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Posters

Topic: Astrogliosis

PP II-1

Genetic fate mapping of polydendrocytes in the mouse cortex after focal cerebral ischemia

P. Honsa[a,b], H. Pivonkova[a,b] and M. Anderova[a,b]
[a]*Institute of Experimental Medicine AS CR, v.v.i., Department of Cellular Neurophysiology, Prague, Czech Republic*
[b]*2nd Faculty of Medicine Charles University in Prague, Prague, Czech Republic*

Polydendrocytes (also known as NG2 glial cells) are the last type of glial cells to be discovered in the adult mammalian central nervous system (CNS). They can be characterized by the expression of NG2 chondroitin sulfate proteoglycan and by their highly branched morphology, and they are uniformly distributed throughout the grey and white matter. It is well known that these cells differentiate into oligodendrocytes *in vivo* and have often been equated with oligodendrocyte precursor cells. Some recent studies have described their differentiation into ventral protoplasmic astrocytes; however, their full differentiation potential in the uninjured or injured CNS still remains elusive.

To follow the differentiation fate of polydendrocytes after CNS injury we used transgenic mice (B6; FVB-Tg(Cspg4-cre)1Akik/J) in which Cre recombinase is expressed under the chondroitin sulfate proteoglycan (Cspg4) promotor. After breeding with reporter mice (Tg(CAG-Bgeo/GFP)21Lbe/J), NG2-positive cells express green fluorescent protein (GFP) and remain GFP-positive (GFP+) during their subsequent differentiation as well. We have used permanent middle cerebral artery occlusion (MCAO), a commonly used model of focal cerebral ischemia, and followed the fate of GFP+ cells using immunohistochemistry and the patch clamp method.

In the non-injured somatosensory cortex GFP+ cells expressed NG2 proteoglycan together with platelet-derived growth factor α receptor. We did not detect any GFP+ oligodendrocytes or neurons nor, in contrast to previous studies, any S100β- or glial acidic fibrillary protein (GFAP)-positive astrocytes in this region. Three days after MCAO, Ki67 staining showed marked proliferation of GFP+ cells at the lesion edge in the somatosensory cortex, and these cells co-expressed nestin together with NG2 proteoglycan. At this time point after MCAO, no GFP+ cells expressed astrocytic or neuronal markers. Seven days after injury, the number of GFP+ cells in the gliotic tissue dramatically increased when compared to non-injured cortex; these GFP+ cells lost their nestin expression but co-expressed the astrocytic marker GFAP. On the other hand, NG2 proteoglycan expression was significantly suppressed. Electrophysiological analysis revealed that GFP+ cells in the non-injured somatosensory cortex display a complex current pattern that is typical for polydendrocytes. This current pattern is maintained 3 days after MCAO; however, 7 days after MCAO, GFP+ cells displayed a passive current pattern resembling that of astrocytes and were widely coupled, presumably via gap junctions. Taken together, our data indicate that GFP+ cells in the somatosensory cortex are activated after MCAO and give rise to an astrocytic population that contributes to the formation of a glial scar.

PP II-2

Rapid behavioural improvement in mice with attentuated reactive gliosis after permanent focal cortical ischemia

M. Porritt[a], U. Wilhelmsson[1], M. Pekna[b], M. Nilsson[a] and M. Pekny[a]
[a]*University of Gothenburg, CBR, Institute for Neuroscience and Physiology, Gothenburg, Sweden*
[b]*University of Gothenburg, Department of Medical Chemistry and Cell Biology, Institute of Biomedicine, Gothenburg, Sweden*

Ischemic stroke results in neuronal injury and reactive gliosis. The hallmark of reactive astrocytes is the up-regulation of the intermediate filaments glial

fibrillary acidic protein (GFAP) and vimentin. Reactive astrocytes are the main component of the glial scar that acts as a barrier, inhibiting neuroregeneration, neurite outgrowth and plasticity. Reactive astrocytes in mice deficient in the intermediate filament proteins (GFAP$^{-/-}$Vimentin$^{-/-}$) are completely devoid of intermediate filaments. GFAP$^{-/-}$Vimentin$^{-/-}$mice has previously been reported to have improved post-traumatic regeneration of neuronal synapses and integration of neural grafts, despite greater synaptic loss at the initial stage after neurotrauma.

Objective: We aimed to explore whether the attenuation of reactive gliosis could modify the acute ischemic infarct volume and functional outcome measured by neurobehavioural testing after focal ischemia.

We aimed to explore whether the attenuation of reactive gliosis could modify the acute ischemic infarct volume and functional outcome measured by neurobehavioural testing after focal ischemia.

Material and methods: Ischemia was induced in male and female mice carrying a null mutation in the GFAP and vimentin gene and the wild-type controls on a C57BL/6 -129Sv-129Ola genetic background. The mice were 5–7 months old (18–30g). Permanent focal cortical ischemia was induced over the left somatosensory cortex by the Rose Bengal photothrombotic method. Neurobehavioural assessment of motor function was performed at 1 and 3 days post ischemia. Animals were killed 1 and 3 days post ischemia and infarct volume and serum estradiol concentration determined.

Results and conclusion: Prior to ischemia, all animals recorded the same baseline levels in neurobehavioural testing. 24 hours after ischemia all animals demonstrated significant motor impairment of the right forepaw with the GFAP$^{-/-}$Vimentin$^{-/-}$ more severely affected. At 24 hours post stroke the majority of GFAP$^{-/-}$Vimentin$^{-/-}$ mice of both sexes were unable to perform many of the neurobehavioural motor tests. All mice had improved neurobehavioural scores at day 3 with the GFAP$^{-/-}$Vimentin$^{-/-}$ mice having the greatest improvement. The time to complete walking both square and round beams, the adhesive removal test and the cylinder test were all returned to near pre stroke testing levels. Cortical infarct volumes were the same for all mice independent of genotype or time point post ischemia. Estradiol concentration did not differ between animals and did not correlate with infarct volume. The absence of intermediate filaments and thus the attenuation of reactive gliosis in the acute period following stroke resulted in a greater functional deficit. This data supports previous findings that reactive astrocytes limit the spread of damage in the acute ischemic period. However, our results suggest that reactive astrocytes also inhibit the post ischemic recovery in the acute period.

PP II-3

Ischemia induces changes in astroglial-synaptic interactions

G. Skibo[a], I. Lushnikova[a], D. Muller[b] and I. Nikonenko[b]
[a]*Bogomoletz Institute of Physiology, Department of Cytology, Kiev, Ukraine*
[b]*Geneva University Medical Center, Geneva, Switzerland*

Objective: It is well known that astrocytes play a critical role in brain homeostasis and functioning by controlling the local environment, participating in neuronal signaling and mediating the strength and kinetics of synaptic activity. Localization of astroglia in close proximity to a synapse may be critical to fulfill these important functions. The aim of this study was to analyze activity-dependent plasticity of excitatory synapses in the hippocampus using 3D EM reconstruction.

Material and methods: Hippocampal organotypic slice cultures were prepared from 7-day-old rat pups and maintained in culture for 10–13 days before experiments. OGD paradigm was applied to induce the so-called anoxic LTP. Tetanic LTP was induced using theta- burst stimulation consisting of five bursts at 5 Hz with each burst composed of four pulses at 100Hz. In a series of OGD experiments D-AP5 – an NMDA- receptor antagonist – was added to the culture medium. In OGD experiments the cultures were fixed and processed for EM using a classic epon embedding protocol. In tetanic LTP experiments slice cultures were processed for EM using the calcium precipitation protocol. Synaptic profiles were photographed, serially aligned, reconstructed and analyzed using Reconstruct software.

Results: 3D reconstructions of synapses showed significant changes following the induction of synaptic potentiation using both protocols, brief OGD and theta-burst LTP induction. The ultrastructural modifications were observed in all three components of a synaptic complex (dendritic spine, presynaptic terminal and associated glial processes). Furthermore, synaptic activity induced a pronounced increase of the glial cover-

age of both pre- and postsynaptic structures. The revealed ultrastructural changes were prevented by the application of NMDA receptor antagonist, D-AP5.

Conclusion: These data provide evidence of dynamic, activity-dependent interactions between processes of glial cells and their pre-and postsynaptic partners and suggest that glia can participate in activity-induced structural synapse remodeling.

PP II-4

Role of STAT-signalling in reactive gliosis: potential implications for endogenous regenerative neurogenesis following cortical injury in mice

S. Tiedt[a], C. Heinrich[a], M. Dichgans[b,c] and M. Götz[a,d]
[a]*Institut für Physiologie, LMU, Lehrstuhl für Physiologische Genomik, München, Germany*
[b]*Klinikum Großhadern, LMU, Neurologische Klinik, München, Germany*
[c]*Ludwig-Maximilians-Universität, Institut für Schlaganfall- und Demenzforschung, München, Germany*
[d]*Helmholtz Zentrum, Institut für Stammzellforschung, München, Germany*

Injury of the adult mammalian cerebral cortex is often accompanied by a reactive astrogliosis, characterised by astroglial hypertrophy and upregulation of GFAP (Glial Fibrillary Acidic Protein) and nestin expression. We have recently found that a subset of reactive astrocytes de-differentiate, resume proliferation, and when isolated in vitro can generate self-renewing and multipotent neurospheres, suggesting that they had acquired stem cell-like properties (Buffo et al., 2008). While few of these astroglia-derived neurosphere cells spontaneously give rise to neurons, forced expression of neurogenic fate determinants can greatly enhance neurogenesis in vitro (Heinrich et al., 2010). However, virtually no neurogenesis occurs in the injured cortex *in vivo*, suggesting that inhibitory mechanisms hamper regenerative neurogenesis. It has been suggested that the STAT (Signal Transducer and Activator of Transcription)-signaling promotes gliogenesis and inhibits neurogenesis in neural stem cells. Here, we hypothesized that following injury STAT proteins (STAT1, STAT3 and STAT5) become expressed in reactive astrocytes and may thereby contribute to the inhibition of regenerative neurogenesis.

Protein expression levels of STATs were analysed following stab wound or MCAO (Medial Cerebral Artery Occlusion) injury in adult mice. While neither STAT1 nor STAT5 were up-regulated or phosphorylation-dependent activated, Western-Blot analysis revealed that 3 days after lesion along with prominent GFAP upregulation, STAT3 protein levels were increased within the cortex ipsilateral to the injury as compared to the contralateral, non-injured side. Subcellular fractioning showed a marked increase in STAT3 expression within the nucleus suggestive of activation of STAT3 signalling. Accordingly, we could detect phosphorylated STAT3 (phospho-STAT3) within the nuclear fraction of proteins prepared from the injured cortical tissue. In order to characterize the cell type in which STAT3 signalling becomes activated we performed co-immunostaining demonstrating that STAT3 is specifically up-regulated in GFAP-expressing astrocytes in the injured cortex. Moreover, 20% of GFAP-positive cells surrounding the injured area were also immunoreactive for phospho-STAT3, supporting the increased activation of STAT3-signaling. In contrast, in the contralateral hemisphere STAT3 expression was mainly restricted to neurons and virtually no phospho-STAT3 expression could be observed.

Our data show that STAT3-signaling becomes activated in a subset of reactive astrocytes in the cerebral cortex following injury. Given the role of STAT signalling in driving neural stem cells to the glial lineage, this injury induced activation may be one of the factors keeping reactive astroglia within the glial lineage despite partial de-differentiation. Thus, inhibition of STAT3-signaling may represent a potential strategy to promote endogenous regenerative neurogenesis.

PP II-5

Analysis of GFAP expression after stroke in bone marrow cell treated SHEEP by two Evaluation methods

T. von Geymüller[a], A. Hoffmann[b], H.-A. Schoon[c], J. Seeger[b], J. Boltze[a] and B. Nitzsche[a,c]
[a]*Fraunhofer Institut für Zelltherapie und Immunologie, Leipzig, Germany*
[b]*University of Leipzig|Faculty of Veterinary Medicine, Deportment of Vet. Anat., Histology and Embryology, Leipzig, Germany*
[c]*University of Leipzig|Faculty of Veterinary Medicine, Institute of Pathology, Leipzig, Germany*

Objectives: Autologous cell treatment is a promising option to induce a better clinical outcome for strokepatients. In this study we tested the influence

of autologous bone marrow cell (aBMC) transplantation on astrocytes in a sheep model of focal cerebral ischemia, focusing on two different evaluation methods for the GFAP expression. Reactive astrocytosis after stroke is of rising interest because of its beneficial or adverse function in regard to the healing processes.

Material and methods: Eight adult rams were subjected to permanent middle cerebral artery occlusion (MCAO). Four animals received 4x10E6 aBMC via i.v. administration 24 h after the MCAO, and four sheep remained untreated. Animals were sacrificed after 7 weeks. Specific regions of ipsi- and contralateral hemisphere of the sheep brains were incubated with polyclonal rabbit anti-GFAP (DAKO) and visualized with fluorochrome-labelled antibody.

Measurements were performed in white and gray matter, with a defined distance to the infarct border using method (I) monochrome, all-encompassing, two-dimensional pictures analysed by ImageJ and method (II) automatic exposure time in a three-dimensional direction of each frame followed by analyses with Volocity-Software (Improvision).

Additionally this method was used for investigations of baseline GFAP measurement in non altered brains of two sheep.

Results: Perilesional GFAP expression in both MCAO groups is significantly increased in gray matter compared to the contralateral hemisphere. The white matter of aBMC animals showed a tendency of increased GFAP expression in respect to the untreated MCAO animals in method (I). This becomes significant in method (II). Normalizing the data to the contralateral hemisphere showed a significant astrogliosis in the perilesional gray matter of both groups compared to non altered brains. In contrast to the astrogliosis of the gray matter an enhancement of the white matter becomes evident only in aBMC animals. Data of method (II) are statistical more significant respect to data acquired by method (I), in which the results themselves are comparable and do not show significant differences in the two MCAO groups.

Conclusions: First, the results of both methods indicate a certain receptivity of astrocytes to a bone marrow cell treatment, especially of astrocytes in the gray matter.

Second, the results demonstrate the importance of different evaluation methods and the necessity for choosing the adequate method for analyzing immunohistochemically stained slices.

The diverse respond of astrocytes depending on their location in white or gray matter maybe caused by their different functions or the specific architecture of the layer.

However, these data reflect that reactive astrogliosis 7 weeks after stroke is still an ongoing process, following maybe more serious astrocytic reactions in white and gray matter at earlier time points.

PP II-6

The neuroprotective/neurodegenerative protease thrombin induces a signaling cascade in astrocytes and neurons involving calcium release and generation of reactive oxygen species, which specifically is connected to the status of ERK1/2 phosphorylation

G. Zündorf and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: Serine proteases activate protease-activated receptors (PAR), a family of G protein-coupled receptors, and thus orchestrate diverse cellular functions, including apoptosis and survival. PAR activation induces an increase of cytosolic Ca^{2+} ($[Ca^{2+}]_c$), activation of extracellular regulated kinase (ERK1/2) and formation of reactive oxygen species (ROS). A cross talk of thrombin signaling and other pathways has been demonstrated. Thus, the targeting of pro-survival thrombin signaling needs evidence for the suggested mechanisms of action and its evaluation in combination with other factors.

Material and Methods: We used Fura-2 and hydroethidine for *in situ* detection of the thrombin-induced changes of $[Ca^{2+}]_c$ and ROS generation in primary astrocytes and neurons under constant perfusion. Different phosphorylation status of ERK1/2 as detected by immunofluorescence was assigned subsequently to single cells with a spectrum of physiological responses.

Results and conclusions: In astrocytes, thrombin induced ERK1/2 phosphorylation *via* activation of phospholipase C. In neurons, varying patterns of ERK1/2 phosphorylation are detectable, independent of any thrombin challenge. Furthermore, thrombin induced in astrocytes a transient average rise of $[Ca^{2+}]_c$ with a concentration-dependent increase in both amplitude and acceleration of extrusion rate. On closer inspectation of the single cells within the entirety of all astrocytes, different types of patterns of responses con-

cerning amplitude and extrusion of $[Ca^{2+}]_c$ were distinguishable which can be correlated with a different spatial distribution of phosphoERK1/2 in nucleus or cytosol. Furthermore, thrombin induced an immediate, transient increase of extracellular hydroxyethidium fluorescence which was interpreted to be caused by a NADPH oxidase-mediated release of $O_2^{\cdot -}$ into the extracellular milieu, since it was decreased by preincubation with the inhibitor apocynin. Similarly, a second phase was seen as a sustained slope of intracellular rise in hydroxyethidium fluoresecence lasting over several minutes, which can be assigned to single neurons and positively correlated with increased ERK1/2 phosphorylation level after thrombin challenge. We conclude that the phosphorylation status of ERK1/2 determines both the cellular vulnerability and the neuroprotective effect of thrombin stimulation in astrocytes and neurons. Furthermore, we show that hydroethidine is suitable for detection of both intracellular and extracellular ROS formation. ROS from NADPH oxidase may be at least in part involved in the thrombin-induced neuroprotection.

Topic: Cell Death and Apoptosis

PP III-13

Analysis of conditional knock-out of calpain small subunit (Capn4) in mouse brain

M. Amini[a], C.-L. Ma[a], J. Vanderluit[b], J.M. Savitt[c], R.S. Slack[a], R. Bergeron[a], P.A. Greer[d] and D.S. Park[a]
[a]*University of Ottawa, CMM/NRC, Ottawa, Canada*
[a]*Memorial University of Newfoundland, BioMedical Sciences, St. John's, Canada*
[c]*Hopkins University, Neurology, Baltimore, United States*
[d]*Queen's University, Cancer Biology and Genetics, Kingston, Canada*

Calcium-dependent calpain proteases have been implicated in many basic cellular processes and neuropathological events. The ubiquitous calpains, μ- and m-calpain, are heterodimers consisting of 80-kDa catalytic subunits encoded by capn1 and capn2, respectively, and a common 28-kDa regulatory subunit encoded by capn4. However, most evidence implicating calpains in physiological and pathophysiological events are based on indirect findings or the use of calpain inhibitors which have insufficient specificity. Germline disruption of capn4, which eliminates both ubiquitous calpain activities, causes early embryonic lethality, hampering the use of those mouse models to explore the physiological and pathophysiological functions of calpains in the CNS. Here we describe the use of a capn4-loxP/nestin-cre conditional mouse mutant to explore calpain functions in long term potentiation (LTP), a mouse model of Parkinson's disease and ischemic stroke. Using the Nestin promoter to drive Cre expression we have targeted capn4 in early neuronal progenitors and selectively eliminated ubiquitous calpain activities in the CNS. In the absence of calpain activity, mice were born at Mendelian ratios and survived to adulthood. They did not show any gross abnormality in morphology in the CNS either at embryonic or adult periods. Interestingly, however, the mutant mice exhibited a significant decrease in LTP formation. Although, in the absence of both calpain activities, there were alterations in expression of some related cytoskeletal components, glutamate receptors subunits and their anchoring proteins, the synaptic physiology appeared unaffected. Moreover, in vitro studies showed that neurons obtained from these mutant mice were resistant to the Parkinson's disease related toxin 1-methyl-4-phenyl-pyridimium (MPP+), hypoxia, and glutamate. We also targeted capn4 using the tyrosine hydroxylase (TH) promoter and showed that dopaminergic neurons obtained from capn4-loxP/TH-cre conditional mouse mutants were resistant to MPP+-induced cell death. In this Parkinson's disease model, conversion of the p35 regulatory binding partner of Cdk5 to p25 was not observed in mutant neurons. In addition, we observed less conversion of p35 to p25 in mutant's neurons after glutamate treatment, an oxidative stress model. In summary, these observations suggest that μ- and m-calpains play more prominent roles in CNS functions and disease than in CNS development. Moreover, this conditional genetic strategy will allow us to examine the precise functions of calpains in LTP generation and neurodegeneration in adult in vivo settings in future experiments.

PP III-14

Korean red ginseng prevents cell death and enhances recovery in ischemic stroke mediating PI3K/Akt pathway

S.-Y. Cheon, K.J. Cho, H.-W. Kim, S.K. Lee, S.-H. Lee, J.E. Lee, B.I. Lee and G.W. Kim

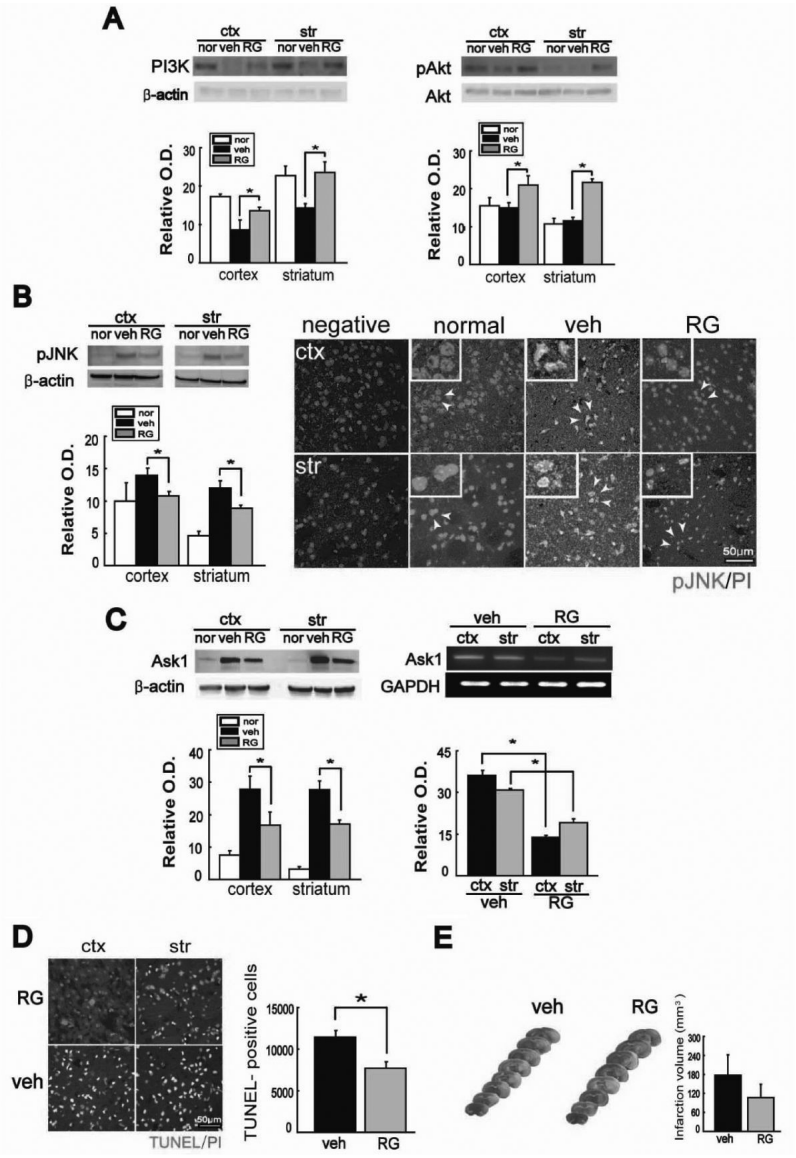

Fig. 1.

Yonsei University, Department of Neurology,Brain Korea 21 Project for Medical Science, Seoul, Korea, Republic of

Objectives: Ginseng has demonstrated beneficial effects for the treatment of neurological injury and disease. Although Asian ginseng (Panax ginseng) is thought to exhibit protective functions when used to treat after cerebral ischemia in vivo, the effects of Korean red ginseng (KRG) on ischemic stroke have not been fully elucidated. Our study was to investigate the role of KRG extract on the ischemic damaged brain mediated (/considered) by PI3K/Akt pathway, which performed before or after stroke onset.

Material and methods: Male C57BL/6 mice used in our study were subjected to 1 h of cerebral ischemia, followed by reperfusion. The pretreatment group was administered crude extracts of KRG (360 mg/kg) and vehicle intraperitoneally for 2 weeks before ischemia, and the post treatment group was given KRG and vehicle for 14 or 28 days by the same route beginning 24 h after ischemia.

