GFP, glial fibrillary acidic protein (GFAP) and doublecortin (DCX). Stereological analysis of BrdU-positive cells in the SGZ revealed only 14% of proliferating cells in aged compared with young controls. After cortical infarcts the total number of BrdU-positive cells remained stable in young animals whereas old mice showed a significant increase in proliferating cells (+40%). Phenotype analysis of the proliferating cells using confocal laser scanning microscopy further showed that cortical infarcts stimulate radial glia-like as well as neuronal subpopulations in the young but only neuronal precursors in the aged brain. Our data demonstrate that hippocampal precursor cells in the aged brain maintain their ability to respond to cortical infarcts even though their number is strongly reduced.

Differential role of VEGF receptors in adult neurogenesis

Wittko, I., Bicker, F., Schänzer, A., Plate, K. H. and Raab, S.
Edinger Institute (*Institute of Neurology*), *JW Goethe-University, Frankfurt/a.M., Germany*

The capability of the adult brain to form new neurons is important to ensure brain homeostasis and endogenous brain repair during physiological and pathophysiological conditions. New neurons originate from neural stem cells, which in the adult brain are mainly located in two neurogenic regions, the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus. From there neural progenitors migrate to target regions, where they replace cells and functionally integrate into existing structures. This process includes cell division, fate choice, migration, survival, differentiation. Both, intrinsic and extrinsic factors influence the different steps involved in adult neurogenesis. Many of these factors are secreted by glial cells. It is known that glial cells influence the migration of neuroblasts towards the olfactory bulb, but the molecules mediating these signals are not identified yet. Recent studies display the importance of the VEGF/VEGF receptor system for neurogenesis in the adult CNS. Most is known about neurotrophic functions of VEGF-A, which is involved in brain angiogenesis, neurogenesis and neuroprotection. VEGFR-2 is supposed to be the main signaling receptor for VEGF-A-mediated effects to cells of neural origin. Additionally, VEGF-B has been shown to be important for neurogenesis. Both, VEGF-A and VEGF-B can activate VEGFR-1. However, the role of VEGFR-1 for the adult brain has not been elucidated so far. In our study, we analyzed and compared the expression and functions of VEGF receptors in the adult CNS and found that both exert different roles in the process of adult neurogenesis.

Modulation of MMP-2 and MMP-9 activity may indicate the possible involvement of these enzymes in neurogenesis after ischemia in the adult gerbil hippocampus

Wójcik, L. and Zalewska, T.
Department of NeuroRepair, Medical Research Institute, Warsaw, Poland

Matrix metalloproteinases (MMPs) are cysteine proteases involved in remodeling of the extracellular matrix (ECM) and modulation of integrin-mediated intracellular signaling pathways associated with the early steps of neurogenesis such as proliferation, differentiation, and migration. In the present work, we investigated whether there is a spatio-temporal relationship between the activity of MMPs and neurogenesis after global cerebral ischemia. The degree of stem cells proliferation was evaluated by BrdU incorporation followed by immunoreaction with specific antibodies. The cellular phenotype of newborn cells was identified by an analysis of neural cell marker expression – NF-200, βIII tubulin and NeuN. The activity of metalloproteinases – MMP-2 and MMP-9 was estimated by gel- and in situ zymography in conjunction with immunohistochemistry. The efficient BrdU incorporation in gyrus dentatus (DG) as well as in CA1 area was observed at 2 and 4 weeks after global ischemia. A few BrdU labeled cells co-expressed a marker specific for mature neurons. Whereas one week after ischemia, when neuronal cells are dying, MMPs activity in CA1 area is comparable to control level, it decreases markedly in pyramidal cells in later time of reperfusion (two and four weeks) due to the loss of neurons. Contrary, a remarkable activation of investigated enzymes was observed in the cells expressing the glial marker (GFAP). The changes of MMPs activity determined in situ has been confirmed by gel zymography. These findings may suggest that the increased activity of MMPs may be involved in neurogenesis in gerbil hippocampus and facilitate the motility of newborn cells.