Results and conclusions: KRG pre-treatment activated the PI3K/Akt pathway and inhibited the Ask1/JNK

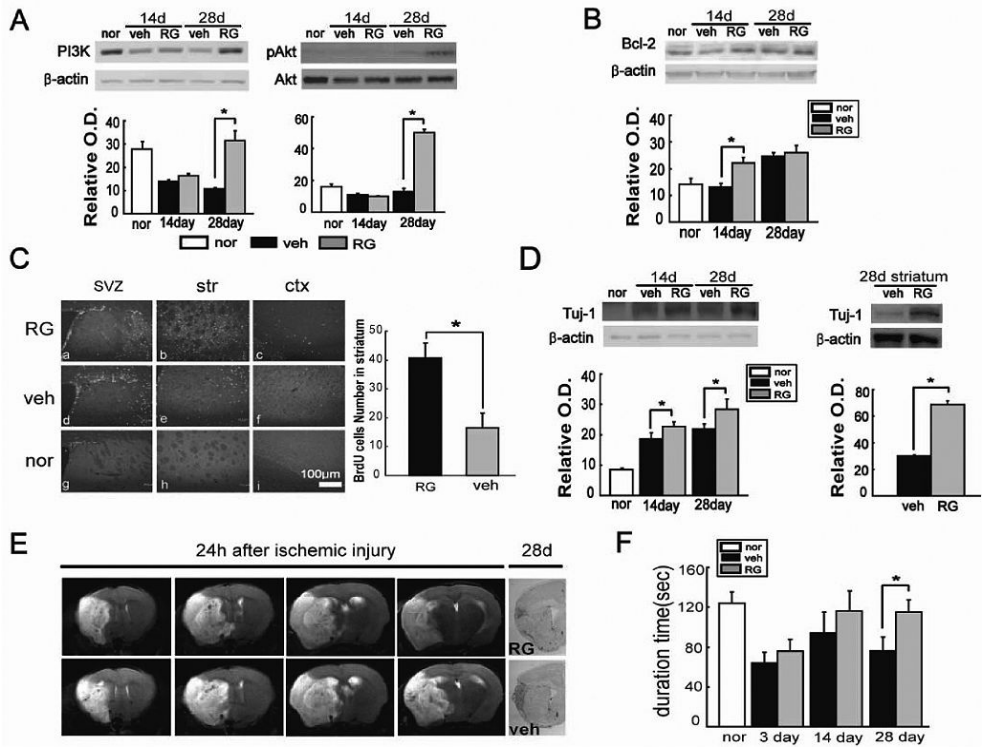

Fig. 2.

pathway by decreasing Ask1 mRNA, leading to decrease DNA fragmentation and infarct volume. KRG treatment, given after ischemic stroke in mice, stimulated the PI3K/Akt pathway and augmented Bcl-2 signaling. Post-treatment of KRG showed to enhance migration of newly generated cells in the ischemic area, reduced glial scar, and improved functional deficits in the rotarod test conducted over periods of 3, 14, and 28 days after ischemia. Collectively, KRG prevents the brain damage by regulating MAPKinase and helps the restoring damage by PI3K/Akt pathway, suggesting that KRG crude extract may be a promising strategy to prevent or rescue brain damage after stroke.

PP III-15

Neuronal nadph oxidase 1 contributes to the death of hippocampal neurons and cognitive impairment in vascular dementia rat model

D.-H. Choi, K.-H. Lee, M. Kim, M.R. Hasan, J.-S. Han, H.Y. Kim, C.Y. Shin, S.-H. Han and J. Lee
Konkuk University, Center for Geriatric Neuroscience Research, IBST, Seoul, Korea, Republic of

Objective: Chronic cerebral hypoperfusion has been characterized as a common pathological status contributing to neurodegenerative diseases such as vascular dementia (VaD). The main clinical outcomes of chronic cerebral hypoperfusion are the cognitive deficits and permanent neural impairment. While oxidative stress has been strongly implicated in the pathogenesis of VaD, the molecular mechanism underlying selective vulnerability of the hippocampal neurons to oxidative damage remains unknown. Recent discovery of the NADPH oxidase (NOX) complex, a specialized superoxide generation system, reveals that overproduction of ROS by NOX activation contributes to neurodegeneration. We previously found that NOX1 is a main superoxide generator in the neurons during neurodegeneration such as dopaminergic neuronal death in Parkinson's disease animal model. However, the role of NOX1 in the pathogenesis of VaD is not known. We investigate to determine whether NOX1-mediated superoxide generation is responsible for an increase in oxidative stress in hippocampal system and subsequent cognitive decline in vascular dementia animal model.

Materials and methods: Male Wistar rats (8 weeks, $n = 6{\sim}8$ each group) were subjected to bilateral common carotid artery occlusion (BCCAo) and were divid-

ed into two groups; sham-operated and BCCAo group. NOX1 immunoreactivity at hippocampal neurons, neuronal cell death and level of superoxide generation were serially determined from 1 week to 10 weeks after BCCAo. NOX inhibitor, apocynin (10mg/kg) ip injection and shRNA Nox1/AAV stereotaxic injection into both hippocampus CA1 subfields were used to investigate the specific role of NOX1 in oxdative damage, neuronal death and cognitive impairment. All rats were subjected to Morris Water Maze Test 5 trials daily for the last 4 days during 10th week before sacrifice.

Results: NOX1 expression gradually increased in hippocampal neurons after 1 week to 4 weeks after BCCAo. NOX1 expression and activity were remained at increased level until 10 weeks after BCCAo. Level of superoxide generation and neuronal cell death in hippocampus CA1 subfield were increased in BCCAo group. Either inhibition of NOX by apocynin or knockdown of NOX1 by shRNA NOX1 reduced ROS generation and decreased hippocampal neuronal cell death. Cognitive impairments were recovered or at least reduced by NOX1 inhibition.

Conclusion: Our date suggest that NOX1 may play an important role in neuronal cell death and cognitive impairment in vascular dementia.

PP III-16

Neuroprotection by activation of K_{Ca} 2 channels in vitro and in vivo

A. Dolga[a], N. Trepolilli[b], N. Plesnila[c] and C. Culmsee[a]
[a]*Philipps-Universität Marburg, Institut für Pharmakologie und Klinische Pharmazie, Marburg, Germany*
[b]*Ludwig-Maximilians-Universität München, Walter Brendel Center for Experimental Medicine, Munchen, Germany*
[c]*Royal College of Surgeons in Ireland (RCSI), Department of Neurodegeneration and Department of Physiology, Dublin, Ireland*

Objectives: Glutamate toxicity and calcium deregulation represent major causes of neuronal death after cerebral ischemia. Since small conductance calcium-activated potassium ($K_{Ca}2$) channels are able to block glutamate receptors they might be an appropriate therapeutic target to restore the compromised calcium homeostasis in the injured brain. In this study we investigated the effects of $K_{Ca}2$ channel modulation in glutamate-induced toxicity in primary cortical neurons *in vitro* and in an experimental model of transient focal cerebral ischemia *in vivo*.

Materials and methods: Primary cortical neurons from embryonic rats were treated with agonists and antagonists of $K_{Ca}2$ channels, with glutamate or NMDA. Cellular viability was determined by the MTT assay. Measurements of intracellular calcium $[Ca^{2+}]_i$ were performed by single-cell fluorescence imaging. *In vivo* brain damage was induced by transient middle cerebral artery occlusion (MCAo) in mice.

Results: In primary cortical neurons, activation of $K_{Ca}2$ channels by the $K_{Ca}2$ channel agonist NS309 reduced excitotoxicity and restored glutamate-induced delayed $[Ca^{2+}]_i$ deregulation. Activation of $K_{Ca}2$ channels mediated neuroprotection when applied up to 3 h after the onset of glutamate exposure. Interestingly, glutamate toxicity induced $K_{Ca}2$ channel degradation which was rescued by NS309. Further, in an experimental model of transient focal cerebral ischemia in mice, activation of $K_{Ca}2$ channels by NS309 reduced the infarct volume and promoted fast and extensive recovery with improved neurological function score.

Conclusions: In conclusion, our findings expose small conductance $K_{Ca}2$ channels as potential therapeutic targets for the treatment of neurodegenerative disorders where glutamate toxicity and disturbed Ca^{2+} homeostasis are prominent.

PP III-17

ATF4-CHOP regulate puma in er stress-induced neuronal death

Z. Galehdar[a], P. Swan[b], B. Fuerth[b], S.M. Callaghan[a], D.S. Park[a] and S.P. Cregan[b]
[a]*University of Ottawa, Cellular and Molecular Medicine, Neuroscience, Ottawa, Canada*
[b]*Robarts Research Institute, University of Western Ontario, Physiology and Pharmacology, London, Canada*

An increasing body of evidence points to a key role of endoplasmic reticulum (ER) stress in neurodegenerative conditions. Extensive ER stress can trigger neuronal apoptosis, however the signaling pathways that regulate this cell death remain unclear. In the present study we demonstrate that Puma is transcriptionally activated in cortical neurons by ER stress and is essential for ER stress-induced cell death. Puma is known to be a key transcriptional target of p53, however we have found that ER stress triggers Puma induction and

cell death through a p53-independent mechanism involving instead the ER stress inducible transcription factor ATF4. Specifically, we demonstrate that ectopic expression of ATF4 sensitizes neurons to ER stress induced apoptosis, and that ATF4-deficient neurons exhibit markedly reduced levels of Puma expression and cell death. However, ChIP experiments indicated that ATF4 does not directly regulate the Puma promoter. Rather, we found that ATF4 induces expression of the transcription factor CHOP, and that CHOP knockdown attenuates Puma induction and neuronal apoptosis. In summary, we have identified a key signaling pathway in ER stress induced neuronal death involving ATF4-CHOP mediated transactivation of the pro-apoptotic Bcl-2 family member Puma.

PP III-18

Prevention of death of neural cells by activation of the uridine nucleotide-specific $P2Y_6$ receptor with a specific agonist

M. Haas[a], B. Fischer[b] and G. Reiser[a]
[a]*Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany*
[b]*Bar-Ilan University, Department of Chemistry, Ramat-Gan, Israel*

Objectives: Protection of tumor necrosis factor α (TNFα) -induced apoptosis in 1321N1 astroytoma cells by activating the uridine nucleotide-specific $P2Y_6$ receptor with the standard agonist UDP was shown recently. This approach later was extended to the analysis of mouse skeletal muscle endogenously expressing the $P2Y_6$ receptor (see [1] and references). The standard agonist UDP and the synthetic UDP derivative MRS2693 were able to prevent TNFα-induced apoptosis. MRS2693 also was able to trigger cytoprotective effects *in vivo* in mouse skeletal muscle after ischemia/reperfusion.

Materials and methods: Here, we extend the analysis of the cytoprotective role of the $P2Y_6$ nucleotide receptor to the protection of neural cells endogenously or exogenously expressing the $P2Y_6$ nucleotide receptor. The TNFα-induced cell death is analysed via measurement of the release of Lactate Dehydrogenase (LDH) and the cytoprotective effect of UDP is compared with that of 5-OMe-UDP, a potent and selective nucleotide agonist of the $P2Y_6$ receptor recently identified by us [2], and its derivatives.

Results: Together with the specific $P2Y_6$ receptor antagonist MRS2578, these nucleotides help to investigate $P2Y_6$ receptor-mediated effects in physiological conditions in the presence of other uridine nucleotide activated P2Y receptors ($P2Y_{2/4/14}$).

Conclusions: We expect to further the knowledge of the cytoprotective role of the $P2Y_6$ receptor to neural cells like microglia, which are known to express the $P2Y_6$ receptor and which can be activated by UDP acting via the $P2Y_6$ receptor.

Furthermore, 5-OMe-UDP and its derivatives will be established as suitable agonists for the exploration of $P2Y_6$ receptor-specific processes in neurodegenerative disease states in brain tissue.

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PP III-19

Macrophage migration inhibitory factor is detrimental following experimental stroke

A. Inacio, T. Wieloch, K. Ruscher and T. Deierborg
Lund University, Clinical Sciences, Lund, Sweden

Aim: Macrophage Migration Inhibitory Factor (MIF) functions as a pleiotropic protein, playing a key role in inflammatory and immune responses as well as in cell growth, including embryogenesis, tumorigenesis, and wound repair. MIF is constitutively expressed in the brain, where it may modulate neuronal activity and glial function. Importantly, MIF is up-regulated in the brain after cerebral ischemia. Hence, we aimed at characterizing the spatial-temporal expression of MIF in the brain during the first week, as well as investigating the putative role of MIF in the pathogenesis of experimental ischemic stroke.

Main methods: We performed transient middle cerebral artery occlusion (tMCAo) in *Mif*$^{-/-}$ (MIF-KO)

male mice on a pure C57BL/6 background and respective *Mif*$^{-+/+}$ (WT) littermates. Spontaneously hypertensive male rats were subjected to permanent middle cerebral artery occlusion (pMCAo), and housed in standard cages (STD) or in an enriched environment (EE) from post-occlusion day 2 to 5. Sensory-motor deficits were assessed by the rotating pole and grip-strength tests. Cortical neurons obtained from Wistar rats (E17) were cultured *in vitro* for 10–15 days and subsequently exposed to combined oxygen-glucose deprivation (OGD).

Results: MIF accumulates in neurons of the evolving core/peri-infarct border zone within hours and gradually over days after tMCAo in mice. This accumulation is particularly evident in the peri-infarct cortex and in cortical parvalbumin-containing interneurons. In addition, we detected an increase in MIF in peri-infarct astrocytes at 7 days post-occlusion. Disruption of *Mif* in mice leads both to a smaller infarct volume and a better sensory-motor function following tMCAo. Likewise, in cultured cortical neurons exposed to OGD MIF levels increase, and inhibition of MIF by (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) protects against cell death, suggesting a role for neuronal MIF in the pathogenesis of stroke. Furthermore, deletion of MIF in mice does not affect the protein levels of several cytokines, including interleukin 1β, in the brain and serum following tMCAo, indicating that the observed MIF-mediated effects are independent of its pro-inflammatory action. Housing rats in an EE improves recovery of function after pMCAo without altering infarct size, and results in a down-regulation of MIF both in the peri-infarct region and the cingulate cortex, when compared to housing rats in STD. Importantly, this down-regulation is accompanied by an increased number of parvalbumin-positive neurons in the peri-infarct cortex.

Conclusion: We conclude that MIF promotes neuronal death by intra and/or inter-neuronal mechanisms likely independent of its pro-inflammatory action. Furthermore, our data suggests that overexpression of MIF in neurons and/or astrocytes hampers the recovery of sensory-motor function following stroke. Our results imply that MIF may be targeted in the development of future stroke therapies.

PP III-20

Inhibition of CDC25 protects neurons from delayed death mediated by hypoxia

G. Iyirhiaro, Y. Zhang, R. Slack and D.S. Park
University of Ottawa, Cellular and Molecular Medicine, Ottawa, Canada

Dysregulation of cell cycle machinery is implicated in a number of neuronal death models including Parkinson's disease, Alzheimer's disease and Stroke. Empirical evidence suggests that cyclin dependent kinases (CDKs), important cell cycle regulators, are inappropriately activated in matured neurons under ischemic stress conditions. A direct role for cell cycle proteins in neuronal death is substantiated by evidence demonstrating that the G1/S regulators cyclin D1 and cdk4 are mediators of delayed neuronal death following hypoxia and cerebral ischemic injury. In the present study we investigate the involvement of the upstream positive regulators of the cell cycle, the cdc25 family of phosphatase, in neuronal death mediated by hypoxic injury.

Primary cerebellar granule neurons (CGN) harvested from CD1 mice were treated with the cdc25 inhibitor, NSC95397 and subjected to hypoxia at 1% oxygen after one week in culture. CGNs were allowed to re-oxygenate at normoxia for 24 hours and assessed for survival. Additionally, CGNs treated with cdc25A shRNA or doubly or singly null for cdc25B/C were also subjected hypoxia/re-oxygenation and assessed for survival.

Here we show that the use of pharmacologic agent that is capable of inhibiting all three cdc25 family members protect neurons from delayed death mediated by hypoxia. We also show that CGNs doubly null for cdc25B and C or singly null for cdc25B or C are not protected from delayed death mediated by hypoxia. However, shRNA mediated knockdown of cdc25A protects CGN from delayed death.

Overall, our results suggest a role for cdc25A in delayed neuronal death mediated by hypoxia.

PP III-21

Role of NR2D-containing NMDA receptors in tissue-type Plasminogen Activator (tPA)-mediated potentiation of excitotoxic neuronal injuries

A. Jullienne[a], A. Montagne[a], D. Monaghan[b], E. Maubert[a], D. Vivien[a] and C. Ali[a]

[a]*INSERM U919 – SP2U, UMR CNRS 6232 – GIP Cyceron, Caen, France*
[b]*University of Nebraska, Dept of Pharmacology and Experimental Neuroscience, Omaha, United States*

The pleiotropic molecule tissue-type plasminogen activator (tPA) is well known for its use in ischemic stroke treatment, based on its ability to cleave plasminogen into active plasmin that degrades fibrin, the major component of blood clots. Above this thrombolytic effect, tPA is also involved in several deleterious processes including aggravation of glutamatergic N-methyl D-aspartate receptors (NMDAR)-mediated neurotoxicity. We have recently suggested that this promotion of neuronal excitotoxicity requires the presence of the NR2D subunit of NMDAR [1] and thus selectively occurs in brain regions expressing NR2D (e.g., cortex, but not in the CA1 subfield of the hippocampus).

Here, to support further our hypothesis, we used UBP145, a more selective antagonist of NR2D-containing NMDAR than the previously tested agent, PPDA [2]. *In vitro*, we observed in primary murine cultures that both PPDA and UBP145 prevent tPA promotion of NMDAR-mediated excitotoxicity in cortical neurons, while in hippocampal neurons, only PPDA provides neuroprotection. These results confirm that UBP145 is more specific for NR2D-containing NMDAR. We then tested the effect of UBP145 *in vivo* in a model of excitotoxicity induced by the intra-cortical injection of NMDA in mice, with or without an intravenous injection of tPA. UBP145 reduced excitotoxic lesions in both conditions, demonstrating its ability to prevent the pro-excitotoxic effect of both endogenous and exogenous tPA at the cortical level.

Further investigations, including those of the effect of UBP145 in excitotoxic challenges to areas that differentially express the NR2D subunit and in a thromboembolic model of stroke should provide support for the potential use of this drug as an adjunct to tPA administration for a safer thrombolysis in stroke patients.

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PP III-22

Hexokinase II mediates endogenous neuroprotection by integrating regulation of glucose metabolism and apoptosis

P. Mergenthaler[a,b], A. Kahl[a], V. van Laak[c], D. Freyer[a], K. Stohlmann[a], S. Thomsen[a], D. Megow[a], J. Priller[d], U. Dirnagl[a,b], D.W. Andrews[e,f] and A. Meisel[a,b,g]
[a]*Charité Universitätsmedizin Berlin, Department of Experimental Neurology, Center for Stroke Research Berlin, Berlin, Germany*
[b]*Charité Universitätsmedizin Berlin, Department of Neurology, Berlin, Germany*
[c]*Charité Universitätsmedizin Berlin, Department of Internal Medicine, Infectious Diseases and Pulmonary Medicine, Berlin, Germany*
[d]*Charité Universitätsmedizin Berlin, Department of Psychiatry, Berlin, Germany*
[e]*McMaster University, Biophotonics Imaging Facility, Hamilton, Canada*
[f]*McMaster University, Department of Biochemistry, Hamilton, Canada*
[g]*Charité Universitätsmedizin Berlin, NeuroCure Clinical Research Center, Berlin, Germany*

All multicellular organisms have evolved mechanisms to protect themselves against noxious stimuli such as substrate deprivation or hypoxia. Preconditioning is a process where a noxious stimulus near to but below the threshold of injury is applied to the tissue and subsequently induces tolerance against stronger injury, such as stroke, thereby evoking protection from damage. The transcription factor hypoxia inducible factor 1 (HIF-1) is a major modulator regulating adaptive processes in response to changes in oxygen-homeostasis and is largely responsible for induced tolerance after hypoxic preconditioning. Among others, HIF1 regulates the expression of the glycolytic enzyme Hexokinase II, one of the hexokinase isoenzymes facilitating the first step of glucose metabolism by phosphorylating glucose.

We found that the expression of Hexokinase II is upregulated upon preconditioning mimicking hypoxia in primary neurons and neurons were subsequently protected from hypoxic cell death. Furthermore, overexpression of Hexokinase II in these cultures protected neurons from apoptotic cell death in an in vitro model of cerebral ischemia (oxygen-glucose deprivation, OGD) in a glucose dependent fashion. In order to investigate the underlying molecular mechanism, we identi-

fied novel interactors of Hexokinase II and confirmed interaction with different methods, including live-cell FLIM-FRET microscopy. Mitochondrial localization of the Hexokinase II multiprotein complex is indispensable, thereby integrating the regulation of apoptosis and pathways of glucose metabolism.

We propose that Hexokinase II serves as a molecular switch in a multiprotein complex with mitochondrial localization, regulating endogenous neuroprotection and apoptosis in a glucose dependent fashion. Thus, Hexokinase II provides a novel link between cellular metabolism and survival.

PP III-23

Decreased oxidative stress during glycolytic inhibition enables astrocytic survival and maintenance of ATP production

C. Nodin, F. Blomstrand and M. Nilsson
University of Gothenburg, CBR, Institute for Neuroscience and Physiology, Gothenburg, Sweden

Ischemic brain injury leads to decreased blood flow and impaired metabolism. The subsequent oxidative stress significantly contributes to cell damage and death. Astrocytes are suggested to be less sensitive to decreased oxygen levels, than e.g. neurons, since they are able to maintain their energy production via glycolysis. Astrocytes serve as the main storage of glutathione and other antioxidants in the brain. Moreover, in the brain, the detoxification and antioxidant systems regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is mainly expressed in astrocytes. Therefore, astrocytes are thought to be of major importance for the defense against oxidative stress in the brain. Nevertheless, it still remains elusive how impaired glycolytic metabolism and oxidative stress contribute to astrocytic cell death.

Objectives: We aimed to explore the involvement of oxidative stress in astrocytic cell death using an *in vitro* model of glycolytic inhibition. The protective effects of Nrf2 activation and exogenous free radical scavengers were also investigated.

Material and methods: Glycolytic inhibition was induced in astrocyte cultured by using iodoacetate (IA), which irreversibly binds to and potently inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Nrf2 system was activated by using sulforaphane, curcumin or tBHQ prior to IA addition. IA was also combined with the respiratory chain inhibitor azide or the exogenous ROS scavengers B27, trolox or tempol.

Results and conclusions: The glycolytic inhibition led to a significant increase in ROS activity, which was accompanied by ATP depression and cell death. By combining IA with azide, the ATP decrease was hastened. Interestingly, the ROS activity remained at control levels and the astrocytes remained viable, although the ATP levels were severely depressed. If the Nrf2-system was pre-activated or if IA was combined with the exogenous ROS scavengers, the levels of ROS were potently decreased, the astrocytes were able to maintain the ATP levels for a longer period of time and cell death was prevented. Importantly, these different combinations did not influence the inhibitory effect of IA on GAPDH. In a wash-out paradigm, the IA-treatment was interrupted (at ATP levels about 70% of control) and the astrocytes were left to recover. In astrocytes treated with IA alone the ATP levels were abolished and massive cell death was observed. Interestingly, by using IA and the combinations mentioned above, the astrocytes were able to recover the ATP levels and remained viable, although the GAPDH activity remained impaired.

Our data indicate that ROS activity during glycolytic inhibition is detrimental for astrocytes *in vitro*. Moreover, cultured astrocytes can endure blockage of glycolysis and maintain ATP production when ROS activity is decreased. Therefore, decreasing oxidative stress during ischemic brain injury may enable astrocytic survival.

PP III-24

The protective role of calcium-independent phospholipase A_2(iPLA_2) under oxidative stress in rat astrocytes in cultures

C. Nordmann, M. Strokin and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: In the central nervous system, Ca^{2+}-independent phospholipase A_2 (iPLA_2) has been shown to be associated with various physiological and pathological processes, including cellular growth, cell death and cell membrane homeostasis. The enzymes of the phospholipase A_2 family control the release of

polyunsaturated fatty acids (PUFAs) from the *sn-2* position of cellular phospholipids [1], thereby initiating various pathways of signal transduction. There are two members of the iPLA$_2$ family, VIA iPLA$_2$ and VIB iPLA$_2$. Both are present in substantial amounts in mitochondria. We suppose that the suppression of iPLA$_2$ in primary rat astrocytes evokes oxidative stress, lipid peroxidation and cell death via induction of mitochondria dysfunction. It was already shown that VIB iPLA$_2$ is required for the prevention and repair of lipid peroxidation. However, the role of iPLA$_2$ in the mechanism of mitochondria dysfunction remains unclear. Here, we investigate whether iPLA$_2$ plays an important role in oxidative stress in astrocytes. Materials and methods: We investigated the impact of the Ca^{2+}-independent phospholipase A$_2$ in primary rat astrocytes by chemical inhibition of iPLA$_2$ with bromoenol lactone (BEL). To determine cell physiological processes we used the specific fluorescence dyes rhodamine 123 and 2′-7′-dichlorofluorescin diacetate as well as TBARS-assay for lipid peroxidation and the release of LDH to detect cell death.

Results and Conclusions: Treatment of rat astrocytes with different concentrations of BEL (2.5 μM to 10 μM) induced lipid peroxidation and oxidative stress. These effects of BEL were clearly concentration-dependent. Additionally, inhibition of iPLA$_2$ activity with BEL under oxidative stress induced by rotenone (24 h pre-incubation) evoked enhanced cell death. Furthermore, we could measure the effect of BEL on mitochondrial membrane potential. This rotenone-induced mitochondria depolarization is potentiated through concentration-dependent inhibition of iPLA$_2$ with BEL (from 0.5 to 5 μM BEL). Our results indicate that members of the Ca^{2+}-independent phospholipase A$_2$ might play a protective role in oxidative stress, which underlines its crucial role for mitochondria functions. Consequently, it was presumed that iPLA$_2$ plays a cytoprotective role in oxidative stress conditions and maintenance of mitochondria functions but the details of the pathways are still not clear. However, the functional difference between the two isoforms of iPLA$_2$, VIA iPLA$_2$ and VIB iPLA$_2$ still has to be determined.

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PP III-25

Critical influence of previous alcohol consumption on ischemic stroke and thrombolysis

M. Rubio, M. Gauberti, H. Villain, D. Vivien and C. Ali
INSERM U919, UMR CNRS 6232, Caen, France

Thrombolysis (injection of recombinant tissue plasminogen activator, tPA, to dissolve the blood clot) is the only approved acute treatment for ischemic stroke. However, it is only given to $< 5\%$ of stroke patients because of strict inclusion criteria and due to a short therapeutic window ($<$ 4.5h post symptoms onset). Nevertheless, although alcohol consumption is a risk factor for stroke, previous alcohol consumption is not an exclusion criterion for thrombolysis. Besides, the impact of alcohol consumption on the progression of ischemic lesions remains barely investigated.

Objectives + Methods: We have first evaluated the influence of different patterns of alcohol consumption (chronic or binge drinking) on the progression of brain lesions after stroke. Adult mice were alcoholised (p.o., alcohol 10% diluted in drinking water) during 6 weeks uninterruptedly (chronic group) or by weekly periods of alcohol/water (4 days/3 days, respectively; binge group). Control mice received only water during the same period. Mice were then subjected to thromboembolic ischemia through direct injection of thrombin into the middle cerebral artery. Twenty min after the stroke onset, a subgroup of mice was thrombolysed (i.v. tPA), and another one was injected with saline.

Results: We determined the time-course evolution of the lesions by first measuring the lesion volumes by MRI at early times (+2h30) post-ischemia. We observed higher lesion volumes after both types of alcohol consumption and a protective effect of thrombolysis in alcoholised animals at early times post-ischemia. We next measured final lesion volumes (+24h post-ischemia) by histological analysis. Mice receiving alcohol chronically showed higher final lesion volumes than control animals (+50%), possibly through an increase in reactive oxygen species production by NADPH oxidase in the brain. Binge-drinking animals showed final lesion volumes slightly higher to those of control animals (30% increase), although this difference was not statistically significant. As expected, thrombolysis reduced final lesion volume in control animals (-65%). Thrombolysis also reduced final lesion volumes in chronic animals, but still, brain damages

were significantly bigger than in control-thrombolysed animals. Finally, tPA completely lost its beneficial effect in binge-drinking animals.

We observed a decrease in LRP and PAI-1 levels in the liver of alcoholised mice. Moreover, near-infrared fluorescence imaging showed higher levels of fluorescent tPA in the brain (iv injection) after alcohol consumption than in control mice. We thus hypothesize that alcohol could reduce the liver clearance of tPA and/or increase tPA's passage into the brain: tPA would then remain longer in the blood and could provoke deleterious effects on brain parenchyma.

Conclusion: Previous alcohol consumption has a deep impact on the progression and final extent of ischemic lesions, as well as on the efficiency of thrombolysis. Here we report the first evidence showing that different patterns of alcohol consumption represent specific sub-populations of stroke patients, for whom acute treatment must be adjusted accordingly.

PP III-26

Combination preconditioning/drug-cocktail required to reverse ceilings of neuroprotection against prolonged OGD

J. Tauskela, J.-P. Thivierge and G. Mealing
National Research Council Canada, Institute for Biological Sciences, Ottawa, Canada

Introduction: Clinical stroke trials conducted to assess neuroprotective drug candidates have failed. Issues primarily of a methodological, biomarker and patient stratification nature have been suggested to improve the performance of clinical trials, as well as improvements in *in vivo* animal models of stroke (STAIRS). *In vitro* models of cerebral ischemia have identified numerous targets and potential therapeutics, but it is not clear how to choose a candidate therapeutic, or why/how combination therapy may be useful. A framework at the *in vitro* model level is required to predict candidate therapeutics providing the highest therapeutic index for translation.

Methods: Neuroprotection was tested in cultured cortical neurons subjected successive increases in the duration of oxygen-glucose deprivation (OGD), well beyond that required to kill neurons (65 min).

Results: Blocking excitotoxicity post-OGD (65 min) provided maximal but limited ($\sim$50%) protection, so some combination of pre-emptive preconditioning and/or acute treatment is required. Diverse preconditioners were screened. Only 10 of 36 preconditioners protected neurons up to (but not beyond) 95 min OGD; remarkably, OGD tolerance correlated with a common ability to suppress a rise in extracellular glutamate. Applying an MK-801/CNQX/nifedipine cocktail at 90 min during OGD to preconditioned cultures preserved neurons up to 120 min OGD. A high-dose L689-560/NBQX/nifedipine cocktail, but not TRPM7 blockers, preserved neurons up to 150 min OGD. This framework outlines what is required for neuroprotection based on toxicity assays, but not what may be clinically possible; i.e., can neurons *tolerate* treatments and is network *function* after OGD truly preserved? These priorities are being addressed using multi-electrode arrays (MEAs) to examine spatiotemporal patterns of network activity and functional connectivity in a chronic and non-invasive manner. Examinations of interactions across pairs of 60 electrodes identified network hubs, which relay information between otherwise isolated regions of the array; planned work will examine if neuroprotection alters the distribution of hubs.

Conclusions: A new framework is proposed: preconditioning buys time before requiring acute intervention during OGD. Key concepts discovered include: (i) multiple ceilings of neuroprotection; (ii) overcoming these ceiling requires combination therapy; (iii) a common end-effector in preconditioning acting at the presynaptic level; (iv) postsynaptic receptor blockade rescue during OGD; (v) a single target, excitotoxicity, must be blocked in an increasingly aggressive manner during an OGD continuum. MEAs will be used to determine therapeutic indices at the *in vitro* OGD model level.

PP III-27

12/15-lipoxygenases play a key role in AIF mediated neuronal cell death induced by oxidative stress

S. Tobaben[a], A. Dolga[a], J. Grohm[a], U. Mamrak[b], M. Conrad[c], N. Plesnila[b] and C. Culmsee[a]
[a]*Philipps-Universität, Institut für Pharmakologie und Klinische Pharmazie, Marburg, Germany*
[b]*Royal College of Surgeons in Ireland (RCI), Departement of Neurodegeneration and Department of Physiology, Dublin, Ireland*
[c]*Helmholtz Zentrum, Institute of Clinical Molecular Biology and Tumour Genetics, München, Germany*

Objectives: This study investigates the role of lipoxygenases (LOX) in reactive oxygen species (ROS) formation and cell death signaling induced by glutamate in a neuronal cell line (HT-22) and in primary cultures of embryonic neurons.

Material and methods: ROS and lipidperoxides were determined using the fluorescent dyes DCF (dichlorodihydrofluoresceine diacetate) and BODIPY (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid). Cell viability was measured using AnnexinV/propidium iodide staining, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and impedance measurements (xCELLigence system). Protein analysis was performed using western blots, transfections and immunostainings. In vivo we performed middle cerebral artery occlusion (MCAO) in mice.

Results and conclusions: Treatment with 12/15-LOX inhibitor PD146176 prevented lipid peroxidation and protected the cells against glutamate toxicity. Investigation of the mitochondrial morphology in HT-22 cells showed that inhibition of 12/15-LOX prevented mitochondrial fragmentation. Furthermore, the 12/15-LOX inhibitor PD146176 prevented translocation of the apoptosis-inducing factor (AIF) to the nucleus after glutamate exposure. In primary cortical rat neurons the 12/15-LOX inhibitors reduced the second increase in intracellular calcium concentration following glutamate exposure and significantly attenuated glutamate-induced cell death. Further, the 12/15-LOX inhibitors significantly reduced cell death induced by oxygen glucose deprivation (OGD). In vivo 12/15-LOX inhibition decreased the infarct area and significantly improved the neuroscore after MCAO in mice.

In conclusion, our data suggest that activation of 12/15-LOX is an important step in neuronal cell death signaling upstream of AIF translocation from mitochondria to the nucleus. Therefore, inhibition of 12/15-LOX could be a promising therapeutic approach reducing cell death in neurological diseases.

PP III-28

Procaine action mechanisms able to counteract cell death processes

I. Turcu, A. Diaconeasa, R.I. Trascu and L. Spiru
Ana Aslan International Academy of Aging, Research and Development, Bucharest, Romania

Objectives: For more than six decades a whole bunch of clinical and preclinical data documented the beneficial effects of different procaine conditioning formulations in age delaying and aging-related diseases. Unfortunately, these data are actually shadowed by worldwide drug market tribulations. Among them, procaine action mechanisms (especially at cellular and molecular level) are lesser understood. Some of these mechanisms seem to be able to protect cells against the cascade of intracellular phenomena following hypoxia.

Material and methods: Preclinical pharmacokinetic studies with double-labeled procaine allowed us to build up the "procainome" (i.e. the in vivo hydrolysis cascade of the procaine molecule and the relationships that exist between its byproducts). Extensive preclinical pharmacodynamic investigations were able to complete the whole panel of these action mechanisms.

Results: The procaine-induced counteraction of natrium pump activity seems to have beneficial effects against glial edema. The well-argued antagonistic effect against free radical formation seems to be connected with a certain amount of protection for DNA, cytoskeleton and protein integrity. Modulation of intracellular, calcium-related events (also well-documented) correlates with the preservation of intracellular calcium homeostasis and furthermore with improvements of receptor dysfunctions; a certain degree of membrane integrity and functionality protection was also noted. Procaine's beneficial effects against excessive glutamate release add supplemental beneficial effects to the whole range of protective mechanisms at cellular level.

Other remarkable procaine interventions seem to be derived from interacting with mitochondrial activity and with the cell energy balance; moreover procaine was proven as a modulator of endogenous methylation processes.

Conclusions: Although many studies seem to validate such conclusions, further studies and thorough research is needed in order to provide important insights for designing new, procaine based drugs. Such drugs would then be expected to act by protecting and supporting normal cell physiology – which is almost always threatened, regardless of the underlying disorder.

Such research would further allow not just therapeutic, curative interventions but also effective preventive measures against quality-of-life-threatening, neurodegenerative disorders.

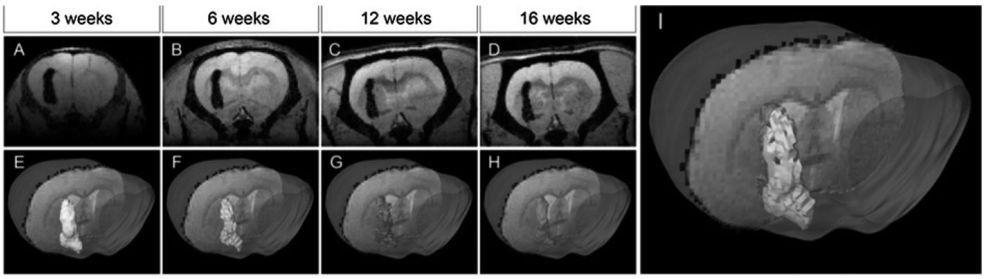

Fig. 1. A-D: Coronal FLASH images from an animal at 3, 6, 12, and 16 weeks post transplantation of SPIO labeled NSCs. E-H: Corresponding three dimensional reconstructions of the graft at each time point.

Topic: Imaging and Cell Tracking

PP II-7

Long-term tracking of human neural stem cells in the neonatal rat brain

T. Farr[a], T. Kallur[a], P. Böhm-Sturm[a], Z. Kokaia[b], M. Hoehn[a]
[a]*Max-Planck Institute for Neurological Research, In Vivo NMR Lab, Cologne, Germany*
[b]*Lund University, Stem Cell Centre, Lund, Sweden*

Objectives: Despite extensive research, sufficient knowledge is lacking regarding the best approach to achieve successful stroke therapy in humans with neural stem cells (NSCs). While detailed immunohistochemical characterization of grafted cell characteristics is necessary, one strategy for long-term noninvasive monitoring of cell grafts is magnetic resonance imaging (MRI). While many groups have grafted several different types of rodent derived cells labeled with superparamagnetic iron oxide (SPIO) nanoparticles into the ischemic rodent brain, few have employed human sources of cells, and most studies have relied only on simple iron stains to detect the label. The aims of the present study were to explore the human NSC response to the contrast agent, and the feasibility of the labeling strategy for long-term monitoring.

Materials and methods: NSCs were derived from the human fetal striatum and labelled with SPIOs (Endorem®). Labelled cells were allowed to proliferate or differentiate *in vitro* and immunocytochemistry was performed for several cell markers and the dextran coating on the SPIOs. Subsequently, labelled NSCs were grafted into the striatum of neonatal rats (post natal day 3) and T_2, T_2*, and FLASH images were acquired for up to 16 weeks with corresponding histology at each timepoint.

Results: The human NSCs readily incorporated SPIOs without lipofectant agents. Proliferation, survival and differentiation capacities *in vitro* were unaffected by the label. In grafted animals, a hypointense signal that corresponded to the graft location was observed at all timepoints (Fig. 1). However, there was a reduction in graft size over time as well as a slight decrease in T_2 values in the graft site. Immunohistochemistry confirmed the existence and survival of transplanted NSCs at all timepoints. The numbers of GFAP+ cells remained constant at all timepoints, whereas the numbers of nestin+ and DCX+ cells were reduced by 16 weeks. However, almost none of the grafted cells at any timepoint were Ki67+.

Conclusion: SPIOs do not change basic NSC properties*in vitro* and, when transplanted *in vivo*, can be monitored for several months with no adverse effects on cell phenotype. It is now feasible to extend these methods to the ischemic rodent brain.

PP II-9

Ischemia-induced blood-brain barrier damage after embolic stroke in rats: Quantification and histochemical typing of affected tissue

W.Härtig[a], J. Grosche[a], J. Pelz[a,b], D. Schneider[b], C. Weise[a,b], U. Bauer[a], J. Kacza[c], U. Gärtner[a], C. Hobohm[b] and D. Michalski[2]
[a]*University of Leipzig, PFI, Leipzig, Germany*
[b]*University of Leipzig, Dept. of Neurology, Leipzig, Germany*
[c]*University of Leipzig, Vet. Med., Dept. Anat., Histol. and Embryol., Leipzig, Germany*

Objectives: Treatment strategies in acute ischemic stroke are still limited, despite numerous efforts focused on preclinical-to-clinical translation. After failure of these attempts, human-like animal models are of steadily growing importance. In addition, a more-complex perspective of tissue salvaging involves endothelial, glial and neuronal components according to the neurovascular unit (NVU) concept. Blood-brain barrier (BBB) alterations – as a main consequence of ischemia – lead to brain edema and hemorrhagic transformation affecting NVU components. Here, we present a novel quantification method of BBB damage and affected tissue following experimental cerebral ischemia.

Material and methods: Wistar rats underwent embolic middle cerebral artery occlusion. Four hours or one day after induction of focal cerebral ischemia, fluorescein isothiocyanate (FITC)-tagged albumin and biotinylated rat IgG were administered intravenously as BBB permeability markers. One hour after injection, rats were sacrificed; 30 μm-thick serial frozen sections were applied to (i) the enhancement of FITC-albumin by carbocyanine (Cy)2-anti-FITC combined with the immunodetection of biotinylated rat IgG using Cy3-streptavidin, (ii) the immunohistochemical conversion of FITC-albumin and biotinylated rat IgG into a permanent diaminobenzidine label, and (iii) the concomitant staining of FITC-albumin, biotinylated rat IgG and neural markers revealed by confocal laser-scanning microscopy.

Results: FITC-albumin and biotinylated rat IgG displayed similar leakage and allowed the quantification of BBB permeability by fluorescence microscopy and at light-microscopical level. NVU components were revealed by using the following markers: Rat endothelial cell antigen-1 (RECA) and laminin for vessels, the lectins from tomato (*Lycopersicon esculentum* agglutinin; LEA) and *Griffonia simplicifolia* agglutinin (GSA) for vessels and microglial subpopulations, ionized calcium binding adaptor molecule 1 (Iba1), CD68 and CD11b for activated microglia, neutrophils and macrophages, S100ß for astroglia, as well as NeuN and HuC/D for neurons.

Conclusions: As a novel approach we describe the simultaneous application of FITC-albumin and biotinylated rat IgG as BBB permeability markers with different molecular weight and high physiological significance, when given after experimental stroke. The presented protocol also allows to quantify the volume of tissue with affected BBB. Furthermore, protocols for concomitant fluorescence labelling of BBB permeability markers and NVU components were established. These newly elaborated techniques might facilitate a more-complex outcome measurement for therapeutical approaches in cerebral ischemia.

PP II-10

In vitro and in vivo detectability of iron labeled stem cells with susceptibility and T2* weighted imaging at 3T.

D. Lobsien[a], A. Dreyer[b], J. Boltze[b] and K.-T. Hoffmann[b]

[a]*University Clinic of Leipzig, Clinic for Diagnostic Radiology Department for Neuroradiology, Leipzig, Germany*

[b]*Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany*

Objective: We examined the detectibility of stem cells labeled with small iron oxide particles (VSOP) with a clinical 3T MR scanner with susceptibility and T2* weighted imaging.

Method and Materials: In vitro measurements were performed in 4 agarose phantoms, each consisting of 3 layers of VSOP labelled ovine stem cells (0; 100; 500 cells or 1,000; 10,000; 100,000 cells). MRI examinations were done at 3T with 6 SWI- and T2*-weighted sequences and 3 RF coils: 8 channel coil: T2 FLASH 3D SWI, TR 60, TE 20, 3 different voxel sizes ($0.37 \times 0.31 \times 0.15/0.6/1{,}2$mm), scan durations of approx. 4h, 50min, 21min. 12 channel coil: T2 FLASH 3D SWI, TR: 40, TE: 20, voxel size $0.39 \times 0.31 \times 0.7$mm, scan duration 32 min. T2* FLASH 3D, TR: 6.2, TE: 20, voxel size $0.39 \times 0.31 \times 0.70$mm, scan duration approx. 9h. Loop coil (diameter 4cm): T2* FLASH 3D, TR 620, TE 20 and T2 FLASH 3D SWI, TR 60, TE 20, voxel size of $0.24 \times 0.2 \times 0.4$ mm, scan durations approx. 8.5h, 4.8h. Images were analyzed by four raters (neuroradiologists) blinded for MR-sequences and stem cell concentrations, and rated for detectability according to a standardized four-step scale. In vivo experiments were carried out in a sheep model. 100,000 and 1,000 stem cells were stereotactically injected into the deep parietal white matter and MRI was performed in vivo under general anasthesia applying both a T2* and a susceptibility weighted sequence, according to the in vitro measurements: 4 channel flexcoil, T2 FLASH 3D SWI, TR: 60, TE: 20, voxel size $0.56 \times 0.39 \times 0.25$mm, scan duration 1h29

min; T2* FLASH 3D, TR: 620, TE: 20, voxel size 0.83 $\times$ 0.66 $\times$ 0.5mm, scan duration 2h7min.

Results in vitro: 100,000 cells were confidently detectable in all MR-sequences. 10,000 cells were confidently detected with SWI with 0.6mm and 0.15mm slice thickness (8 channel RF coil) and with T2* (12 channel RF coil). Confidently visible were 1000 cells when examined with SWI with 0.15mm slice thickness (8 channel RF coil) and with T2* (both 12 channel and small loop RF coils). 500 cells were confidently visible with SWI with 0.15mm (8 channel RF coil), however, this sequence showed a false positive visibility at a concentration of 0 cells as well. No sequence definitely supported the detection of 100 cells. 0 cells were "definitely not visible" in no sequence. In vivo: Both 100,000 and 1,000 cells could be detected with T2* and with susceptibility weighted imaging.

Conclusion 1,000 VSOP labelled stem cells could be confidently detected with SWI- as well as T2*-weighted MRI at 3T in vitro. An in vivo experiment in a sheep model showed detectability of 1,000 stereotatically injected cells as well. The results indicate the usability of this models to monitor the behaviour of labeled stem cells in experimental stroke therapy.

PP II-11

Dynamic, semi-quantitative optical imaging of intracellular ros levels and redox status in hippocampal neurons

F. Funke, F. Gerich and M. Müller
Universität Göttingen, Zentrum Physiologie und Pathophysiologie, Göttingen, Germany

Objectives: The cellular redox status is determined by various extra- and intracellular factors, and contributes to cytosolic signaling as well as oxidative stress. Especially mitochondria modulate the cytosolic redox balance by oxidizing NADH and $FADH_2$ and generating reactive oxygen species (ROS). Whereas cellular NADH and FAD levels can be monitored reliably as autofluorescence, quantifying cellular ROS production is more demanding because the various redox-sensitive dyes share major disadvantages such as irreversible oxidation, autooxidation and photosensitivity. As an alternative, we therefore took advantage of a genetically engineered redox-sensitive green fluorescent protein (roGFP1) and carefully evaluated its response properties.

Materials and methods: The genetically encoded redox-sensitive optical probe roGFP1 was developed at the University of Oregon by the group of S. J. Remington. It responds reversibly to oxidation/reduction and is ratiometric by excitation (395 nm/ 470 nm), thereby enabling semi-quantitative analyses of cytosolic ROS levels and redox status. So far, roGFP1 has mostly been used in various cell lines but only rarely in primary cultured neuronal preparations. Therefore, we now elucidated its response properties in rat hippocampal neurons, performing ratiometric CCD-camera imaging as well as 2-photon microscopy. Dissociated cell cultures and organotypic slice cultures were transiently transfected with roGFP1 expressing vectors using electroporation or lipofectamine.

Results and conclusions: Cytosolically expressed roGFP1 readily responded to hydrogen peroxide, superoxide as well as hydroxyl radicals, and calibration of its response range allowed for the direct comparison of different preparations. Furthermore, roGFP1 was only negligibly affected by changes in intracellular pH or Cl^- content and well suited also for 2-photon excitation. The roGFP1 was sufficiently sensitive to detect changes in endogenous ROS production during impaired mitochondrial respiration (hypoxia, rotenone, 3NPA, antimycin-A, cyanide, FCCP) or neuronal stimulation (glutamate, 50 mM K^+) and to visualize the formation of perimitochondrial ROS microdomains. We conclude that roGFP1 is well suited for dynamic, compartment specific, subcellular analyses in even complex neuronal networks such as organotypic slice preparations. The ability to correlate dynamic changes in cellular ROS content with mitochondrial metabolism and neuronal network activity is an absolute requirement for a detailed mechanistic understanding of redox and ROS signaling in normal and diseased brain function.