Rho kinase inhibitor-fasudil regulates the *in vitro* and *in vivo* production of neuronal progenitors

Xiao, B.-G., Ding, J. and Lu, C.-Z.
Institute of Neurology, Fudan University, Shanghai, China

Rho-kinase is thought to play a role in the mechanisms underlying the occurrence of hemodynamic dysfunctions, such as vasoconstriction, endothelial injury and hyperviscosity, and inflammatory processes. Fasudil, a Rho-kinase inhibitor, shows promise as being of benefit in the treatment of ischemic brain injury. Here, we reported that Fasudil appears to act directly on neural stem cells (NSCs), promoting the Neurosphere generation and neuron proliferation *in vitro. In vivo* experiments show that the upregulation of Rho-kinase II was observed in the central nervous system after hypoxia. The administration of Fasudil promoted the proliferation of neuronal progenitors in the adult subventricular zone after hypoxia. Further studies exhibit that Fasudil not only acts on directly NSCs, but also indirectly promotes the production of NSCs through regulating astrocyte functions. These findings suggest that Fasudil is capable of regulating the production of neuronal progenitor cells by different mechanisms.

VEGF-A and VEGF-D gene therapy and functional recovery in cortical photothrombotic model of rat stroke

Yanev, P.[a,b], Wirth, T.[a,b], Ylä-Herttuala, S.[a,b] and Jolkkonen, J.[a,b]
[a]*Department of Neurology, University of Kuopio, Finland*
[b]*A.I. Virtanen Institute, University of Kuopio, Finland*

Background: Treatment of cerebral ischemia is still restricted to immediate trombolysis and later physiotherapy. The brain has a potential to induce and execute self-repair mechanisms such as neuronal sprouting, neurogenesis and angiogenesis. It was previously shown that vascular endothelial growth factors (VEGFs) are powerful mediators to increase angiogenesis and neovascularization as well as promote neuronal survival. Here we studied the possible effects of angiogenesis using VEGF-A and VEGF-D distributed in the brain via the CSF following brain ischemia. The treatment is targeted to repair mechanisms rather than acute neuroprotection. *Methods and Results:* Four different gene constructs were used – VEGF-A165, VEGF-D (full length) and VEGFΔNΔC (proteolytically processed), LacZ, packed into adenovirus vector and baculovirus vector. The use of baculovirus vector brings an alternative to the well-established adenoviral transfection. Baculovirus preferentially transfects the choroid plexus, whereas adenovirus transfects ependymal cell lining, when they are delivered in the ventricle. Gene transfer into the rat brain was performed after Rose

Bengal cortical photothrombosis by stereotactical injection to the lateral ventricle. Different viral transfection efficacy is compared as well as the construct expression was controlled. Angiogenesis is evaluated by semi-quantitative assessment of the PECAM-1(CD31) stained adjacent brain sections. Since the peak of viral gene production is several days after transfection, VEGFs induced decrease in initial lesion size is not expected. The neurological deficit was evaluated at 7, 14 and 21 days after operation by beam-walking, limb-placing and cylinder tests. The set of behavioral tests did not yield any statistically significant difference between the transfected animals and the control group. *Conclusions:* The study tested neuroreparative function of VEGF-A and -D through perilesional angiogenesis and other brain repair mechanisms. In the current experimental settings, where the focus is set on neurological outcome, no improvement in recovery was observed. Further research is needed to understand the therapeutic value of angiogenic factors in improving stroke recovery.

Effect of edaravone, a neuroprotectant approved in Japan for indications of acute ischemic stroke, in rodent and primate stroke models

Yuki, S.[a], Akira, T.[a], Kondou, H.[b], Osajima, T.[b], Tashiro, T.[c] and Honjo, K.[d]
[a]*Pharmacology Lab., Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan*
[b]*DMPK Research Lab., Mitsubishi Tanabe Pharma Corporation, Kazusa, Japan*
[c]*Mitsubishi Chemical Safety Institute LTD., Kumamoto, Japan*
[d]*Honjo Bioscience, Inc., Osaka, Japan*