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PP II-12

In vivo imaging after stroke investigated by intracranial 2-Photon microscopy

M. Riek-Burchardt[a], J. Neumann[b], G. Orlando[c], M. Gunzer[c], K.G. Reymann[a,d]
[a]*Leibniz Institute for Neurobiology, Project group Neuropharmacology, Magdeburg, Germany*
[b]*Otto-von-Guericke University, Department of Neurology, Magdeburg, Germany*

[c]*Otto-von-Guericke University, Institute for Molecular and Clinical Immunology, Magdeburg, Germany*
[d]*Institute Applied Neurosciences gGmbH, Magdeburg, Germany*

The CNS damage caused by stroke is accompanied by an acute inflammatory answer. The inflammatory process during stroke consists of activation of resident brain microglia and recruitment of leucocytes, namely neutrophils and monocytes/macrophages. However, just what is being sampled by these microglial processes has not been demonstrated *in vivo*, and the nature and function of any interactions between microglia and the invading immune cells is incompletely understood. Recently, we could identify a new neuroprotective mechanism of the CNS whereby microglia guards neurons by engulfment of toxic neutrophil granulocytes invading brain slices in an *in situ* stroke model. In our current work we investigate the *in vivo* relevance of these findings. For induction of cerebral ischemia we use a model of permanent middle cerebral artery occlusion combined with an occlusion of the common carotid arteries for 20 min. Crossing Lys-EGFP (green fluorescent neutrophils) mice with CX3CR1-EGFP (green fluorescent microglia) mice allows the visualization of both cell types in the same animal, using large differences in morphology and migration characteristics as a marker for the identification despite similar colour. We investigate the post-ischemic changes of microglia/neutrophil morphology and behaviour using intracranial live imaging via two-photon microscopy at different time points (6, 12, 24 and 48h) after the insult. The technical possibilities of two-photon fluorescence microscopy enable a look, up to 500μm into live tissue.

Our examinations demonstrate that the described method is very powerful to investigate the *in vivo* situation of post-ischemic microglial activation and neuroimmune cross-talk. We observed an early extravasation of neutrophils after ischemia. In the first 24 h local microglia changed their morphology dramatically. We will present the first post-ischemic *in vivo* studies from different microglial activation phases with time-lapse movies directly from the infarct area.

Intracranial *in vivo* 2-photon microscopy is a useful method to investigate immune cell interactions directly within vital tissue. These imply a new quality and new possibility to examine the inflammation processes in the brain after cerebral ischemia.

PP II-13

In vivo monitoring of tissue viability and lesion growth in focal cerebral ischemia in rats using ^{201}TlDDC small-animal SPECT

M. Zappe[a], U.H. Schröder[a], O. Großer[b], H. Amthauer[b], H. Scheich[c], K.G. Reymann[a], J. Goldschmidt[c]
[a]*Leibniz Institut for Neurobiology, Project Group Neuropharmacology, Magdeburg, Germany*
[b]*Otto v. Guericke University, Clinic for Radiology and Nuclear Medicine, Magdeburg, Germany*
[c]*Leibniz Institut for Neurobiology, Auditory Learning & Speech, Magdeburg, Germany*

In vivo monitoring of lesion size and lesion growth in focal cerebral ischemia in rodents is usually done using magnetic resonance imaging. But the diffusion- and perfusion-weighted images obtained with MRI cannot provide direct information on tissue viability in acute cerebral ischemia. In particular, it has remained highly controversial whether or to what degree irreversible damage in the ischemic core can be detected using diffusion weighted imaging. We introduce a novel method for in vivo monitoring of tissue viability and lesion growth in focal cerebral ischemia in rodents, based on high-resolution SPECT-imaging of brain potassium metabolism using the thallium isotope ^{201}Tl as a tracer.

We induced cerebral ischemia in rats by endothelin-mediated reversible occlusion of the middle cerebral artery. After induction of ischemia rats were intravenously injected with the lipophilic chelate complex ^{201}thallium diethyldithiocarbamate (^{201}TlDDC) and the time course of the ^{201}Tl distribution was monitored with a dedicated small-animal SPECT/CT-scanner. In addition, we used a histochemical technique – a modified Timm-technique or autometallographic method – for mapping, with cellular resolution, the thallium distribution in the brain.

We provide evidence, using the histochemical technique and in vivo monitoring of ^{201}Tl redistribution, that Tl^+ is released from TlDDC into the brain extracellular space, from which neurons and glial cells take up the tracer. Upon MCAO induction with both methods we find a core region, in which Tl^+-uptake is reduced to background levels. This core region expands over time and can be monitored with submillimeter spatial resolution in vivo using small-animal multipinhole SPECT-imaging.

We conclude from these findings that ^{201}TlDDC small-animal SPECT can be used for in vivo monitor-

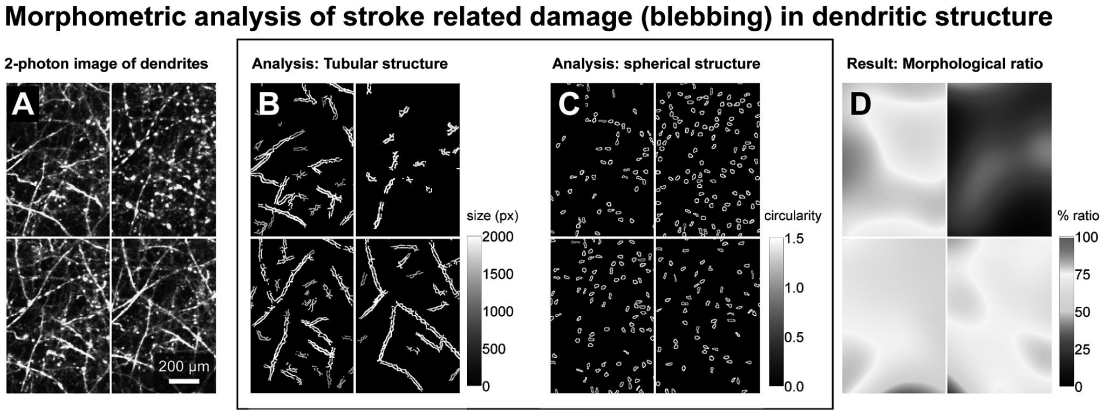

Fig. 1. Quantification of the degree of structural damage in neurons (blebbing) by determination of the morphological ratio. A, Two-photon images of YFP labeled dendrites recorded *in vivo* in a mutant mice before (upper left panel), during (upper right panel) and 2 min/10 min after (lower panels) transient ischemia. B, Projection of identified tubular and C, spherical structure across multiple thresholds. D, Two-dimensional polt of the ration of local densities of pixels that are edge of tubular and spherical structure, defining the morphological ratio.

ing of tissue viability and lesion growth in cerebral ischemia. Tl^+ is a well-established K^+-analogue, and K^+-uptake as well as the maintenance of intra- to extracellular K^+-gradients are closely linked to cellular viability. The use of ^{201}Tl for imaging brain potassium metabolism, however, was limited due to the poor blood-brain barrier K^+-permeability. Application of ^{201}TlDDC, from which $^{201}Tl^+$ is released after bypassing the blood-brain barrier, makes it possible to use ^{201}Tl for SPECT-imaging of tissue viability in the brain in the same manner as has been done previously in myocardial imaging.

Small-animal multipinhole SPECT-imaging can be performed at higher spatial resolution than small-animal PET-imaging. In addition, due to the long half-life of ^{201}Tl (73h) the spatiotemporal patterns of ^{201}Tl redistributions can be monitored, depending on the dose injected, over a period of about three days after a single injection of ^{201}TlDDC. ^{201}TlDDC small-animal SPECT is a convenient metabolic imaging technique that offers substantial advantages over both PET and MRI in assessing tissue viability in rodent models of cerebral ischemia *in vivo*.

PP II-14

Imaging morphological changes and redistribution of cortical activity after acute ischemic damage by targeted ministrokes in mice

A. Sigler[a,b,c] and T. Murphy[a]
[a]*University of British Columbia, Kinsmen Laboratory for Neurological Research, Vancouver BC, Canada*
[b]*MPI for Experimental Medicine, Molecular Physiology and Imaging, Göttingen, Germany*
[c]*Universität des Saarlandes, Institut für Physiologie, Homburg, Germany*

After damage by an ischemic stroke, the function loss is not necessarily irreversible because of the brain's ability to redistribute function. Typically, related brain regions take over the function of lost regions within weeks to months. To address whether patterns of brain activity could in part redistribute more rapidly through pre-existing pathways, we examined in mice patterns of sensory-evoked and spontaneous within the first two hours after stroke that we induced in a subregion of the somatosensory cortex by photothrombosis with Rose Bengal. We determined neuronal activity using the voltage sensitive dye RH1692 and fast epifluorescence imaging of the brain surface. For quantitative determination of neuronal tissue damage, we imaged with *in vivo* two-photon microscopy the fluorescence of YFP labelled neurons in C57BL/6 transgenic mice before and after ischemia. We examined characteristic stroke-related changes in dendritic morphology (blebbing) to assess the spatial distribution of the neurons' degree of structural integrity using computer-based morphometric tools. After stroke, responses to forelimb sensory stimulation became re-centred by 200–500 μm towards the adjacent hindlimb regions. Within the forelimb map spared by stroke, forelimb-stimulated responses became delayed in kinetics and tended to invade other sensory territories at prolonged points after stimulation. We conclude that the focus of forelimb-specific

somatosensory cortex activity can be in part rapidly redistributed after ischemic damage, most likely through pre-existing synaptic pathways given that this can occur within hours.

PP II-15

Lipid-coated iron oxide: A multimodal nanoparticle for cellular imaging

G. van Tilborg[a], W. Mulder[b], S. van der Pol[c], L. van Bloois[d], A. van der Toorn[a], G. Storm[d], E. de Vries[c] and R. Dijkhuizen[a]
[a]*University Medical Center Utrecht, Image Sciences Institute, Utrecht, Netherlands*
[b]*Mount Sinai School of Medicine, Translational and Molecular Imaging Institute, New York, United States*
[c]*VU University Medical Center, Department of Molecular Cell Biology and Immunology, Amsterdam, Netherlands*
[d]*Utrecht University, Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, Netherlands*

Introduction and objective: In recent years, various studies have shown that labeling of leukocytes with superparamagnetic iron oxide particles (SPIO) enables MRI of neuroinflammation after experimental stroke [1]. Labeling of leukocytes has been performed via several approaches, including intravenous injection of the superparamagnetic material. Using this approach it is claimed that circulating leukocytes engulf the nanoparticles and infiltrate the brain after stroke. However, others have suggested that contrast enhancement may at least partly originate from extravasation of SPIO through impaired blood-brain barrier [2]. Additional fluorescent labeling of MRI contrast agents would enable detection of the true origin of SPIO-induced MRI contrast, as this allows for identification of labeled cells by means of flow cytometry or fluorescence microscopy. Therefore, the objective of this study is to develop a novel methodology for simultaneous magnetic and fluorescent cell labeling using a single nanoparticulate agent.

Materials and methods

Contrast agent: Multiple iron oxide nanocrystals ($\sim$ 5 nm) were incorporated in a lipid monolayer composed of PEG2000-DSPE/DSPC/rhodamine-PE. The resulting nanoparticle had an average hydrodynamic diameter of 109 nm.

Cell labeling: Murine macrophages (RAW) were incubated with the contrast agent containing final concentrations of 0, 50, 100 and 200 mg Fe/ml. Cells were incubated for 3 hours, followed by MRI, flow cytometry and fluorescence microscopy measurements. Particle-induced toxicity was evaluated using an MTT assay, while a 1,10-phenantroline colorimetric assay for iron was performed to quantify cellular uptake of the nanoparticles.

Results: RAW cells were successfully labeled with the lipid-coated iron oxide particles, which allowed their detection with both MRI and optical techniques. Signal-to-noise ratio on T_2-weighted MR images decreased with increasing incubation concentrations, as a result of an increase in intracellular iron content. Consequently, the detection limit of labeled cells with MRI was reduced for cells with high nanoparticle content. MRI results were corroborated by fluorescence microscopy and flow cytometry. Importantly, no particle-induced toxicity was observed within the used range of incubation concentrations.

Conclusions and discussion: Lipid-coated iron oxide particles allow for simultaneous magnetic and fluorescent labeling of RAW cells, without inducing toxicity. The approach is facile and flexible as it allows adjustment of particle size and surface properties, such as charge and PEG content. This can be used to improve nanoparticle uptake and pharmacokinetics. In conclusion, our study demonstrates that lipid-coated iron oxide particles represent an attractive, versatile and potent contrast material for simultaneous fluorescent and magnetic labeling of leukocytes.

Reference

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[2] R. Oude Engberink et al., JCBFM 2008.

Topic: Inflammation and Microglia

PP III-29

Cross-talk between peroxisome proliferators activated receptors (PPARs) and their ligand-synthesizing enzymes, Ca^{2+}-dependent phospholipase A_2 and cyclooxygenase-2.

S. Aleshin[a], M. Sergeeva[b] and G. Reiser[a]
[a]*Medizinische Fakultät der Otto-von-Guericke Univer-*

sität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany
[b]*Moscow State University, Belozersky Institute of Physico-Chemical Biology, Moscow, Russian Federation*

Objectives: Synthetic agonists of peroxisome proliferator-activated receptor (PPAR) are promising therapeutic drugs for inflammatory diseases, also in the brain. However, inflammatory activation of phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2) induces synthesis of endogenous ligands of PPAR, which may interfere with exogenous therapeutic ligands for PPAR activity. Previously we described cross-talk between synthetic PPAR ligands, and their influence on PPAR expression levels [1]. Nevertheless, the cross-talk between endogenous PPAR ligands and PPAR expression levels and functions is still unclear. We examined this question in naïve and lipopolysaccharide-(LPS)- stimulated rat primary astrocytes, using COX-2 expression as PPAR-dependent index of inflammation.

Materials and methods: All experiments were performed on naïve and LPS-stimulated primary rat astrocytes, protein quantification was performed by Western blot, activity of PPAR isoforms by EIA-based PPAR α, δ, γ Complete Transcription Factor Assay Kit, mRNA levels of PLA_2 and COX-2 by Real-time PCR, all as described [1].

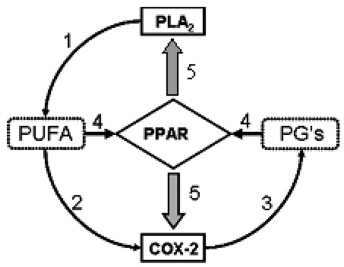

Fig. 1. Proposed schema of PPAR ligand synthesizing enzymes involvement in regulation of PPAR function. PLA_2 produce polyunsaturated fatty acids (PUFAs) (pathway 1), which are a substrate for COX-2 (pathway 2). COX-2 converts PUFAs to prostanoids (PGs) (pathway 3). PUFAs and PGs both act as PPAR agonists and activates different isotypes of PPAR (pathway 4), these activation leads to changes in PLA_2 and COX-2 expression levels (pathway 5).

Results: Inhibition of PLA_2 changes expression levels of PPAR isotypes. Moreover, activation of PPARg by rosiglitazone potentiated the LPS-induced COX-2 expression. This potentiation was eliminated after PLA_2 or COX-2 inhibition, but was again recovered by PPARb agonist L-165041. Thus, L-165041 mimicked the effect of endogenous PPAR ligand, released by PLA_2 and COX-2. PPARb agonist L-165041 did not induce COX-2 expression in control cells, only in rosiglitazone-pretreated LPS-stimulated astrocytes. This difference is due to the fact that rosiglitazone causes significant increase in PPARb level in LPS-stimulated astrocytes. Thus, we conclude that PPARg increases COX-2 expression in LPS-stimulated astrocytes via increasing PPARb level.

Conclusions: Results, presented in this study, as summarized in Fig. 1, demonstrate an important feedback loop between PPARs and their ligand-synthesizing enzymes, when ligands, produced by these enzymes and regulate their expression via PPARs.

Reference

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PP III-30

The brain-specific protein p42^{IP4} (ADAP1) and shedding of amyloid precursor protein (APP) via the interaction-partner nardilysin and interaction of p42^{IP4} with nardilysin, which is enhanced by tubulin

C. Borrmann, R. Stricker and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: Neuronal protein p42^{IP4} (ADAP1) can be found in neuritic plaques in Alzheimer disease brains. The interaction of p42^{IP4} and the metalloendopeptidase nardilysin (NRD) was investigated biochemically and functionally in SH-SY5Y-cells. Previously, it was reported that NRD enhances the α-cleavage of amyloid precursor protein (APP) by the TNF-α-converting enzyme. This leads to reduced production of Amyloid – β peptide (Aβ). The interaction of NRD alone with β-tubulin was reported. We previously found an interaction of p42^{IP4} with α-tubulin. We were therefore interested to study whether the interaction of p42^{IP4} and NRD with tubulin might have

an impact on the direct interaction between p42^{IP4} and NRD. We recently found that p42^{IP4} could influence the catalytic activity of full-length NRD. This prompted us to study whether NRD-mutants, lacking important catalytic motifs, have different binding properties to p42^{IP4}.

Materials and methods: Culturing of neuroblastoma cells (SH-SY5Y), Western Blot, Aβ40-ELISA, Far-Western Blot, tubulin polymerization, nocodazole treatment and confocal microscopy were carried out by standard methods. Western Blot detected changes in the neuroprotective soluble α-fragment of APP (sAPPα-release), and Aβ40-ELISA measured Aβ-40 release

Results: Although the overexpression of p42^{IP4} can influence the time-dependent upregulation of NRD, this overexpression has no effect on the shedding of APP – sAPPα, as well as on Aβ40-release. SH-SY5Y-cells, stably transfected with p42^{IP4}-GFP, showed a co-localization of NRD, p42^{IP4} and tubulin in the cytosol and at the plasma membrane. Treatment with nocodazole did not change the co-localization of p42^{IP4} and tubulin. However, the co-localization of all three proteins is visible only in vesicular structures. Furthermore, we show here, using Far-Western Blot that polymerized tubulin enhances the binding of NRD to p42^{IP4}. For NRD mutants, where the Zn^{2+} – binding motif was changed, the binding to p42^{IP4} was also increased in the presence of tubulin. Although the mutant of NRD, lacking the acidic domain (DAC) is able to bind p42^{IP4} the addition of tubulin does not enhance the binding to p42^{IP4} in this case.

Conclusions: Shedding of APP via α-secretases results in the release of non-amyloidogenic sAPPα, and APP shedding via β-secretase (BACE1) leads to the formation of neurotoxic Aβ-peptides. Protein interaction partners of p42^{IP4} were shown to influence the α-secretase (NRD) as well as the β-secretase (Ran binding protein in microtubule-organizing center RanBPM). We found that p42^{IP4} does not directly influence the APP-shedding – it does not increase the Aβ-40 release and it does not enhance the sAPPα-release via α-secretase and NRD. Furthermore, the interaction of p42^{IP4} and NRD can be enhanced by tubulin. Additionally, p42^{IP4} does not exclusively bind to the DAC of NRD. Tubulin does not influence the binding of NRD-mutant, lacking the DAC, to p42^{IP4}, which points towards a binding position of tubulin outside the DAC. These results are most interesting findings, because they support the concept that the neuron-specific protein p42^{IP4} is an adaptor protein, which is regulated by its ligands. Since p42^{IP4} can bind directly to actin, the results shown here connect not only NRD and p42^{IP4} with tubulin but possibly also the actin cytoskeleton via p42^{IP4}.

PP III-31

Absence of mfg-e8 decreases apoptotic cell clearance, increase inflammation and worsens infarct lesions in focal permanent murine cerebral ischemia

N. Deroide[a,b], L.R. Nih[a], D. Lerouet[c], J. Vilar[d], C. Guerin[d], B. Lévy[b,e], I. Margaill[c], M. Pocard[a], Z. Mallat[d] and N. Kubis[a,b]
[a]*INSERM, U965, Paris, France*
[b]*Lariboisière Hospital, Physiology Department, Paris, France*
[c]*Paris Descartes University, EA 4475, Paris, France*
[d]*INSERM, U970, Paris, France*
[e]*Institut Vaisseaux Sang, Paris, France*

Objective: Systemic and local inflammatory processes play a key role, mainly detrimental, in the evolution of ischemic stroke. Deficient clearance of apoptotic cells after ischemia leads to increased inflammation. Apoptotic cell phagocytosis by macrophages alters their cytokine expression towards an anti-inflammatory profile. Milk fat globule EGF-factor 8 (MFG-E8), or lactadherin, links phosphatidylserine of apoptotic cells to integrin αvβ3 and αvβ5 of macrophages and is a potent opsonin for the clearance of apoptotic cells. Moreover, MFG-E8 seems to have an important role in VEGF dependent neovascularisation. The aim of our study was to evaluate the role of MFG-E8 in cerebral ischemia, in terms of inflammation and angiogenesis, both crucially involved in stroke repair.

Material and methods: Permanent focal cerebral ischemia was induced by transtemporal coagulation of the middle cerebral artery in MFG-E8 $-/-$ and WT littermate mice (n = 7–8/group) and in recombinant MFG-E8 and vehicle treated C57Bl/6 mice (n = 9–10/group). Intra-cerebro-ventricular (i.c.v.) injection of recombinant MFG-E8 (1 μg/d) was realized daily from day 0 to day 6. Infarct volume (cresyl violet staining), apoptosis, apoptotic cell clearance (TUNEL and double CD68-TUNEL immunostaining), inflammatory response to ischemia (Iba1 immunostaining, and IL1β, TNFα, TGFβ and IL10 RT-QPCR analysis) and angiogenesis (double CD31-BrdU immunostaining) were all

assessed at day 7, except the RT-QPCR analysis (day 4).

Results: Infarct volume at day 7 was significantly increased in MFG-E8 $-/-$ mice, compared to WT mice (17.5 ± 2.8 vs. 12.6 ± 2.4 mm^3, $p < 0.05$, $n = 8$). Apoptotic cells were more numerous in MFG-E8 $-/-$ mice as shown by TUNEL staining (68.1 ± 14.6 vs. 44.1 ± 17.9 TUNEL+ cells/ROI, $p < 0.05$, $n = 7$). There was also an increase of the association between apoptotic cells (TUNEL+ cells) and microglia/macrophages (CD68+ cells) (22.1 ± 6.5 vs. 14.2 ± 4.7 /ROI, $p < 0.05$, $n = 7$), without internalization of apoptotic cells, suggesting a reduction of apoptotic cells clearance by default of engulfment. Microglia/macrophages infiltration was significantly increased in MFG-E8 $-/-$ mice (54.8 ± 11.2 vs. 40.2 ± 9.8 Iba1+ cells/ROI, $p < 0.05$, $n = 7$) associated with a pro-inflammatory cytokine profile with a significant rise of IL1β and TNFα ($p < 0.01$) and a decrease of TGFβ ($p < 0.05$). Angiogenesis was not significantly different between groups. Daily i.c.v. injection of recombinant MFG-E8 reduced infarct volume at day 7 (13.7 ± 3.2 vs. 17.2 ± 3.5 mm^3, $p < 0.05$, $n = 9$).

Conclusion: Our results show MFG-E8 is a key protein for apoptotic cell efferocytosis in murine cerebral ischemia. The absence of MFG-E8 induces a dysfunction of phagocytosis leading to a pro-inflammatory state and to an enlarged infarct lesion. The effects of i.c.v administration of MFG-E8 recombinant protein are currently under study. Preliminary results show a reduction of infarct volume after MFG-E8 administration.

PP III-33

Species differences in origin of reactive microglia

K.L. Lambertsen[a], T. Deierborg[b], R. Gregersen[a], B.H. Clausen[a], M. Wirenfeldt[a], H.H. Nielsen[a], I. Dalmau[a], N. Diemer[a], F. Dagnaes-Hansen[c], F.F. Johansen[d], A. Keating[e] and B. Finsen[a]
[a]*Institute of Molecular Medicine, Department of Neurobiology, Odense, Denmark*
[b]*University of Lund, Department of Experimental Medical Science, Lund, Sweden*
[c]*University of Aarhus, Department of Medical Microbiology and Immunology, Aarhus, Denmark*
[d]*University of Copenhagen, Department of Molecular Pathology, Copenhagen, Denmark*
[e]*Princess Margaret Hospital, Ontario Cancer Institute, Toronto, Canada*

Microglia serve homeostatic functions in normal CNS and they sense threats to and modulate neuronal function in the injured and diseased CNS. At present, our understanding of microglial biology and involvement in disease is influenced by the observation that exogenous immigrating bone marrow (BM)-derived cells may contribute to reactive microgliosis in the mouse. We set out to investigate possible species-differences in microglial recruitment and transformation of BM-derived cells into microglia in the injured CNS. We analyzed microglial origin in the brain of irradiated BM chimeric mice and rats subjected to transient global cerebral ischemia, known to elicit a predictable, strong microglial reaction. Both species displayed the characteristic microglial hyperplasia and rod cell transformation in the regio superior of the hippocampus 6–7 days after surgery. In the mouse a subpopulation of lesion-reactive microglia originated from transformed BM-derived cells, in contrast to BM chimeric rats, where no recruitment and microglial transformation of BM-derived cells were observed. The results suggest that reactive microglia in the rat originate from resident microglia, while they have a mixed BM-derived and resident origin in the mouse.

PP III-34

Effect of 710nm visible light irradiation on neuroprotection and immune function after experimental stroke

K.-H. Lee, D.-H. Choi, M.Y. Kim, M.R. Hasan, J.H. Lim and J. Lee
Konkuk University, Center for Geriatric Neuroscience Research, IBST, Seoul, Korea, Republic of

Objective: Immunosuppression after stroke is known to be controlled by regulatory T cell (Treg), which is reported to be related with reduction of secondary brain tissue injury and neuroprotection by the inhibition of inflammatory changes and immune reaction. On the other hand, immunosuppression is thought to induce generalized T cell mediated immunity that ends up with vulnerability to opportunistic infection. Recently, phototherapeutic effects of low level infrared laser irradiation (808nm) on brain neuronal cell protection after stroke have been reported. We previously

reported that 710nm wavelength visible light increased total lymphocyte counts in vivo, especially T lymphocytes, more selectively CD4+ T lymphocytes. Therefore, we investigated to determine the effects of 710nm visible light irradiation on neuronal protection and cellular immunity in stroke animal model.

Materials and methods: Male Wistar rats (8 weeks, 275–300 g, $n = 5\sim6$ each group) were subjected to 90 min middle cerebral artery occlusion (MCAo) followed by reperfusion and were randomly divided into two groups: no irradiation (MCAo control) and irradiation (MCAo+irradiation). Sham operated rats were also divided into control (Sham control) and irradiation group (Sham+irradiation). After establishing MCAO or sham operation irradiation groups were exposed to 710nm wavelength visible light right by light-emitting diode for 4 weeks. At the several time points, whole blood samples were collected from tail vain and activation of Treg and helper T cell were determined by flow cytometry. Infarct volume, neuronal cell death and synaptophysin immunoreactivity were measured.

Results: MCAo+irradiation group showed decreased infarct volume in comparison with MCAo control. Synaptophysin immunoreactivity was increased in irradiation groups (both Sham+irradiation and MCAo+irradiation groups). While population of CD4+ T-cell was decreased and population of CD8+ T-cell was increased after 14 days MCAo, population of CD4+ T-cell was increased in MCAo+irradiation group.

Conclusion: Our data suggest that 710 nm visible light irradiation may modulate cellular immunity and play a role in neuroprotection in stroke animal model.

PP III-35

Macrophage phenotypes in the injured rat central nervous system

C. Loy[a,b], J. Favaloro[a,b], V. Perreau[c], M. Porritt[a], M. Katz[a], S. Dieni[a], N. Kerr[a,b], P. Batchelor[a,b] and D. Howells[a,b]
[a]*University of Melbourne* (*Austin Health*), *Medicine, Heidelberg Victoria, Australia*
[b]*Florey Neurosciences Institute, Melbourne, Australia*
[c]*University of Melbourne, Melbourne, Australia*

Introduction. Macrophages, the first cells to respond to injury, have been studied extensively, and are recognized as highly dynamic and heterogeneous cells. They exhibit different phenotypes, changing and responding to environmental fluctuations. Macrophage behaviour has a dual role in the aftermath of injury. In the injured mammalian CNS, macrophages have been implicated in both exacerbating, and ameliorating damage to tissue at the injury site. They produce both pro-inflammatory and neurotrophic factors, and appear to support axonal sprouting.In the injured mouse striatum, axons sprout in the periphery of the macrophage-filled wound. The fibres course towards the wound edge, forming varicosities with microglia along the way. These sprouting fibres terminate in dense plexuses,within which lie macrophages. The fibres do not extend into the wound cavity, where macrophages of apparently identical morphology and immunoreactivity are found less than 100μm away. This suggests the presence of at least two macrophage phenotypes: one that supports regenerating fibres and one that does not.

Aims: The aims of this study are to (1) verify that different macrophage phenotypes exist after injury to the CNS in the rat, and (2) develop immunohistochemical markers of the different macrophage phenotypes.

Hypothesis: At least two different macrophage phenotypes exist within the injured CNS – a cytotoxic macrophage capable of causing cell death in the core of the wound, and a reparative macrophage capable of stimulating wound repair at the wound edge.

Methods: Adult male rats were given bilateral brain axonal injury using a Scouten wire-knife. These rats were perfused two weeks after injury with 4% paraformaldehyde. Tissue was processed for immunohistochemistry and laser microdissection. Ox42-positive macrophages located at the wound core or edge were laser-catapulted into Trizol. RNA was prepared from the two populations of cells and purified with a Qiagen RNeasy kit. cDNA was prepared using the Invitrogen Superscript III Amplification Kit and the gene expression profiles compared on Affymetrix Rat Genome 230 2.0 Array.

Results: 30000 genes were analyzed and many genes characteristic of macrophages were expressed as anticipated. 928 genes had a minimum 2-fold change in expression, between macrophage populations from the two locations. We identified potentially targetable families of genes. These included transcription factors and regulators of axonal growth.

Conclusion: The macrophages from two different locations at the wound exhibited considerable differential gene expression, even though they were isolated from locations less than 100μm apart. Limiting the development of cytotoxic or non-supportive macrophages,

or directing macrophage activation towards a growth-supportive phenotype immediately after trauma, is likely to increase axonal sprouting. We have shortlisted possible immunohistochemical markers that may identify, and differentiate between the supportive and non-supportive macrophages.

PP III-36

Tumour necrosis factor-α and tnf-α receptors in cerebral artaries following cerebral ischemia in rat

A. Maddahi and L. Edvinsson
Experimental Vascular Research, Clinical Sciences, Lund, Sweden

Objective: Tumour necrosis factor-α (TNF-α) is a pleiotropic pro-inflammatory cytokine, which is rapidly upregulated in the brain after injury. TNF-α is involved in increased production of vasoconstrictor receptors, induces expression of adhesion molecules in cerebral endothelial cells, increases release of matrix metalloproteinase and cytokines such as interleukin-6 (IL-6) and interleukin-1β (IL-1β) after cerebral ischemia. Mitogen activated protein kinase (MAPK) pathways, such as MEK-ERK1/2 pathway is involved in regulation of cytokine expression. The aim of this study was first to investigate induce expression of TNF-α and TNF-α receptors in cerebral arteries following global or regional ischemia, and after organ culture. Secondly, we asked if the enhanced expression was regulated via activation of the MEK-ERK1/2 pathway.

Material and methods: We used two *in vivo* models, subarachnoid haemorrhage (SAH) and transient middle cerebral artery occlusion (MCAO), and *in vitro* organ culture of isolated cerebral arteries. The localization and amount of TNF-α, TNF-R1, TNF-R2, and proteins were analyzed with immunohistochemistry using confocal microscopy and western blot analysis at 48 h after SAH or MCAO, and after 24 h and 48 h in organ culture.

Results: Immunohistochemistry revealed enhanced expression of TNF-α, TNF-R1 and TNF-R2 in the cerebral arteries wall after MCAO and SAH at 48 h when compared with their control groups. TNF-R1 and TNF-R2 were upregulated in the culture after 24 h and 48 h, and this upregulation was time-depended, reaching a maximum at 48 h of organ culture. Co-localization study revealed that TNF-R1 was localized in the cell membrane of the smooth muscle cells (SMC) of cerebral arteries while TNF-R2 was located in either the nucleus or the cell membrane in both endothelial and smooth muscle cells. Treatment with a specific MEK1/2 inhibitor (U0126) injected immediately after reperfusion in MCAO, reduced the enhanced expression of TNF-α.

Conclusion: The present study shows that both cerebral ischemia and organ culture induce the expression of TNF-α, and its receptors (TNF-R1 and TNF-R2) in the wall of cerebral arteries.

PP III-37

Time-dependent superoxide generation in nox2 knockout mice following middle cerebral artery occlusion and reperfusion

S. McCann, G. Dusting and C. Roulston
University of Melbourne, O'Brien Institute, Fitzroy, Australia

Objective: NADPH oxidase-derived reactive oxygen species (ROS) contribute to the progression of brain injury following stroke. Currently, thrombolysis to achieve reperfusion is the only approved therapy for ischaemic stroke. However reperfusion may contribute to excessive ROS generation in neurons, cerebral vessels and inflammatory cells, exacerbating brain injury. We examined the role of the Nox2 NADPH oxidase in the generation of the ROS superoxide following ischaemia and reperfusion in the brain using knockout mice.

Materials and methods: The middle cerebral artery was occluded in Nox2 $-/-$ and Nox2 $+/+$ mice for 60 min using an intraluminal filament. Separate groups of mice were allowed to recover for 6 h, 24 h or 72 h. Infarct size was evaluated using MCID analysis of unstained sections. Superoxide was detected *in situ* using superoxide-sensitive dihydroethidium (DHE) fluorescence. The types of cells generating superoxide were identified using double labelling with DHE and antibodies against cells including neurons and microglia.

Results: Following 6 h of reperfusion, DHE-detected superoxide was increased in the stroke-affected cortex and striatum of both Nox2 $-/-$ ($P < 0.001$) and Nox2 $+/+$ ($P < 0.001$) mice compared with the appropriate contralateral control regions. This increase was significantly greater in Nox2 $+/+$ mice than Nox2 $-/-$ mice ($P < 0.001$). Infarct volume was not different between Nox2 $+/+$ (12.9 ± 4.3 mm^3) and Nox2 $-/-$ ($9.5 \pm$

2.1 mm^3) mice at 6 h post-stroke ($P = 0.471$). There was however significantly greater infarct in Nox2 $+/+$ (35.6 ± 4.3 mm^3) than Nox2 $-/-$ (15.1 ± 3.1 mm^3) mice at 24 h post-stroke ($P < 0.01$). After 24 h of reperfusion, superoxide had returned to control levels in all regions except for the cortex of both Nox2 +/+ ($P <0.001$) and Nox2 $-/-$ ($P < 0.05$) mice; however there was no difference between genotypes. After 72 h of reperfusion superoxide was increased to a lesser degree in the cortex of Nox2 $+/+$ mice ($P < 0.01$) and decreased in the striatum ($P < 0.05$). There was no difference in the stroke-affected cortex or striatum of Nox2 $-/-$ mice compared to control tissue. Double-labelling revealed the source of detected superoxide to be both neurons and inflammatory cells.

Conclusion: Nox2 NADPH oxidase contributes to brain damage following ischaemia and reperfusion. Increased superoxide generation seen in Nox2 $+/+$ mice after 6 h of reperfusion may contribute to increased infarct size at 24 h through several cell types. Nox2 could present a target for intervention to improve outcome following ischaemic stroke and reperfusion, either independently or in conjunction with current therapies such as thrombolysis.

PP III-38

Analysis of the inflammatory response after stroke in spontaneously hypertensive rats by multicolour flow cytometry

K. Moeller[a,b], D.-C. Wagner[b], J. Seeger[a], J. Boltze[b,c], M. Kamprad[c] and T. Stahl[d]

[a]*University of Leipzig, Department of Veterinary Anatomy, Histology and Embryology, Leipzig, Germany*

[b]*Fraunhofer Institute of Cell Therapy and Immunology, Department of Cell Therapies, Ischemia Research Unit, Leipzig, Germany*

[c]*University of Leipzig, Translational Centre of Regenerative Medicine, Leipzig, Germany*

[d]*IDT Biologika GmbH, Dessau-Rosslau, Germany*

Objective: In recent years of experimental stroke research it has become evident that the pathophysiology of brain ischemia is a multiphasic process that not only leads to inital neuronal death but also induces secondary immune alterations. Those complex immunologic modifications including peripheral immunodepression with subsequent susceptibility to infection as well as severe delayed inflammation of the central nervous system crucially influence the long-term outcome after stroke. According to recent studies in different animal species and inbred strains particularly T-lymphocytic subpopulations (regulatory T-Cells, autoreactive effector T-Cells) as well as dendritic cells seem to play a central role for the constitution of the immun state. To characterize specific cell populations multicolour flow cytometry is a highly suitable method as it allows the parallel analysis of several subpopulations in one sample.

Material and methods: To characterize post stroke immune reaction we used Spontaneously Hypertensive Rats (SHR) to induce permanent Middle Cerebral Artery Occlusion (pMCAO). We established and performed multicolour flow cytometric analysis of brain tissue homogenates and peripheral blood to characterize and quantify immune cell subpopulations and their specific activation 96h post stroke as compared to control animals. Thereby we put the main focus on T-Cell subpopulations and T-Cell related activation of antigen presenting cells (APC). Alongside we determined the influence of different enzymatic cell preparation protocols on yield, viability and epitope stability.

Results: With the flow cytometric panels of up to 7 markers used in one sample we are currently able to clearly define 8 different subsets of immune cells in brain cell suspensions. Differences in between control and infarct animals are most evident within the lymphocytic compartment (CD45+, CD11b-) and in monocytic subpopulations. The setup of an activation panel for the rat comprising antibodies against CD80, CD86 and MHCII allows to depict the interaction of infiltrating T-Cells and specifically activated APC.

Additionally we were able to show that the enzymatic digestion of brain tissue has a strong impact on the preservation of epitopes and hence the characterization of certain markers specifically in the T-cell compartment.

Conclusions: The modulation of the immune response is the latest and most promising approach for new therapeutic strategies in stroke research. However, to model such therapeutic trials it is indispensable to elucidate the exact nature of immunologic properties and post stroke immune alterations in the specific animal strain chosen for the experimental setup. Thus, we present a methodology that allows to qualitatively and quantitatively delineate immunologic processes after brain ischemia in the SH-rat and depict its limitations as well.

PP III-39

The role of CD8+ cytotoxic T cells in acute experimental cerebral Ischemia

E. Mracsko, A. Liesz, W. Zhou, S. Karcher and R. Veltkamp
University Heidelberg, Department of Neurology, Heidelberg, Germany

Introduction: Both human and experimental studies indicate that leukocyte-mediated inflammation is a major contributor in the pathogenesis of neuronal death after ischemic stroke. Although, less is known about the contribution of specific leukocyte subpopulations to post-ischemic neuroinflammation. CD8+ T cells may play deleterious roles either through molecules released from their cytotoxic granules such as perforin, receptor-mediated cytotoxicity, or indirect pathways including release of inflammatory cytokines that recruit other leukocyte subsets. Here we investigated the downstream mechanism by which CD8 T cells contribute to ischemic brain injury.

Materials and methods: Cytotoxic CD8+ T cells were depleted by i.p. injection of monoclonal anti-CD8 antibodies 24h prior to stroke-induction, and the depletion was verified by flow cytometry. Permanent focal cerebral ischemia was induced by transtemporal middle cerebral artery occlusion (MCAO) distal from the lenticulostriatal arteries. For the examination of effector mechanisms perforin-deficient ($Prf^{-/-}$) mice were used. Neurological outcome was assessed by infarct volumetry and behavioural testing (Corner test) at 1, 3 and 7 days following stroke induction. Leukocyte invasion was analyzed by immunohistology, systemic and cerebral cytokine expression were measured by ELISA, RT-PCR and WB.

Results: CD8-specific antibodies caused 70% depletion of the CD8+ T cell population in blood, spleen and mesenteric lymph nodes and remained diminished during the time of investigation. T cell depletion was specific for CD8+ cells and did not reduce other leukocyte subpopulations. Infarct volumes were significantly reduced in CD8+ depleted mice at 7d compared to controls ($11{,}9 \pm 3{,}3 mm^3$ vs. $16{,}1 \pm 3{,}1\ mm^3$) but not at 24h and 3d. Furthermore, behavioural outcome was significantly improved. In terms of the investigated effector mechanisms, $Prf^{-/-}$ mice had significantly smaller infarcts than C57BL/6 control animals 7d after MCAO. We futher investigated the invasion of leukocyte subpopulations into the ischemic brain at several time points after MCAO.

Conclusion: Our findings suggest that perforin mediated cytotoxicity is the key mechanism for neuronal damage caused by CD8+ cytotoxic T cells during postischemic neuroinflammation.

PP III-40

Influenza virus infection aggravates stroke outcome

S. Muhammad[a], E. Haasbach[b], M. Kotchourko[a], A. Strigli[a], A. Krenz[a], D.A. Ridder[a], A.B. Vogel[b], H.H. Marti[c], Y. Al-Abed[d], O. Planz[b] and M. Schwaninger[a]
[a]*University of Heidelberg, Institute of Pharmacology, Heidelberg, Germany*
[b]*Friedrich-Loeffler-Institut, Institute of Immunology, Tübingen, Germany*
[c]*University of Heidelberg, Department of Physiology, Heidelberg, Germany*
[d]*The Feinstein Institute for Medical Research, Department of Medicinal Chemistry, New York, United States*

Stroke is triggered by several risk factors including respiratory tract infections such as influenza. However, it is unknown how and in which way influenza infection affects stroke pathophysiology and outcome. In a mouse model of stroke we show that intranasal infection with influenza virus H1N1 leads to larger infarcts. Changes in blood pressure, blood gases and body temperature did not explain the increased infarct size in influenza infection. After influenza virus infection the respiratory tract released cytokines into the blood, such as RANTES, that induced MIP-2 and other inflammatory mediators in the ischemic brain and thereby stimulate the invasion of neutrophils expressing the metalloproteinase 9. In concomitant influenza infection neuroinflammation was accompanied by severe disruption of the blood-brain barrier and an increased rate of intracerebral hemorrhages after tPA treatment, the standard therapy of ischemic stroke. To investigate the role of cytokines, we blocked cytokine release by using GTS-21, a selective agonist of the $\alpha 7$ nicotinic acetylcholine receptor. GTS-21 ameliorated ischemic brain damage and improved survival. In conclusion, influenza virus infection triggers a cytokine cascade that contributes to brain damage in stroke and increases the risk of intracerebral hemorrhage after tPA treatment. Blockade of cytokine production by $\alpha 7$nAchR agonists is a novel therapeutic option to treat complications of influenza.

PP III-41

Promoting endogenous protective mechanisms at the blood-brain barrier to reduce neurovascular inflammatory injury following stroke

A. Stowe[a,b], R. Hu[b], R. Gilchrist[b], R. Klein[b] and J. Gidday[b]
[a]*UT Southwestern, Neurology, Dallas, United States*
[b]*Washington University, Neurological Surgery, St. Louis, United States*

Objective: In rodents, one exposure to systemic hypoxia protects the brain from subsequent ischemic injury, though the window for cerebroprotection lasts only a few days. Recently, we showed that a repetitive hypoxic preconditioning (RHP) stimulus prolonged the window of 'ischemic tolerance' to 8 wks, with cerebroprotection associated with reductions in infarct volumes and postischemic leukocyte adherence to cortical venules. As chemokines regulate leukocyte dynamics, we investigated how RHP may alter chemokine expression to promote long-term ischemic tolerance. We found that RHP elevated message and protein levels of CXCL12 – a chemokine associated with maintenance of blood-brain barrier (BBB) integrity – for weeks prior to stroke onset. We therefore hypothesized that RHP strengthens the BBB by localizing the increased CXCL12 expression to the perivascular space of cortical microvessels following stroke to improve BBB integrity and reduce leukocyte diapedesis within the area of infarct.

Materials and methods: Adult, male SW/ND4 or transgenic mice with EGFP-expressing myelomonocytic cells were exposed to RHP (9 exposures over 2 wks; 8% or 11% O_2; 2 or 4 h duration). Two or 4 wks following the final hypoxic exposure, a 60-min transient middle cerebral artery occlusion (tMCAo) was induced via the intraluminal suture method. Control mice had tMCAo but no prior hypoxia. After 24 h of reperfusion, animals were sacrificed to determine 1) percent of total microvessels with CXCL12 colocalization ($n = 3$/group, 58–117 vessels/animal), 2) the extent of BBB leak to endogenous IgG ($n = 5$–6/group), and 3) leukocyte diapedesis within the ischemic zone ($n = 5$–6/group). One-way ANOVA, Student's t-test, or linear regression analysis determined significance ($p < 0.05$).

Results: In control animals with no prior RHP, tMCAo reduced the number of CXCL12-positive vessels within the cortex when compared to detection in naïve mice ($39 \pm 2\%$ vs. $47 \pm 4\%$; $p < 0.05$). In contrast, the fraction of CXCL12-positive vessels was greater than naïve levels when RHP preceded stroke by either 2 wks ($54 \pm 0.4\%$) or 4 wks ($79 \pm 13\%$; $p<0.05$). Post-stroke leukocyte diapedesis was reduced from control values by 44% ($p < 0.05$) or 75% ($p < 0.001$), respectively, in mice with RHP completed 2 wks or 4 wks prior to stroke. Furthermore, RHP abrogated BBB leak following stroke ($p < 0.0001$) at both time points. In correlating CXCL12 microvessel expression to indices of post-stroke inflammation, the number of CXCL12-positive vessels in the post-stroke cortex inversely correlated with both leukocyte diapedesis into the infarcted region ($R^2 = 0.94$; $p < 0.01$) and BBB disruption ($R^2 = 0.73$; $p < 0.05$).

Conclusions: We show that RHP induces an unprecedented and long-lasting cerebroprotective phenotype that is characterized by post-stroke reductions in vascular inflammation weeks after the termination of preconditioning. The animals with prior RHP have an enhanced deposition of CXCL12 at the BBB which may help to maintain a structural coupling between astrocytic end-feet and endothelial barrier proteins, thereby 'trapping' and preventing CXCR4-expressing leukocytes from diapedesing into brain parenchyma. Causal studies to confirm the contribution of CXCL12 to RHP-induced neurovascular protection are underway. Understanding and translating these endogenous protective mechanisms into therapeutics could afford sustained periods of cerebroprotection in subpopulations of individuals at identified risk for stroke.

PP III-42

Temporal and spatial dynamics of post-ischemic microglia/macrophage activation in rat embolic stroke evaluated by [11C]PK11195-PET

M. Walberer[a,b], H. Backes[b], M.A. Rueger[a,b], B. Neumaier[b], G.R. Fink[a,c], R. Graf[b] and M. Schroeter[a,b]
[a]*University Hospital of Cologne, Department of Neurology, Cologne, Germany*
[b]*Max-Planck-Institute for Neurological Research, Cologne, Germany*
[c]*Research Centre Juelich, Cognitive Neurology Section, Institute of Neuroscience and Medicine, Juelich, Germany*

Objectives: Neuroinflammation evolves as a multifacetted response to focal cerebral ischemia involving

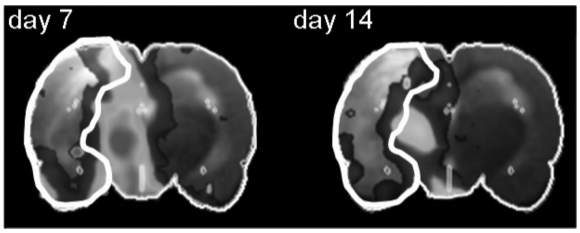

Fig. 1. [^{11}C]PK11195-binding is fused on T2-MRI at day 7 and day 14 after stroke. The infarct is circumscribed by a white line.

the recruitment of monocytes/macrophages and activation of resident microglia cells. Those cells expressing the peripheral benzodiazepine receptor can be visualized by [^{11}C]PK11195-Positron Emission Tomography (PET). This *longitudinal* study uses the macrosphere stroke model to characterize the time-course of microglia/macrophage (m/m) activation in subareas of embolic stroke on an *intraindividual* basis.