Introduction: Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger partly exerting its effect by inhibiting lipid peroxidation that proceeds in the ischemic brain. Edaravone was approved for use in Japan on patients with acute ischemic stroke (AIS) and has since been widely used under the trade name RADICUT®. Its approval and extensive usage in AIS patients has encouraged its global development. A new dosing regimen was developed for use in the EU/US considering differences in the therapeutic environments between the two regions (e.g. the length of stay in acute care hospitals, etc.). The objective of this study was to define doses that will be explored in clinical trials taking into consideration the pharmacokinetic profile of Edaravone, using two different animal models. *Method:* We used an intraluminal suture model (2 h occlusion) in rats and a permanent MCA occlusion (pMCAO) model in monkeys. In rats, edaravone was administrated immediately after reperfusion. Bolus intravenous injections (0.05, 0.1, and 0.2 mg/kg) were followed by 24 h continuous infusions (0.25, 0.5, and 1.0 mg/kg/h). The infarct volume was evaluated using 5 TTC stained brain slices, 24 h after reperfusion. In monkeys, edaravone was administered 2 h after pMCAO. Bolus intravenous injections (0.1, 0.2 and 0.4 mg/kg) were followed by 22 h continuous infusions (0.5, 1.0 and 2.0 mg/kg/h). After assessment of neurological deficits, the brain was removed under anesthesia and the infarct volume was determined by the TTC stain. *Result:* In the rat model: Edaravone reduced infarct volume dose-dependently, especially in striatum [control ($n = 12$): 105.02 ± 4.67 mm^3, low dose ($n = 13$): 85.03 ± 4.64 ($p < 0.05$), middle dose ($n = 13$): 83.94 ± 6.69 ($p < 0.05$), high dose ($n = 14$): 71.26 ± 6.24 ($p < 0.01$)]. In the monkey model: Edaravone ameliorated the neurological deficits significantly, [control ($n = 10$): 51.2 ± 4.7, low dose ($n = 10$): 40.5 ± 5.3, middle dose ($n = 10$): 32.0 ± 3.6 ($p < 0.05$), high dose ($n = 10$): 33.7 ± 5.3 ($p < 0.05$)] and reduced the infarct volume of striatum significantly at middle and high doses. Steady state plasma concentration (CSS) of unbound edaravone was almost at the same level in the two species. *Conclusion:* Edaravone was administered to different AIS models, a rat intraluminal suture model, and a monkey pMCAO model, using a new dosing regimen. Edaravone evidently reduced the infarction volumes, especially in the striatum and ameliorated neurological deficits in both models. These results were considered encouraging enough to move on to clinical trials for the purpose of global development.

Hypoxia induced oxidative stress in mitochondria ameliorated with immunosupressant

Yusuf, S. and Agrawal, S.
Department of Surgery, Section of Neurosurgery, University of Nebraska, Medical Center, Omaha, USA

The detrimental effects of hypoxic oxidative stress to mitochondria in central nervous system are well known. The present study evaluates the effect of tacrolimus (FK-506) against hypoxic oxidative stress in spinal cord mitochondria of rats *in vitro*. Spinal tis-

sue was pre-treated with FK-506 (0.1 mM) and subsequently exposed to hypoxic conditions by placing it in Ringer's solution saturated with 95%N2-5%CO2 for 1 h. ATP content, calcium uptake in non-synaptic mitochondria myeloperoxidase (MPO) activity (to measure neutrophil infiltration), mitochondrial lipid peroxidation (LPO), and glutathione (GSH) content were measured in various groups. As a result of spinal cord hypoxia, a significant decrease in mitochondrial ATP and GSH content was observed by 30.64% and 60.14% respectively over sham values. In addition, a significant increase in mitochondrial LPO level (57.77%) and MPO activity (461.24%) was observed in hypoxic group over sham values. Calcium uptake in non-synaptic mitochondria was found to be significantly increased in hypoxic group as evidenced by the percent decrease in absorbance by 90.0%. Pre-treatment with either FK-506 showed a significant restorative response in the status of various parameters studied in hypoxic groups as compared to respective control group. FK-506 pre-treatment showed a significant increase in ATP (11.19%) and GSH (66.46%) content. Conversely, a significant decrease in mitochondrial LPO (18.97%) level and MPO (42.86%) activity was observed because of FK-506 pre-treatment. Calcium uptake was also decreased in mitochondria as exhibited by the increase in absorbance by 11.19% after FK-506 treatment. In conclusion, present study demonstrates the neuroprotective effects of FK-506 pretreatment against oxidative stress of mitochondria after spinal cord hypoxia.