Material and methods: Wistar rats ($n = 12$) underwent middle cerebral artery (MCA) occlusion induced by TiO_2-macrospheres. T2-weighted magnetic resonance imaging (MRI) and [^{11}C]PK11195-PET were performed before, as well as 2, 7, 14, 21, and 42 days after induction of ischemia. Additionally, at days 7 ($n = 6$) and 42 ($n = 6$), immunocytochemistry against CD68 and CD11b was performed. PET, MRI as well as immunocytochemical stainings were matched to analyse temporal and spatial dynamics of m/m accumulation.

Results: In all animals, T2-MRI verified an ischemic lesion in the MCA territory. After a peak at day 2, infarct volume and vasogenic edema decreased and atrophy occurred. As visualized by [^{11}C]PK11195-PET and immunocytochemistry, m/m accumulated at the infarct margin and in peri-infarct tissue with a delay of several days. The maximum of local m/m activation was interindividually found at day 7 or day 14 after induction of ischemia and decreased afterwards. From day 7 onwards, m/m infiltrated the ischemic core. At later time points, m/m accumulation in varying degrees occurred in areas distant from focal ischemia, e.g. descending fibre tracts or thalamic nuclei. Only minor binding of [^{11}C]PK11195 was visible within the infarct core due to a lack of reperfusion in this model of permanent focal ischemia.

Conclusions: Using [^{11}C]PK11195-PET, neuroinflammation can be analyzed non-invasively and repeatedly over time. M/m accumulation is an ongoing process for weeks after ischemia with complex dynamics and spatial patterns in subareas of the infarct. Moreover, it extends beyond the infarcted tissue to remote areas affected by secondary damage and degeneration. In addition to degeneration, m/m responses may also have an impact on regeneration, reorganization, and recovery of function after stroke.

PP III-43

ODG – induced activation of MMPs in organotypical hippocampal slice cultures.

L. Wojcik, A. Sarnowska, L. Srojek, M. Ziemka-Nalecz and T. Zalewska
Mossakowski Medical Research Centre PAS, NeuroRepair, Warsaw, Poland

Objective: Matrix metalloproteinases (MMPs) belong to a family of endopeptidases, known to play important roles in the extracellular matrix (ECM) remodeling in various physiological and pathological processes. Although up-regulation of MMPs have been investigated in the context of detrimental roles in brain ischemic injury, their involvement in neurogenic response of neural stem/progenitor cells after ischemic insult has only been considered recently. The purpose of our study was to check if activation of MMPs coincides with the generation of new neurons in hippocampal slices subjected to oxygen-glucose deprivation (OGD). In the first step of our investigation we determined the activity of MMP-2 and MMP-9 in the hippocampal organotypical slice cultures.

Materials and methods: Hippocampal slice cultures was prepared from 7-days old Wistar rats in line with the method of Stoppini, as slightly modified in our laboratory. Hippocampal slices were transposed to Millicell-CM (Milipore) membranes in a 6-well plates. Cultures were started in a horse serum-containing medium, which was gradually replaced between days 4 and 7 in culture by a serum-free, defined solution based on DMEM-F12. Culturing was took place in a moist atmosphere of air and 5% CO_2, at 37°C up to 7 days in vitro. Then, slices were exposed for 40 min to a conditions deprived of glucose and oxygen. After transferring to a standard medium slices were then cultivated for 24h, 48h and 72h. Control slices were cultured in the same conditions but were not subjected to OGD treatment. Activity of MMP-2 and -9 was estimated by in situ zymography. Double fluorescent labeling was performed in order to identify the cell types expressing gelatinolytic activity. Neural cell types was determined with proteins specific for

either astrocytes (GFAP), microglia (Iba-1) or neurons (NF-200 and NeuN).

Results and conclusions: OGD results in the only slight activation of MMPs in the dentate gyrus as compared to the control conditions. In this structure MMPs activity was localized in cells of the granular layer as well as in the hilus. Double staining shows the presence of MMPs in some cells NF-200(+) and GFAP(+). In contrast, in the CA1 pyramidal layer gelatinolytic activity was significantly elevated and was mostly associated with the microglial cells. Therefore, we can assume that MMP-2 and-9 may be involved in inflammation associated with degeneration of neurons caused by OGD.

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Topic:Mitochondria and Metabolism

PP IV-1

Inhibition of p53 preserves mitochondrial morphology and function and protects against glutamate-induced cell death.

S. Diemert[a], S. Tobaben[a], J. Grohm[a], R. Hartmannsgruber[a], N. Plesnila[b] and C. Culmsee[a]
[a]*Philipps-Universität Marburg, Institut für Pharmakologie and Klinische Pharmazie, Marburg, Germany*
[b]*Royal College of Surgeons in Ireland* (*RCSI*), *Department of Neurodegeneration and Department of Physiology, Dublin, Ireland*

Objectives: This study investigates the role of Pifithrin-alpha mediated neuroprotection in HT-22 cells and synaptosomal preparations with special focus on effects of p53 at the level of mitochondria.

Material and methods: Cell viability was measured by MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and impedance measurements (xCELLigence system). Mitochondrial morphology was evaluated and categorized manually using MitoGFP – dependent stainings of mitochondria. Measurement of mitochondrial membrane potential was conducted using Rhodamin 123 and JC-1 fluorescence. NF-kB activity was determined using luciferase reporter constructs. Standard protocols were applied for protein analysis. Results and conclusions: In HT-22 cells glutamate-induced cell death follows a marked translocation of p53 to mitochondria and is accompanied by a decrease of mitochondrial membrane potential and a drastic change in mitochondrial morphology. The p53 inhibitor pifithrin-alpha (PFT) not only averts cell death but also enhances mitochondrial fusion and preserves mitochondrial membrane potential. Interestingly, PFT markedly increases the mitochondrial membrane potential over control level. Furthermore, PFT significantly attenuated mitochondrial ROS formation and preserved mitochondrial membrane potential in synaptosomal preparations, supporting the idea that at least part of the pro-apoptotic activity of p53 was mediated directly at the mitochondrial level and therefore is independent of p53-mediated transcription. In line with these findings we could not observe any involvement of NF-kB dependent transcription in the observed neuroprotective effects of Pifithrin-alpha in HT-22 cells. Consequently neither transcriptional activity of NF-kB nor expression of any of its target proteins affecting mitochondrial function was influenced by Pifithrin in HT-22 cells.

In conclusion, inhibition of p53 preserved mitochondrial morphology and mitochondrial membrane integrity, and enhanced neuronal survival following oxidative stress. These protective effects of PFT likely result from a direct inhibition of p53-dependent activity at the level of mitochondria.

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PP IV-2

Dynamin-related protein-1 as a therapeutic target to prevent mitochondrial pathways of neuronal cell death in vitro and in vivo

J. Grohm[a], S.-W. Kim[b], U. Mamrak[b], A. Cassidy-Stone[c], J. Nunnari[c], N. Plesnila[b] and C. Culmsee[a]
[a]*Philipps-Universität Marburg, Institut für Pharmakologie und Klinische Pharmazie, Marburg, Germany*
[b]*Royal College of Surgeons in Ireland* (*RCSI*), *Department of Physiology, Dublin, Ireland*
[c]*University of California, Department of Molecular and Cellular Biology, Davis, United States*

Question: Mitochondria are highly dynamic organelles that undergo permanent fission and fusion, a process that is important for mitochondrial function and cellular survival. Emerging evidence suggests that in neurons intrinsic death pathways are associated with detrimental mitochondrial fragmentation. Therefore,

we addressed the question whether regulators of mitochondrial fission serve as targets for neuroprotective strategies in models of glutamate toxicity *in vitro* and in a model of focal cerebral ischemia *in vivo*.

Material and methods: For *in vitro* studies we applied models of glutamate toxicity in immortalized mouse hippocampal neurons (HT22 cells) and embryonic rat cortical neurons. Cell viability and apoptotic cell death were evaluated by MTT assays, ATP measurements and Annexin/Propidium Iodide staining, respectively. Fluorescence microscopy was used to detect changes of mitochondrial morphology after transfection with mitochondria-targeted GFP (mitoGFP) or Mitotracker staining. Apoptotic cell death and mitochondrial outer membrane potential (MOMP) were further quantified by flow cytometry. Inhibition of Drp-1 was achieved by siRNA approaches and specific small molecule compounds. Focal cerebral ischemia was performed in male C57BL/6 mice that were subjected to transient middle cerebral artery occlusion in deep isofluorane anaesthesia under control of physiological parameters and laser-doppler blood flow measurements in the ischemic brain area.

Results: In cultured neural cells, glutamate toxicity correlated with mitochondrial fragmentation and peri-nuclear accumulation of the organelles. Significant mitochondrial fragmentation occurred within a few hours after the glutamate challenge and clearly preceded the neuronal cell death. Such glutamate-induced mitochondrial fission was accompanied by ATP depletion and loss of mitochondrial outer membrane potential (MOMP). Novel small molecule inhibitors of dynamin-related protein-1 (Drp-1), an established regulator protein involved in mitochondrial fission, prevented ATP depletion, loss of MOMP and mitochondrial fragmentation, and protected the neural cells from glutamate toxicity. Additional experiments using siRNA confirmed the neuroprotective effects by Drp-1 inhibition *in vitro*. Moreover, the pharmacological Drp-1 inhibitors also reduced the infarct volume in a model of transient focal ischemia in mice, suggesting a major impact of the mitochondrial fission machinery in neuronal cell death after acute brain injury *in vivo*.

Conclusion: Our data expose Drp-1 as a potential therapeutic target to prevent mitochondrial fragmentation and subsequent key mechanisms of neuronal cell death induced by glutamate toxicity and cerebral ischemia. Thus, inhibiting the regulators of pathological mitochondrial fragmentation is proposed as a novel strategy of neuroprotection.

PP IV-3

Evaluation of cyclosporine a in a stroke model in the immature rat brain

P.-L. Leger[a,b], D. De Paulis[c], S. Branco[a], P. Bonnin[d], S. Renolleau[a,b], A. Gharib[c] and C. Charriaut-Marlangue[a,b]
[a]*INSERM, U676, PARIS, France*
[b]*CNRS, UMR7102, PARIS, France*
[c]*INSERM, U886, LYON, France*
[d]*INSERM, U965, PARIS, France*

Objective: Mitochondria play essential roles in energy metabolism, generation of reactive oxygen species, and regulation of apoptosis in response to neuronal brain injury. Damage to the bioenergetic integrity of mitochondria plays a critical role in adult ischemia, and the formation and opening of the mitochondrial permeability transition pore (mPTP) is one of the core mediators of this process [2]. The effects of ischemia-reperfusion on opening of the mitochondrial permeability transition pore (mPTP) and its blockade in the immature brain are not fully understood.

Material and methods: Ischemia was performed in Wistar P7 rats [3]. Briefly, anesthetized rats were exposed to left middle cerebral artery electocoagulation (MCAo) followed by a 50 minutes occlusion of the left common carotid artery. Ischemic animals ($n = 123$) were randomly administered with either saline buffer or Cyclosporine (CsA, Sandimmum, Novartis®, Basel, Switzerland) dissolved in saline. Lesion volumes were evaluated at 48 hours post-injury on cresyl violet-stained sections. Mitochondria were isolated from cortical tissues and both measurement of the mitochondrial Ca^{2+} retention capacity (CRC) and mitochondrial oxygen consumption were measured as previously described [1].

Results: CsA (10 mg/kg) was administered 14 hours before induction of ischemia and effects were analyzed at 30–40 min and 48 hours after reperfusion. CsA administration reduced infarct size, DNA fragmentation and apoptotic bodies, and inflammatory responses in mild but not severe injury. CsA increased the Ca^{2+} load required to open the mPTP (78.4 ± 19.2 *vs* 50.2 ± 19.9 nmol.mg^{-1} protein, $p < 0.05$) in limiting the decoupling of the respiratory chain by unchanged state 3 but reduced state 4, and attenuated early calpain-mediated alpha-spectrin proteolysis.

Conclusion: CsA mediates inhibition of mPTP opening and has a tendency to protect immature rat brain against mild ischemic injury.

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PP IV-4

Abnormal Ca^{2+} signaling in astrocytes caused by inflammation is normalized after suppression of VIA Ca^{2+}-independent phospholipase A_2 (VIA $iPLA_2$).

M. Strokin and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: Many Ca^{2+}-regulated intracellular processes are involved in the development of neuroinflammation. However, the changes of Ca^{2+} signaling in brain under inflammatory conditions were hardly studied. Proper ATP-induced Ca^{2+} signaling is a central event for the communication of the cells among the astrocytic network, which functions in the tight concert with the synaptic network. We investigated the changes in Ca^{2+} signaling in primary astrocytes after pro-inflammatory stimulation with lipopolysaccharide (LPS) and the role VIA phospholipase A_2 in the observed changes.

Methods: *Cell culture*: Primary astrocytes were prepared from the whole brains of new born rats and used for experiments on 12–14 day in culture; Intracellular Ca^{2+} measurements: Ca^{2+} concentration in single cells was measured using digital fluorescence microscopy using Fura-2 as fluorescence dye and alternative excitation wavelength 340/380 nm; *Silencing with siRNA:* VIA $iPLA_2$ was silenced in astrocytes using magnet assisted transfection with specific siRNA; *Western blot analysis:* Cell lysates were subjected to SDS/PAGE (4–12%) followed by Western blot analysis using specific antibodies against VIA $iPLA_2$.

Results: We reveal that Ca^{2+} responses to purinergic ATP stimulation are significantly increased in amplitude and duration after onset of inflammatory conditions (LPS 100 ng/ml for 6–24 h). We detected that increased amplitudes of Ca^{2+} responses to ATP in LPS-treated astrocytes can be explained by substantial increase of Ca^{2+} load in stores in endoplasmic reticulum. The mechanism implies enhanced Ca^{2+} store refilling due to the amplification of capacitative Ca^{2+} entry. Also for the increased duration of Ca^{2+}responses in LPS-treated cells amplified capacitative Ca^{2+} entry is the reason. Next, we established that the molecular mechanism for the LPS-induced amplification of Ca^{2+} responses in astrocytes is the increased expression and activity of VIA phospholipase A_2 (VIA $iPLA_2$). Indeed, either gene silencing with specific siRNA or pharmacological inhibition of VIA $iPLA_2$ with S-bromoenol lactone reduced the load of the Ca^{2+} stores and amplitudes of Ca^{2+} responses in LPS-treated astrocytes to values, which were comparable to those in untreated cells.

Conclusions: i) ATP-induced Ca^{2+} responses in astrocytes challenged with lipopolysaccharide (LPS) are strongly amply amplified. ii) VIA $iPLA_2$ is overexpressed in astrocytes after LPS stimulation. iii) VIA $iPLA_2$ plays a key role in development of pathological Ca^{2+} signaling. We propose that the regulation of VIA $iPLA_2$ activity is an instrument for neuroprotection during brain inflammation condition in brain after stroke [1], brain bacteria invasion or encephalitis.

Reference

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Topic: Others

PP I-14

Changes in motor behaviour of hemiparkinsonian rats after intrastriatal injection of botulinum neurotoxin A

V. Antipova[a], A. Hawlitschka[a], C. Holzmann[b], R. Benecke[c], E. Mix[c] and A. Wree[a]
[a]*University of Rostock, Institute of Anatomy, Rostock, Germany*
[b]*University of Rostock, Medical Genetics, Rostock, Germany*
[c]*University of Rostock, Clinic of Neurology, Rostock, Germany*

Objectives: Botulinum neurotoxin A (BoNT) is a bacterial enzyme which inhibits the release of acetyl choline from presynaptic vesicles through its proteinase activity cutting the SNARE complexes. Due to its durable inhibitory effects BoNT is mainly used in the treatment of different neuromuscular diseases such as dystonias, spastic palsies and different pain types.

To test the potential of an intracerebral botulinum neurotoxin A (BoNT) treatment as a therapeutic option of Parkinson's disease (PD) we have investigated the consequences of intrastriatal injections of BoNT on motor functions in an animal model of PD. The rational of intrastriatal BoNT-injection is to decrease the hypercholinism of the striatum in PD by local injection.

Material and methods: Adult Wistar rats, which had received 6-hydroxydopamine (6-OHDA) in the right medial forebrain bundle (Hemiparkinson model) 4 weeks before BoNT-treatment and healthy control rats were injected with 100 pg, 1 ng or 2 ng BoNT into the right striatum. Animal behaviour due to 6-OHDA and/ or BoNT were investigated by following tests: 1) apomorphine induced rotation test, 2) cylinder test (forelimb preference), 3) activity test in open field, and 4) RotaRod test.

Results: Hemiparkinsonian rats showed more than 8 apomorphine induced rotations per minute in anticlockwise direction. These rotations were completely abolished by ipsilateral injection of 1 ng and 2 ng BoNT, this effect lasts for at least 3 months. Six months after BoNT application a reduction of the BoNT effect in hemiparkinsonian rats still occurred and a reappearance of pathological apomorphine rotations was noted. However, BoNT injection did not alter the outcome of the cylinder test, the open field activity and the RotaRod tests during 6 months post injection significantly.

Conclusion: In conclusion, BoNT seemingly reduced the inhibitory cholinergic signals of the striatum, which leads to apomorphine-induced rotations towards the injection side (clockwise). Intrastriatal BoNT-injection annual the apomorphine-induced rotations in 6-OHDA lesioned rats and thereby reverse the 6-OHDA induced motor deficit that becomes visible after pharmacological provocation. The intrastriatal BoNT applicaton could be a new option for a therapeutic strategy of PD. Intrastriatal BoNT application could avoid undersirable side-effects and adverse pathogenetic effects of systemically administered anticholinergics.

PP I-15

Simultaneous TIDDC-SPECT, MRI and histological studies in SHSP rats without salt-loaded diet

C. Büche[a], H. Braun[b], J. Goldschmidt[b], F. Angenstein[b], H.-J. Heinze[a], M. Görtler[a], K.G. Reymann[b] and S. Schreiber[a]

[a]*Universitätsklinikum, Neurologie, Magdeburg, Germany*

[b]*Institut für Neurobiologie (IfN), Magdeburg, Germany*

Background: Beside few basic histological works (Fredriksson et al., 1985) little is known about the natural progression of cerebral small vessel disease including its microbleeds and microthromboses in association with corresponding imaging results in spontaneously hypertensive stroke-prone (SHSP) rats without a salt-loaded diet.

Methods: 4.7 Tesla MRI T_2-weighted image and TOF-MRA were perfomed in SHSP rats without a salt-loading diet within their 12th and 32nd week of life. Two 32-weeks-old rats additionally underwent simultaneous thallium diethyldithiocarbamate (TIDDC) Single Photon Emission Computed Tomography (SPECT). TIDDC, here applied in a lipophilic complex, serves as a potassium metabolism analogue and is taken up into neurons and can so be used as a marker for neuronal activity (Goldschmidt et al., 2010). After perfusion coronal brain slices of animals at different ages were stained with HE.

Experiments were carried out after approval by the state animal care and local ethics committee.

Results: T_2-weighted hyperintensities have been detected at the earliest time in 32-weeks-old animals; MRA did not show any macroangiopathy in none of the animals. Concerning SPECT, TIDDC dispersal was reduced in those regions correspondingly being MRI-positive. From the age of 12 weeks on accumulation of erythrocytes in the small vessels of the basal ganglia and cortical regions could be detected (Figure). In the 32nd week of life the animals showed microbleeds and microthromboses in those regions being MRI-positive (Figure).

Conclusion: The histological data are compatible with early autoregulatory disturbances of the small brain vessels. MRI lesions occur far later than it has been described in animals fed with a salt-loaded diet and are associated with a progressed microangiopathy including microbleeds and microthromboses. The

SPECT changes show disturbances of neuronal potassium metabolism in corresponding T_2-hyperintensive lesions.

PP I-16

Structural kidney damage predicts cerebral microbleeds in SHSP rats

C. Büche[a], S. Schreiber[a], G. Cornelia[a], C. Zhu[b], P. Mertens[b], H.-J. Heinze[a], M. Görtler[a], K.G. Reymann[c], H. Braun[c]
[a]*Universitätsklinikum, Neurologie, Magdeburg, Germany*
[b]*Universitätsklinikum, Klinik für Nieren – und Hochdruckkrankheiten, Magdeburg, Germany*
[c]*Institut für Neurobiologie (IfN), Magdeburg, Germany*

Background: Spontaneously hypertensive stroke-prone (SHSP) rats develop brain and kidney damage due to a malign arterial hypertension as a consequence of genetic alterations in the renin-angiotensin and endothelin system. Little is known about the natural progression of cerebral small vessel damage in temporal association with the occurrence of kidney damage in that model of microangiopathy without feeding a salt-loaded diet.

Methods: The kidneys and brains of 31 male SHR-SPs without salt-loaded diet were stained with HE at different stages of age (12th until 32nd week). Wistar rats served as control animals.

Results: Concerning the kidney, progressive glomerular and tubular bleedings, severe hyalinosclerosis of the glomeruli with interstitial inflammation and fibrous thickening of arterioles and interlobular arteries have been detected starting in the age of 22 weeks.

In different brain regions (cortical regions, the basal ganglia and to a lesser extend in the hippocampus of both hemispheres) accumulation of erythrocytes in the lumen of arterioles and capillaries were seen from the 12th week on. Vascular remodeling, microbleeds and microthromboses were first seen in the 32-week-old group.

Conclusion: The histological data are compatible with early autoregulatory disturbances of the small brain vessels occurring independently from structural kidney damage and its associated arterial hypertension. The later kidney damage is not only the cause of an arterial hypertension but might also predict independently from traditional risk factors (Khella et al., 2007) a progressed cerebral microangiopathy including microbleeds and microthromboses

PP I-17

Synergistic antinociceptive effects of n-methyl-d-aspartate antagonist and electroacupuncture in the complete freunds adjuvant-induced pain model

J.-Y. Jang[a], S.-T. Koo[a], H.-K. Shin[a], E.-S. Choe[b], J.-U. Baek[a] and B.-T. Choi[a]
[a]*Pusan National University, School of Korean Medicine, Gyeongnam, Korea, Republic of*
[b]*Pusan National University, Department of Biology, Busan, Korea, Republic of*

Objectives: This study examined the synergistic antinociceptive effects associated with signaling pathway proteins in the dorsal horn of the spinal cord in a complete Freunds adjuvant (CFA)-induced pain model when electroacupuncture (EA) and N-methyl-D-aspartate receptor (NMDAR) antagonist were administered in combination.

Material and methods: Bilateral EA stimulation (2 Hz, 1 mA) was needle-delivered for 20 min once daily at acupoints corresponding to Zusanli and Sanyinjiao with intrathecal injection of NMDAR antagonist dizocilpine (MK801) under isoflurane anesthesia.

Results: When the thermal sensitivity was measured after 30 min of anesthesia, hindpaw sensitivity induced by CFA was strongly inhibited by both dizocilpine injection and EA stimulation. Co-treatment with EA and dizocilpine showed a synergistic antinociceptive effect in the inflammatory pain model. On day two of the experiment, we examined phosphorylation of the NMDAR NR2B subunit, extracellular signal-regulated kinase (ERK), p38 and cAMP response element-binding protein (CREB) in the ipsilateral dorsal horn of L4-5 segments by Western blot analysis. Phosphorylation of the NMDAR NR2B subunit induced by CFA was markedly inhibited by co-treatment with dizocilpine and EA, but not by dizocilpine or EA treatment alone. CFA-induced phosphorylation of the ERK was inhibited by both dizocilpine and EA, but that of p38 was inhibited by EA only. A noticeable change in these proteins was observed in response to EA alone and co-treatment with dizocilpine and EA. CFA-induced phosphorylation of the CREB was inhibited by dizocilpine, but did not show marked changes. Immunohistochem-

ical analyses confirmed that there was a significant difference in NMDAR NR2B subunit and ERK phosphorylation, which may have been involved in the synergistic antinociceptive effects that occurred in the dorsal horn of the spinal cord.

Conclusion: It is possible that the combined treatment with EA and the NMDAR antagonist dizocilpine resulted in synergistic antinociceptive effects in an inflammatory pain model via inactivation of both the NMDAR NR2B subunit and ERK of the spinal cord.

PP I-18

Common mechanisms in neurodegenerative disorders

A. Diaconeasa and L. Spiru
Ana Aslan International Academy of Aging, Research and Development, Bucharest, Romania

Objective(*s*): At first sight, neurodegenerative disorders are different in terms of biomarkers, symptoms and affected tissues, although there are some overlapping symptoms in such disorders (i.e. depression is common in psychiatric disorders as well as in Alzheimer's disease, Parkinson disease etc). Neurodegenerative disorders may however have even more important common features in terms of mechanisms.

Regeneration in vertebrates implies cell dedifferentiation and proliferation followed by cell differentiation. Regeneration actually refers to tissue repair; however, when one or more regenerative processes are impaired, degeneration occurs. We'll try to identify which processes are commonly altered in neurodegenerative disorders.

Material and methods: Neurodegenerative disorders, as well as degenerative disorders of other tissues are more common in humans than in other related species. Humans suffer from autoimmune diseases, and have a greater risk of developing type II diabetes and cancer than captive chimpanzees. The hugely-sized human brain also hosts particular pathology (which includes, besides Alzheimer's disease, some psychiatric disorders, autism etc). A valid hypothesis capable of explaining the mechanisms of human evolution should also explain the occurrence and mechanisms of human-specific brain diseases.

Our hypothesis originates in the assumption of King and Wilson (1975), stating that the human evolution is more the result of genetic regulation changes than a result of gene structure alterations.

Some particular ecological changes, documented in the history of our species, may result in epigenetic modifications (hereditarily transmittable) that could be responsible for further genetic changes leading to alterations of gene expression and function. As a result, some important biochemical pathways, playing a crucial role in organism development, could suffer changes in terms of regulation and activity.

Results: The IGF1/insulin signaling pathway (involved in metamorphosis in some species, as well as in cell proliferation and cell differentiation) is altered in selected human pathological conditions (i.e. psychiatric disorders, autism, Alzheimer's disease, diabetes, cancer, psoriasis etc). Changes in the activity of this pathway could determine the development of some human unique traits.

Alterations of the IGF1/insulin pathway affect the cell proliferation and the survival/differentiation ratio. In certain disorders (i.e. psychiatric disorders) the cell proliferation and survival is affected. In other disorders (i.e. autism) the cell differentiation is affected. There are however some disorders (i.e. Alzheimer's disease) in which both processes are disturbed.

In those neurodegenerative disorders that are characteristic to later stages of life cell differentiation is the most affected disturbance, since aging is associated with impaired insulin signaling.

Conclusion(*s*): Such processes are responsible for some degenerative disorders affecting various tissues. Under these circumstances, it becomes obvious that adjusting this metabolic pathway may prevent or even cure certain degenerative disturbances. This opens new perspectives in neurodegenerative disorder pharmacological research; moreover, a better understanding of such disturbances may allow effective strategies for preventing neurodegenerative disorder.

PP I-19

Effects of silver nanoparticles in primary mixed neural cultures: Oxidative stress and interference with calcium signalling

A. Haase[a], S. Rott[b], A. Mantion[c], P. Graf[d], A. Thuenemann[c], W.P. Meier[d], A. Taubert[e,f], A. Luch[a] and G. Reiser[b]
[a]*Federal Instiute of Risk Assessment* (*BfR*), *Department of Product Safety, Berlin, Germany*

[b]*University of Magdeburg, Institute of Neurobiochemistry, Magdeburg, Germany*
[c]*University of Basel, Department of Chemistry, Basel, Switzerland*
[c]*BAM – Federal Institute for Materials Research and Testing, Berlin, Germany*
[e]*University of Potsdam, Institute of Chemistry, Potsdam, Germany*
[f]*Max – Planck – Institute of Colloids and Interfaces, Potsdam, Germany*

Background: Nanoparticles gain an enormous commercial interest. The number of products is steadily increasing, ranging from consumer products, advanced building materials, coatings or paints to applications in nanomedicine for bioimaging or drug transport purposes. Concerns about putative adverse effects on human health are increasing. It is well known that nanoparticles can be systemically distributed and cause additional effects on secondary target organs. The central nervous system (CNS) is especially vulnerable. Transport of nanoparticles into the CNS seems possible via different routes. However, only very few studies have investigated the effects on neural cells so far. Here, we analyze the effects of silver nanoparticles (SNP), which are among the most commercialized nanoparticles, in primary mixed cortical neural cultures.

Objectives: Cellular effects of SNP of two different sizes (20 nm and 40 nm) were studied with respect to cytotoxicity, development of oxidative stress and calcium responses. Gold nanoparticles were included for comparison reasons.

Methods: Primary neural cultures were obtained from cortex of 1- to 3-day-old Wistar rats. The cytotoxicity was assessed with WST-1 and LDH assay (Roche Diagnostics). Protein carbonyls were detected with OxyBlot Kit (Millipore). For measurement of ROS we used 1 μM dihydroethidium in NaHBS and followed the reaction at 520 nm at single cell level. For analysis of calcium responses the cells were preincubated with 2 μM Fura-2AM in NaHBS and responses were followed by measuring the ratio at 340 nm/380 nm at single cell levels.

Results: We observed a strong cytotoxicity towards SNP, which was dependent on the SNP size and also on the differentiation state of the neural cells. In addition, we could visualize ROS formation directly in living cells. We support this with biochemical studies. We detect fast formation of protein carbonyls and induction of heme oxygenase 1 (HO-1), which are both established stress markers. The formation of oxidative stress is of special concern since in the CNS it is tightly linked with neurodegeneration and with the onset and progression of diseases, such as Alzheimer or Parkinson. Furthermore, we observe an acute calcium response after SNP addition, suggesting that SNP interfere with intracellular signalling processes. Gold nanoparticles in comparison prove mainly inert.

Conclusions: Our results show that neural cells are highly sensitive towards treatment with silver nanoparticles. We demonstrate that responses will occur within individual but different time frames. The generation of ROS or the calcium responses will occur already within a few minutes, while the formation of protein carbonyls or induction of HO-1 can be detected only after several hours. Morphological changes are observed as a very late response. Further investigations are required to understand the underlying molecular mechanisms of these responses.

PP I-20

Tissue-type plasminogen activator induces plasmin-dependent proteolysis of intracellular neuronal nitric oxide synthase.

Y. Hommet, A. Baron, F. Casse and D. Vivien
Inserm U919, Caen, France

Objective: Despite its pro-fibrinolytic activity, tissue-type plasminogen activator (tPA) is a serine protease known to influence a number of physiological and pathological roles in the central nervous system (CNS). We previously found that by interacting with NMDA receptors, tPA could increase NMDA-evoked calcium influx in neurons. Since NMDA receptor activity is also known to activate the production of nitric oxide (NO), we sought to determine if tPA could influence NO release under physiological conditions.

Material and methods: Neuronal cultures: Mouse neuronal cultures were prepared as previously described (Rose et al, 1993).

Nitrate/Nitrite assay: Nitrite/nitrate were quantified using the Griess method and a DAF-2 diacetate (Sigma Aldrich).

Immunocytochemistry: We used anti-NOS1 (Santa Cruz Biotech.) and anti-MAP2 (Abcam) antibodies.

Immunoblotting: We used anti-NOS1 (Santa Cruz Biotech.), Protein G-Sepharose beads (GE-Healthcare).

2D electrophoresis: We used Ettan IPGphor (GE-Healthcare), pH strips 3-11NL (GE-Healthcare).

Results: tPA reduces Nitric Oxid production in neurons. We first observed that a 1 hour treatment with tPA reduced by 20% the amount of NO released by cultured neurons. Confocal imaging, 2D-PAGE, and immunoprecipitation analyses revealed that tPA induced a proteolysis of nNOS. Pharmacological approaches demonstrated that tPA-mediated nNOS proteolysis was independent of NMDA receptors, calpains and LRP, but rather, mediated by the conversion of plasminogen into plasmin. In agreement with our in vitro studies, we measured reduced levels of proteolysis of nNOS in both the cortex and the hippocampus of tPA deficient mice.

Conclusion: tPA is known to have differential effects, depending on its interactions with different players in various pathways. Our present data show a new function of tPA/plasmin in the brain through their ability to promote nNOS proteolysis, thus regulating the release of nitric oxide, an important neural messenger.

Here we demonstrate that tPA is able to induce NOS1 proteolysis under physiological conditions, a reaction mediated by activation of plasminogen into plasmin, independently of NMDA receptor activation or calpain-dependent processes. Since nNOS-derived NO and tPA are involved in neurogenesis and synaptic plasticity, the tPA-dependent proteolysis of nNOS could represent a new mechanism through which tPA influences neuronal physiology.

PP I-21

Down-regulation of the GABA$_A$ receptor subunit δ following transient focal cerebral ischemia

C. Frahm, N. Jaenisch and O.W. Witte
University Hospital, Hans Berger Clinic for Neurology, Jena, Germany

Objectives: One consequence of stroke is the appearance of epileptic seizures in $\sim$ 20% of stroke patients. This might be due to a post-ischemic imbalance of inhibitory and excitatory transmission. Though several studies pointed to an impairment of GABAergic inhibition following stroke the special impact of tonic inhibition has not been addressed yet. Tonic inhibition regulates neuronal excitability via extra-synaptically located GABA$_A$ receptors. They respond to low concentration of GABA and exhibit a reduced desensitization. We investigated the regulation of the GABA$_A$ receptor subunit δ following a transient focal cerebral ischemia.

Material and methods: Mild and severe ischemia were induced in rats by a transient occlusion of the middle cerebral artery for 30 and 120min, respectively. GABA$_A$ receptor δ mRNA (qPCR) and protein expression (western blotting) were studied at different reperfusion times (2h, 1d, 7d and 30d).

Results: We found a transient decrease of GABA$_A$ receptor δ mRNA expression in the injured hemisphere in the first post-ischemic week following mild as well as severe ischemia. Investigation of the GABA$_A$ receptor δ expression on protein level revealed a down-regulation at 1d up to 7d following severe ischemia and a decreased expression restricted to 7d post-injury following mild stroke. Severe ischemia led to a stronger down-regulation of GABA$_A$ receptor δ mRNA and protein. Normalization of the data to ß-III-Tubulin as a neuronal marker displayed a neuron-specific down-regulation of GABA$_A$ receptor δ expression.

Conclusions: Our study revealed a transient neuron-specific down-regulation of GABA$_A$ receptor δ following focal cerebral ischemia. The decreased expression of GABA$_A$ receptor subunit δ provides first hints of an impaired tonic inhibition following stroke. Further studies will need to assess functional consequences.

PP I-22

Effects of ziziphus jujuba fruit extract on oxidative stress and spatial memory impairment induced by chronic ethanol treatment in the rat.

M. Moghaddasi Jahromi[a] and M. Taati[b]
[a]*Lorestan University of Medical sciences, Physiology, Khoramabad, Iran, Islamic Republic of*
[b]*University of Lorestan, Physiology, khoramabad, Iran, Islamic Republic of*

Introduction: Long term or excessive drinking alcohol damages retrospective memory, that is, the learning, retention and retrieval of previously presented materials. The present study investigated the effect of aqueous extract of Ziziphus jujuba fruit [ZJF], which has been suggested as an antioxidant, against ethanol-induced oxidative damage and spatial memory impairment. Methods: Male wistar rats were divided into four groups of 10 rats each as follows: the normal control group was administered saline via gavage, the ethanol control and ZJF group was administered ethanol (4 gr/Kg, 40% V/V in saline) and aqueous extract of ZJF (200mg/Kg) respectively. The combined

ethanol and ZJF group, was administered ZJF daily via gavage for 60 consecutive days 30 minutes prior the ethanol administration. Spatial learning performances was assessed after 60 days in the Morris Water Maze and the level of TBARS in the hippocampus was measured.Results:Chronic ethanol administeration increased TBARS levels in the hippocampus compared to control group ($p < 0.05$) and Combined ZJF extract-ethanol treatment caused a significant ($P < 0.05$) decrease of TBARS levels compared to the ethanol group. Extract of ZJF significantly ($p < 0.05$)improved the deficit in the spatial memory (escape latency and distance swam to find the submerged platform) due to chronic ethanol exposure and fruit extract alone had also a significant effect ($P < 0.05$) on water maze performances. conclusion: this study suggest protective role of ZJF in preventing memory loss and oxidative damage after chronic ethanol administeration and may also be useful as supportive adjuvant in the treatment of impaired memory function.

PP I-24

Correcting the OMEGA-6/OMEGA-3 ratio in the mice diet protects from mcao-induced lesion.

C. Nguemeni Yonga[a], N. Simon-Rousseau[b], C. Heurteaux[a] and N. Blondeau[a]
[a]*C.N.R.S, Institut de Pharmacologie Moléculaire et Cellulaire, Sophia-Antipolis, France*
[b]*ONIDOL, Paris, France*

Objectives: Populations of Western countries are severely deficient in omega-3 polyunsaturated fatty acids. The ratio between linoleic acid (LA) and α-linolenic (ALA) is higher than 10:1 in the dietary intake and the recommended ratio of 5:1 is never achieved. This imbalance is suspected to be a risk factor for stroke. Stroke is a major cause of mortality and morbidity associated with a significant socioeconomic cost and a marked patient/families burden. Because neuroprotective drugs failed in clinical trials, testing alternative strategies, such as nutraceuticals, are of considerable interest. Several epidemiologic studies in cerebral diseases advocated beneficial effects of diets rich in seafood and vegetable oils, rich in long-chain omega-3. Few things are known on the role of the precursor, alpha-linolenic acid (ALA). Therefore, our work aimed to design and compare preventive strategies by ALA supplementation either by injections or by diets on MCAO-induced lesions.

Material and methods: ALA supplementation was given by i) 500 nmole/kg of ALA injected on days 1, 3 and 7 or ii) experimental diets (ALA represents 0, 0.4, 0.8 or 1.4% total weight). Infarct size was measured 24h post-MCAO. Since, neuroplasticity stimulation and reduction of post-stroke depression improve stroke recovery, their pre-existing level may modify the impact of stroke. The potential effects of ALA on neuroplasticity were evaluated through the expression of proteins involved in synaptic functions and glutamatergic neurotransmission. For the sensibility to depression, Porsolt Forced Swim Test and Tail Suspension Test that are commonly accepted to predict antidepressant efficiency were used. To analyze whether ALA action was direct or not, neuroprotection and neuroplasticity were also tested *in vitro*.

Results: Three repeated ALA injections reduced by approximately 30% the post-ischemic infarct volume 24 hours post-MCAO. Diet with a ratio between LA and ALA below 3:1 achieved the same level of protection. Below this ratio, an increase in the ALA content of the diet didn't significantly increase the protection (0.8–1.9% of ALA). A significant reduced mortality rate, infarct size and increased probability of spontaneous reperfusion in the post-ischemic period were also observed with the 0.8% diet. Supplementation by injection induces neuroplasticity and antidepressant-like effects. These effects were only seen comparing diet lacking ALA with the richest in ALA content. ALA also promoted protection and neuroplasticity *in vitro*.

Conclusion: A direct role of ALA in stroke protection and neuroplasticity was established *in vitro* and *in vivo*. While the brain protection was also obtained with ALA supplementation through experimental diet, the effect on neuroplasticity and depression were slightly different of those observed with ALA injections. Taken together our findings provide new insights into the potential of ALA as nutraceutical aiding in stroke prevention and protection.

PP I-28

The influence of neuroprotective therapy on improvment of cognitive functions in patients with vascular dementia

I. Suvorova
Irkutsk State Institute Postgraduate Education, Neurology and Neurosurgery, Irkutsk, Russian Federation

Introduction: Vascular cognitive impairment and vascular dementia are important causes of cognitive decline in the elderly. The main aim of vascular dementia treatment to avoid and prevent the progression of cognitive impairment and progression of vascular dementia.

Objective: To study efficacy and safety of prolonged course of Neuroprotective therapy as Cerebrolysin in patients with vascular dementia.

Material and methods: 48 patients with mild and moderate vascular dementia aged from 55-79 years (20 man and 28 female, average age 64,3 $\pm$ 5,9 years) have been studied. Diagnosis as Vascular Dementia was determined in accordance with ICD-10 and NINDS-AIREN criteria. Clinical-neurological and MRI-investigation were performed for all patients. The study of clinical efficacy and safety of prolonged course of Cerebvrolysin therapy in vascular dementia was conducted during the open study. All patients with dementia were divided on two groups with mild and moderate dementia. The infusion of Cerebrolysin were assigned for them. The study period was 36 months and during the study period a five course of Cerebrolysin therapy were performed for 4 weeks of each course. Efficacy and safety of Cerebrolysin were assessed clinically and with a battery of widespread scales and neuropsychological tests.

Results: The results of our study are demonstrated a well safety and high tolerability of prolonged course Cerebrolysin therapy. The improvment of cognitive, functional and motor activities in patients with mild and moderate dementia indicate the high effectiveness of Cerebrolysin. A prolonged Neuroprotective therapy allows to prevent the progression of cognitive impairment in patients with vascular dementia, so, by the finish of 3-years study in 8 patients (34,8%) with mild dementia the MMSE summary point was more then 24 point, consequently, the moderate cognitive impairment were diagnosed for these patients; in 6 patients (26,1%) with moderate dementia the MMSE summary point was more then 19 point, thus, the mild dementia was diagnosed for these patients.

Conclusion: A prolonged Neuroprotective Cerebrolysin therapy allows to prevent the progression of cognitive impairment in patients with vascular dementia and performed of timely therapeutic actions for patients with vascular cognitive impairment will allow to avoid of vascular dementia progression.

PP I-29

Middle cerebral artery occlusion: Confounding behavioural effects and welfare issues of external carotid transection.

R. Trueman[a], D. Harrison[a], D. Dwyer[b], S. Dunnett[a], M. Hoehn[c] and T. Farr[c]
[a]*Cardiff University, School of Biosciences, Cardiff, United Kingdom*
[b]*Cardiff University, School of Psychology, Cardiff, United Kingdom*
[c]*Max-Planck-Institute for Neurological Research, In-vivo-NMR Laboratory, Cologne, Germany*

Objective: The most common method of performing intraluminal middle cerebral artery occlusion (MCAo) requires permanent cauterisation or ligation of the external carotid artery (ECA) for insertion of the occluding filament. The ECA is extracranial and has several branches which supply the mastication and neck muscles, mandible, tongue, pharynx, scalp and ears. To date, few groups have examined the effect of ECA transection on outcome. Therefore, the objective of the current study was to assess the effect of ECA transection on behaviour and animal welfare following MCAo.

Materials and methods: Three different surgical methods for MCAo were compared: one with transection of the ECA, one which left the ECA perfused but required permanent ligation of the common carotid artery (CA), and a novel method that achieved reperfusion through both the ECA and CA. These MCAo lesion groups were compared to three corresponding sham groups, and a naive control group was also included to assess the effect of the sham surgery. Weight loss following surgery was recorded daily as a welfare measure, and all animals were assessed on battery of behavioural tests for three months following MCAo. These tests included the disengage task, adhesive removal, adjusting steps, vibrissae evoked paw placing, apomorphine induced rotations, skilled paw reaching and detailed analysis of drinking behaviour. Lesion volume was determined by both MRI and standard histological methods and further histological analysis on mastication muscles was also carried out.

Results and conclusions: As expected, ECA transection did not influence lesion size amongst the groups. However, clear detrimental effects of ECA transection were seen on some behavioural and welfare measures in both the MCAo and sham animals with ECA transection. This indicates that this surgical method may add

confounding factors to behavioural studies and compromise animal welfare.

PP I-30

Adverse reactions of pregabaline at pain policlinic patients

C. Usta and M. Akbas
akdeniz universty, pharmacology, antalya, Turkey

Introduction: Pregabaline is a new agent used for the treatment of pheripheral neuropathic pain. In this study for 9 months period we aimed to investigate the advers effects of pregabaline on the neuropathic pain patients.

Methods: After the examination of patients at pain policlinic, advers effects were asked on their first control.

Results: Pregabalin's advers effect has been determined 38 of . . . patients whom were admitted to policlinic. These advers effects are; (1) , dizziness (6), vertigo (15), somnolance (2), nausea vomiting (3), hypotension (2), headache (2), weakness (3), hallucination (2), extremity edema, and constipation (1).

Conclusion: Pregabaline's advers effects are important to determined because it is used for a long time for chronic pain patients. In this study pregabaline's adverse effects were detected but those were not very important but in some patients pregabaline administration had to be ceased.

PP I-31

Transthyretin: Regulation of 14-3-3ζ levels- a role in neuroprotection?

M. Vieira[a,b] and M.J. Saraiva[a,b]
[a]*Instituto de Biologia Molecular e Celular, Molecular Neurobiology, Porto, Portugal*
[b]*Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal*

Background: Transthyretin (TTR) is a 55-kDa homotetrameric protein synthesized mainly in the liver, eye, and choroid plexus, and its main function is the transport of thyroxin (T_4) and vitamin A (retinol) associated with the retinol binding protein (RBP). Recent findings have shown that transthyretin is a potent scavenger of Abeta peptide deposits, suggesting a possible neuroprotective role for TTR in neurodegenerative processes, such as Alzheimer Disease (AD).

14-3-3 is a family of highly conserved, ubiquitously proteins that are encoded by separated genes. There are seven known mammalian 14-3-3 isoforms (β, ε, γ, η, σ, τ, ζ). These proteins participate in a large amount of biological processes, including neuronal development, cell growth control, differentiation, apoptosis and regulation of ion channels. In Alzheimer disease 14-3-3 was positively marked in neurofibrillary tangles (NFTs).

Two-dimensional electrophoresis studies showed that the hippocampus of TTR null mice, have less 14-3-3ζ compared to WT mice.

Objective: The aim of this study was investigate the regulation of 14-3-3ζ levels by TTR.

Material and methods: TTR WT, TTR KO and heterozygous animals of different ages were used. We analyzed the levels of 14-3-3ζ in the hippocampus by immunohistochemistry, western blot, RT-PCR and in primary cultures in presence/absence of TTR.

Results: Levels of 14-3-3ζ in the hippocampus by western blot of 6 month old WT, KO and Heterozygous mice showed a dose/response relation, because the levels of 14-3-3ζ were lower in TTR KO than in TTR heterozygote/s which were lower than in TTR WT mice. These results showed that TTR is important to the expression of 14-3-3ζ; however this regulation does not occur at the transcriptional level, as comparative RT-PCR analyses between the 3 strains of mice showed no differences.

To investigate if the regulation of expression of 14-3-3ζ was age dependent we compared protein levels by western blotting studies in 3, 6 and in $>$ 12 month animals; in 3 month animals there is reduction of 50% in the expression of 14-3-3ζ levels in hippocampus of TTR KO mice as compared to WT littermates. At 6 months the reduction was approximately 32%. In 12 month animals no difference between WT and TTR KO mice was evident. These results were confirmed by semi-quantitative immunohistochemistry.

To explore the relationship between 14-3-3ζ and TTR in hippocampus we used primary cultures of hippocampal neurons from TTR KO animals. Cells were treated with TTR WT serum, TTR KO serum, and TTR KO serum + recombinant TTR WT. In TTR KO neurons, cells treated with KO serum had lower levels of 14-3-3ζ than cells treated with WT serum; addition of TTR WT to KO serum resulted in increased of 14-3-3ζ levels.

Conclusions: We suggest that expression of 14-3-3ζ is age dependent and can be influenced by TTR. Though

the mechanisms underlying TTR neuroprotection are largely unknown, they might encompass regulation of 14-3-3ζ expression.

PP I-32

Chronic hyperperfusion and angiogenesis follow sub-acute hypoperfusion in the thalamus of rats subjected to focal cerebral ischemia

P. Yanev
University Medical Center Utrecht, Image Sciences Institute, Utrecht, Netherlands

After focal ischemia in rats, cerebral blood flow (CBF) is disrupted in peri-infarct regions. Remote brain areas connected to the primary infarct are, however, also affected.