Neuron functional impairment in response to oxygen and/or glucose deprivation

Zagrean, A.-M.[a], Ceanga, M.[a], Spataru, A.[a], Le Duc, D.[a] and Toescu, E. C.[b]
[a]*Center for Excellence in Neuroscience, Department of Physiology, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania*
[b]*Department of Physiology, Medical School, University of Birmingham, UK*

Aim: Identifying the progress of neuronal functional impairment in response to deficient energy metabolism, as in hypoxia/ischemia, is important for an effective intervention during the therapeutic window for neuroprotection. In this study we have used primary cultures of cerebellar granule neurons to assess the neuronal damage resulting from 3 hours exposure to oxygen-deprivation (OD–5% dissolved oxygen), glucose-deprivation (GD), or both in combination (oxygen-glucose deprivation; OGD). *Methods:* Neuronal survival was assessed both morphologically, by use of a combined fluorescent staining with cytosolic calcein-AM and nuclear dyes Hoechst33342 and propidium iodide (PI), and functionally by ATP measurements (luminescent assay). *Results:* The dynamic response of the neuronal cultures showed significant differences during the 3 hours of metabolic stress. Exposure to OGD induced a degree of cellular death (%PI-positive neurons), larger than that evoked by OD and GD combined ($20.6 \pm 0.05\%$ in OGD vs $9.9 \pm 0.03\%$ in GD and $2.9 \pm 0.05\%$ in OD). Furthermore, morphological inspection of the 3 culture conditions showed that while in OD and GD there were less than 10% and 40% morphologically compromised cells, in OGD these accounted for more than 80%. To investigate this process, we defined, using the cytosolic calcein and nuclear Hoechst33342 fluorescent dyes, a nuclear/cytosolic ratio (NCR) parameter allowing us to identify and separate a population of neurons still alive but metabolically impaired (i.e., swollen, with decreased NCR). We also showed that the temporal dynamics of this population in OGD cultures reflected the differential decrease in ATP during this period. *Conclusion:* Exploring the salvage potential of these metabolically impaired cells could open new therapeutic opportunities.

Signaling pathways involved in thrombin-induced neuroprotection

Zündorf, G. and Reiser, G.
Institute of Neurobiochemistry, Medical Faculty, Otto-von-Guericke University Magdeburg,, Germany

The protease thrombin was shown to be an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations (Striggow et al. 2000). However, little is known about the inter- and intracellular pathways involved. To gain further insight into PAR-mediated signaling we investigated several thrombin-induced effects in a hippocampal mixed culture containing astrocytes and neurons. Using fluorescence methods we detected cell specifically thrombin-induced changes in intracellular calcium, mitochondrial potential, reactive oxygen species, NO and ERK-phosphorylation in situ. In astrocytes, thrombin induced intracellular calcium release with concentration dependent maximal amplitude and extrusion rate, and

NO release, decreased by specific inhibitors of iNOS, and ERK1/2, but not by the PI3 kinase inhibitor wortmannin. In contrast, in neurons no thrombin-induced release of calcium and NO were measurable, although iNOS was clearly detectable by immunofluorescence. In these cells, however, a concentration-dependent thrombin-induced formation of ROS was found which was decreased by wortmannin but not by MAPK inhibition. Furthermore, thrombin-induced phosphorylation of ERK1/2 was detected in astrocytes. The opposite effect was found in neurons, where pERK1/2 was dephosphorylated by exposure to thrombin. Neither in astrocytes nor in neurons was a thrombin-induced depolarization of mitochondria detectable.