Here we examined long-term hemodynamic and cerebrovascular changes in the thalamus after middle cerebral artery occlusion (MCAO) in rats. CBF as quantified by ASL MRI decreased in the ipsilateral and contralateral thalamus 2 days after MCAO. Partial recovery of thalamic CBF occurred by day 7 and thereafter the ipsilateral thalamus was chronically hyperperfused compared to its contralateral side, at 30 days and 3 months after MCAO. This was in contrast to permanent hypoperfusion in the ipsilateral cortex.

Angiogenesis was indicated by endothelial cell (RECA-1) immunohistochemistry showing an increased number of blood vessel branching points in the ipsilateral thalamus at the end of the 3 month follow-up. Only transient IgG extravasation was observed in the thalamus, which indicates that the blood-brain barrier did not leak beyond day 2 after MCAO. Angiogenesis was preceded by a transient increase in expression of angiogenesis related cadherin adhesion molecules, cadherin-7 and protocadherin-1. Parallel increases in thalamic angiogenesis, CBF and impaired forelimb use in MCAO rats suggest restorative function for angiogenesis.

In conclusion, thalamic pathology after MCAO seems to involve long-term hemodynamic changes and angiogenesis through upregulation of developmental vascular adhesion factors. Post-ischemic angiogenesis in the thalamus represents a novel form of remote plasticity, which may support removal of necrotic brain tissue and also aid functional recovery.

Topic: Regeneration and Recovery

PP I-2

Repeated transient sulforaphane stimulation in astrocytes leads to prolonged nrf2-mediated gene expresion and protection from superoxide-induced damage

H. Andersson[a], P. Bergström[b], Y. Gao[b], J.-O. Karlsson[c], C. Nodin[a], M.F. Anderson[a], M. Nilsson[a] and O. Hammasten[b]
[a]*Center for brain repair and rehabilitation, Dep Neuroscience and Physiology, Gothenburg, Sweden*
[b]*Intstitute of Biomedicine, Dep of clinical chemistry and transfusion medicine, Gothenburg, Sweden*
[c]*Intstitute of Biomedicine, Dep of Medical Chemistry and Cell Biology, Gothenburg, Sweden*

Oxidative stress is one of the main causes of tissue damage following ischemic insults in the brain. Nrf2 is a transcription factor responsible for much of the inducible cellular defense against oxidative stress. Nrf2 can also be activated by xenobiotics like sulforaphane, a component highly enriched in cruciferous vegetables such as broccoli. Sulforaphane is cleared from the body within a few hours but can induce long-term protection from oxidative stress.

Objective: Here we have examined whether this prolonged protection is explained by a slow down-regulation of the Nrf2 response. Furthermore, we examined the hypothesis that repeated transient sulforaphane stimulation results in an accumulation of Nrf2-mediated gene expression.

Material and methods: The kinetics of sulforaphane-induced Nrf2 response was studied in astrocytes, a cell type known to be highly involved in the defense against oxidative stress in the brain.

Results and conclusion: Sulforaphane stimulation for 4 h induced an Nrf2-dependent increase of Nqo1 and Hmox1 mRNA that remained elevated for 24 h, and the corresponding proteins remained elevated for over 48 h. In addition, peroxide-clearing activity and the levels of glutathione were elevated for more than 20 h after stimulation for 4 h with sulforaphane, resulting in an increased resistance to superoxide-induced cell damage. Repeated sulforaphane stimulation resulted in an accumulation of mRNA and protein levels of Nqo1 and a persistent cell protection against oxidative damage. These findings indicate that brief stimulation of the

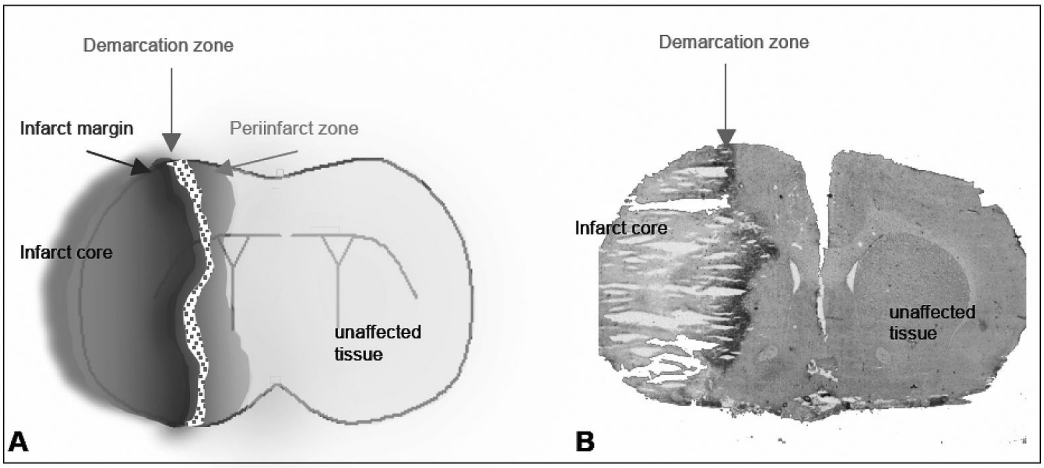

Fig. 1. **A:** Sketch of the demarcation zone separating vital from necrotic tissue; **B:** Immunohistochemical NG2-staining reveals the demarcation zone after permanent MCAO.

Nrf2 pathway by sulforaphane results in long-lasting elevation of endogenous antioxidants in astrocytes and that part of this response can be built up by repeated transient stimulation.

PP I-3

NG2-positive cells separate vital from necrotic tissue after focal cerebral ischemia

H. Claus[a,b], M. Walberer[a,b], M.-L. Simard[a], B. Emig[a], M.A. Rueger[a,b], G.R. Fink[a,c] and M. Schroeter[a,b]
[a]*University Hospital of Cologne, Department of Neurology, Cologne, Germany*
[b]*Max-Planck-Institute for Neurological Research, Cologne, Germany*
[c]*Research Centre Juelich, Cognitive Neurology Section, Institute of Neuroscience and Medicine, Juelich, Germany*

Objectives: Focal cerebral ischemia induces neuroinflammatory responses that cause secondary tissue alterations beyond the primary site of lesion. Aim of this study was to specify the region differentiating vital from ischemic tissue. This newly defined 'demarcation zone' may discriminate between primary and secondary neurodegeneration.

Materials and methods: Male Wistar rats underwent transient (suture model) or permanent middle cerebral artery occlusion (embolisation of TiO2 macrospheres). Three animals per group were sacrificed for immunohistochemical analysis at day 3, 7, 14, and 28 after induction of ischemia. Active macrophages were detected by immunohistochemical stainings against CD68, CD8 and Iba1. Neuronal cells were identified by anti-NeuN and astrocytes by anti-GFAP. Proliferating or immature cells were visualized by anti-vimentin (VIM) and anti-NG2 (chondroitin sulphate proteoglycan 4).

Results: As revealed by NeuN-staining, no neuronal cells were obtainable in the infarct core. During the observation time a lessening extent of neurons appeared in the emerging infarct-surrounding scar of astrogliosis. At the pannecrotic infarct-margin a rim of CD8-positive cells was facing towards the 'demarcation zone' between necrosis and gliosis. Within the demarcation zone, we detected NG2-positive cells. These were most prominent at day 7 and disappeared by day 28 after induction of ischemia (see figure). Simultaneously, there was evidence of VIM-, Iba1- and CD68-positive cells in the demarcation zone. However, in contrast to NG2-positive cells, they invaded the necrotic infarct core in the course of 8 weeks post ischemia.

Conclusions: Our findings characterize multiple infarct and periinfarct subareas, distinguished by specific patterns of neuroinflammation. Macrophages invade the infarct core to phagocytose necrotic debris. CD8-positive cells line up at the severely damaged margin of necrosis. Vital gliotic tissue contains the primary site of lesion. Most interestingly, we newly describe a population of NG2-positive cells in the demarcation zone that dynamically separate vital from necrotic tissue. Those proliferating cells most likely impact on regenerative processes and stabilize the lesion's contain-

ment. We thus speculate that events occurring within the demarcation zone affect processes of secondary neurodegeneration.

PP I-4

Nigral 6-OHDA-lesion in mice leads to a phenotypic shift of striatal neurons into tyrosine hydroxylase containing cells

M. Duckert, O. Schmitt, A. Wree and S. Haas
Medical Faculty/University of Rostock, Institute of Anatomy, Rostock, Germany

We established a mouse model of Parkinson's disease by injecting 6-OHDA into the right medial forebrain bundle. In these mice, contrary to Hemiparkonsian rats, we documented the appearance of tyrosine hydroxylase (TH) containing neurons in the dopaminergic deafferentiated striatum. This is of special interest, because TH is the pacemaker enzyme of dopamine synthesis. In this study we investigated whether these TH-ir neurons were newly generated or if reprogramming mechanisms of existing striatal neurons were leading to a phenotypic shift.

C57BL/6-mice ($n = 16$) were lesioned by a stereotactic 6-OHDA-injection into the right medial forebrain bundle. Three days prior to the lesion all animals received daily injections of the S-phase marker BrdU for up to one week. One group of animals was perfused four days after lesion. Three months after lesion apomorphine-induced rotations were evaluated in the second group to document successful lesions and finally this mice were also perfused with 3.7% PFA and immunohistochemistry to visualize TH-ir neurons or glial cells in combination with BrdU-ir cell nuclei in brain sections was performed with both groups.

Four days after lesion the unilateral dopaminergic cell loss in the substantia nigra and the loss of TH-ir terminal fibers in the striatum was clearly detectable. At this time point numerous TH-ir neurons with various processes could be observed in the lesioned striatum. Three months post lesion the unilateral dopaminergic cell loss in the substantia nigra and the decreased amount of TH-ir fibers in the striatum was more pronounced. This unilateral striatal dopaminergic deafferentiation was also demonstrated by the amounts of apomorphine-induced contralateral rotations (16.4 ± 2.5 rotations per min). Whether at 4 days postlesion nor at three months postlesion TH-BrdU co-localized neurons were detectable in the lesioned striatum. Here, only glial cells co-expressed BrdU. The amount of BrdU-ir cells in the lesioned hemisphere was significantly higher in the lesioned striatum, compared to the intact striatum where only single glial cells contained BrdU.

TH-ir neurons in the lesioned striatum were detectable very early post-lesion and did not contain BrdU. Therefore, these now appearing cells have not been generated by adult neurogenesis. However, the fact that postmitotic neurons in the adult brain perform a phenotypic shift is a very interesting phenomenon. Moreover, because TH is the pacemaker enzyme of dopamine synthesis the further question is if these cells express more dopaminergic markers and are able to compensate the lesion effect. This issue and the question from which pool of existing striatal neurons the TH-ir neurons were derived remains unclear and will be further investigated.

PP I-5

MICE differ from rats: Detection of tyrosine hydroxylase containing neurons in the mouse striatum after 6-OHDA-injection

S. Haas, A. Hilla, D. Reinhardt, O. Schmitt and A. Wree
Medical Faculty/University of Rostock, Institute of Anatomy, Rostock, Germany

Our objective was to establish a mouse model of Parkinson's disease by transferring the well known 6-OHDA-injection protocol from rats to mice. A mouse model is of special interest, because by using transgenic or knock-out animals the effects of various factors on such a dopaminergic deafferentiation could be investigated.

C57BL/6-mice were lesioned by stereotactic 6-OHDA-injection (5 μg/2 μl) into the right medial forebrain bundle. One, 2 and 3 months after lesion apomorphine- and amphetamine-induced rotations were evaluated and forepaw preferences of lesioned mice were compared with age matched intact controls. Three months after lesion, mice were perfused with 3.7% PFA and immunohistochemistry with various primary antibodies to visualize tyrosine hydroxylase immunoreactive neurons (TH-ir) in brain sections containing the substantia nigra and the striatum was performed. TH, the pacemaker enzyme of dopamine synthesis, is widely used to detect dopaminergic neurons.

All different primary antibodies in combination with appropriated secondary antibodies showed specific labelling and no cross reaction.

In the apomorphine test we observed significantly different rotational behaviours during one to three months post lesion: one group demonstrated rapidly increasing numbers of high contralateral rotations over time, whereas the other group showed low and stable amounts of rotations. However, to our surprise the ipsilateral rotations in the amphetamine test were not as high as expected and finally all animals performed contralateral rotations. Lesioned animals used their right forepaws significantly more often as compared to the intact mice. Unbiased stereologic counting of TH-ir neurons in the substantia nigra revealed a significant unilateral dopaminergic cell loss of about 90% in all lesioned mice. There was no significant difference between high or low rotating mice. As expected, the dopaminergic deafferentiation of the ipsilateral striatum resulting in loss of TH-ir nerve terminals was also found. However, to our surprise in the dopaminergic deafferentiated striatum we observed now TH-containing neurons. In the striatum of high rotating animals we found about 7594 ($\pm$ 607) TH-ir neurons, whereas in the low rotating mice only 5297 ($\pm$ 1141) TH-ir cells could be detected. In Hemiparkinsonian rats we never observed such TH-ir perikarya in the dopamine-deafferentiated striatum.

If the striatal TH-ir neurons release dopamine and if they are able to compensate the loss of dopaminergic neurons from the substantia nigra will be further investigated. We conclude, that plasticity of striatal neurons in the mouse brain considerably differs from that of the rat. The underlying mechanisms are yet unknown.

PP I-6

Transplantation of human umbilical cord blood neural stem cells supported by gelatin/laminin scaffolds into focal ischemic brain injuried rats

A. Jablonska[a], A. Sarnowska[a], H. Winiarska[a], E. Wanacka[a], M. Dainiak[b], F. Plieva[b], H. Jungvid[b], K. Domanska-Janik[a] and B. Lukomska[a]
[a]*Mossakowski Medical Research Centre Polish Academy of Sciences, NeuroRepair, Warsaw, Poland*
[b]*Protista Biotechnology AB, Lund, Sweden*

Neural stem cells are potentially promising treatment strategy for neurological disorders. Among variety of proposed procedures, one of the most promising seems to be artificial tissue transplant preparation which would support proper cell-cell interactions prior to- and after grafting. Recently, we showed that human umbilical cord blood neural stem cells (HUCB-NSC) forming three-dimensional (3D) aggregates with human keratin scaffolds differentiated *in vitro* to the level enabling them to create functional neuronal network (Jurga, et al, 2009). *The goal of the studies* was to analyze HUCB-NSC behavior in the milieu of brain tissue after their transplantation on gelatin/laminin scaffolds.

Materials and methods: HUCB-NSC (5×10^5cells/ml) were cultured in serum free medium (DMEM/F12+ B27+EGF) for 3–5 days in the presence of high cross-linked gelatin scaffolds covalently linked with laminin (size $2 \times 2 \times 2$mm). Then 3D-formed aggregates have been transplanted into the cortex of normal or focal ischemic brain injuried rats. The brain ischemic insult was performed by stereotactic injection of Na/K ATP-ase inhibitor - ouabain (OUA) (1ul/50nmol) into the striatum of adult Wistar rats. HUCB-NSC 3D aggregates were transplanted 3 days after the OUA application. At 7 day thereafter rat brains were removed and immunohistochemical analysis was performed.

Results: Phase contrast microscopy images of HUCB-NSC 3D aggregates based on scaffolds revealed high efficiency of cells attached to the scaffold (460cells/mm^2) and migrated into the core of it. Within 5 days of *in vitro* culture HUCB-NSC present in 3D aggregates displayed mitotic activity (Ki67$^+$) and expressed neuronal markers (NF200$^+$). 7 days after transplantation into the cortex of intact rats engrafted HUCB-NSC remained inside the scaffolds with no sign of migration into the brain parenchyma. The donor cells survive and proliferate within the scaffolds but they do not show neural markers. HUCB-NSC transplanted as 3D aggregates into the brain of OUA-injured rats revealed high mitotic activity. The subsets of HUCB-NSC visible inside the scaffolds expressed undifferentiated neural progenitor marker (Nestin$^+$) and more mature neuronal marker (NF200$^+$). However, HUCB-NSC transplanted as 3D aggregates into the brain of intact or OUA-injured rat brain stimulate immune reaction of the host. The accumulation of macrophage/microglia (ED1$^+$) in the tissue and activation of astrocytes (GFAP$^+$) was observed.

Conclusions: Gelatin/laminin scaffolds provide the platforms to guide cellular organization of transplanted HUCB-NSC. They also protect seeded inside exogenous cells from the harmful effect of host immune cells and may allow the diffusion of nutrients and other fac-

tors to propagate cell survival, proliferation and differentiation. Though, transplanted cells as 3D aggregates did not migrate outside the scaffolds nor differentiate into mature neurons.

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PP I-7

Validation of neurofunctional tests for stroke in spontaneously hypertensive rats

A. Kranz[a], S. Braun[a], J. Okoniewski[a], J. Boltze[a,b] and D.-C. Wagner[a]
[a]*Fraunhofer Institute, Department of Ischemia Research, Leipzig, Germany*
[b]*Translational Centre for Regenerative Medicine, Leipzig, Germany*

Objectives: Animal models of stroke were regularly used for the preclinical evaluation of novel treatment approaches. Neurofunctional tests constitute the translational correlate for a clinical improvement of stroke patients. Hence, the quantification of neurological deficits is one of the most important endpoints in preclinical stroke research. However, a systematic validation of the commonly used behavioral tests is still lacking, especially for premorbid animals such as spontaneously hypertensive rats. Here we aimed to validate the accurateness of three sensorimotor test systems to detect neurological deficits after cerebral ischemia in premorbid rats.

Methods: Thirty young adult spontaneously hypertensive rats were randomly distributed to the stroke or the sham group. Animals from the stroke group were subjected to permanent middle cerebral artery occlusion (MCAO) and sham animals were exposed to the same procedure except the artery occlusion. An investigator who was blinded to the group allocation performed regular measurements of the neurological deficits using the following tests: (1) adhesive removal test (ART) and (2) cylinder test (CYT) at days 3, 10, 17, 24 and 31; (3) ladder rung test (LR) at days 3 and 31. An area under the curve (AUC) was calculated from each test result and averaged for the according experimental group. Differences between the experimental groups were tested using a t-test or u-test. Using the obtained standard deviations and postulate an expected difference of the mean of 15%, we further calculated the required group sizes for attaining a test power of 80%.

Results: Each of the three behavioral tests showed a significant difference between the MCAO and the sham group. This effect was more pronounced for the ART and the LR compared to the CYT. The power calculation revealed the necessity to include large group sizes in order to detect a 15% difference of the mean. The determined power curves further provides a second quality survey for behavioral tests since the required group sizes varied by the factor 10 between the different test systems.

PP I-9

Liver X receptor beta activation promotes lipid microdomain formation and apolipoprotein e secretion after experimental stroke

E. Kuric, K. Ruscher and T. Wieloch
Lund University, Experimental Brain Research, Lund, Sweden

Objective: Mechanisms are required to scavange the massive release of cellular lipids which otherwise significantly contribute to the progression of neuronal death and infarct expansion after stroke. The transcription factor Liver X receptor (LXR) regulates the expression of lipid transport proteins and is up-regulated following experimental stroke. The present study was conducted to evaluate if LXR is involved in lipid transport mechanisms in animal models of stroke.

Methods: Male spontaneous hypertensive rats were subjected to permanent occlusion of the middle cerebral artery (MCAO). After selective sorting, animals were assigned into standard cages or enriched environment cages for 3 days. Complementary, primary cultures of cortical astrocytes were exposed to combined oxygen glucose deprivation (OGD). DNA binding activity and levels of LXR was analyzed from the infarct core/ peri-infarct border zone and posthypoxic astrocytes by gel shift assay and Western blotting, respectively. Lipid microdomains were extracted to determine the integration of CD63 and apolipoprotein E.

Results: Here we show that activation of the transcription factor liver X receptor beta (LXRβ) promotes the formation of lipid microdomains in astrocytes after combined oxygen glucose deprivation. Accompanied, we observed an accumulation of the cholesterol binding protein ApoE in astrocytic galactosyl-

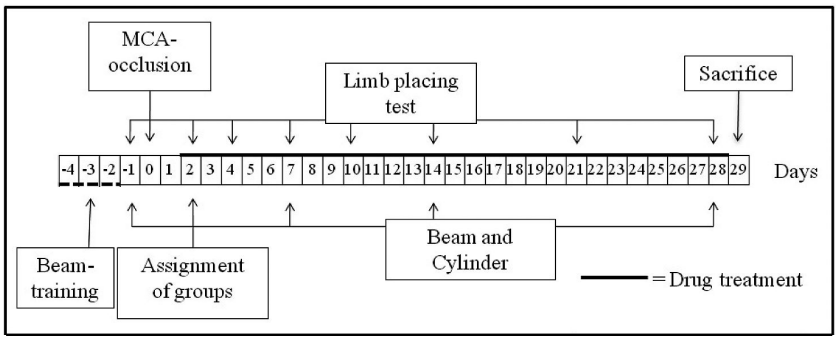

Fig. 1.

cerebroside enriched lipid microdomains and an increased secretion of ApoE to CD63 enriched exosomes, both facilitated by the specific LXR agonist N-(2,2,2-Trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl] benzenesulfonamide (T0901317). In rats housed in an enriched environment which significantly improves functional recovery, elevated LXRb activation and increased levels of lipoprotein particles in the peri-infarct area strongly corroborate an optimized clearance of free lipids in the ischemic hemisphere.

Conclusion: Our data support the notion that lipid clearance mechanisms involving LXRb and the cholesterol binding protein ApoE are activated in the peri-infarct infarct core border zone and associated with an improved recovery after experimental stroke.

PP I-10

Bepridil, a non-selective calcium channel blocker, augments thalamic pathology and improves functional recovery in mcao rats

A. Lipsanen, T. Sarajärvi, M. Hiltunen and J. Jolkkonen
University of Eastern Finland, Clinical medicine-neurology, Kuopio, Finland

Objectives: We have previously shown increased amyloid precursor protein (APP) and b-amyloid (Ab) and calcium depositions in the ipsilateral thalamus in rodents following focal cerebral ischemia [1,3]. Interestingly, calcium and Ab showed an overlapping distribution pattern in the thalamus of rats subjected to middle cerebral artery occlusion (MCAO) [2]. The aim of this study was to investigate whether chronic treatment with bepridil, a non-selective calcium channel blocker, would decrease calcium and amyloid load in the thalamus and improve behavioral outcome in MCAO rats.

Material and methods: Eighteen male Wistar rats (250–340 g) were subjected to sham-operation or transient MCAO (120 min). Bepridil (50 mg/kg, p.o., once a day) was administrated for 26 days starting the administration two days after MCAO ($n = 5$). Sham-operated ($n = 6$) and MCAO control rats ($n = 7$) were treated with the vehicle. Behavioral impairment was assessed using the cylinder, tapered/ledged beam and limb placing tests (Fig. 1). After the follow-up, animals were sacrificed for analysis of calcium, Aβ_{40} and Aβ_{42} levels.

Results: Bepridil decreased calcium ($p < 0.05$), soluble Aβ_{40} ($p < 0.05$) and Aβ_{42} ($p < 0.05$) in the ipsilateral thalamus compared to vehicle treated MCAO rats. Bepridil treatment did also improve forelimb use in MCAO rats ($p = 0.05$). Other behavioral tests showed no significant differences between experimental groups.

Conclusion: Prevention of secondary pathology in the thalamus by calcium channel blockers may provide a novel treatment to facilitate functional recovery after stroke.

Reference

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PP I-11

Stimulation of posttraumatic regeneration of peripherial nerve and cytoactive composite material LitAr

I.I. Markov[a], S.D. Litvinov[b,c] and S.M. Sergeyev[a]
[a]*samara State University of Medicine, Anatomy, Samara, Russian Federation*
[b]*samara State Aerospace University named after S.P. Korolyov, Materials of Science, Samara, Russian Federation*
[c]*Russian Agrarian University* (*The K. Timiryazev Moscow Agricultural Academy*), *Center of Nanobiotechnologies, Moscow, Russian Federation*

Ideà: The restoration of damaged peripheral nerves in the area of critical diastases is one of the most complex problems of modern reconstructive microsurgery. The present study was performed for the study of the possibility of stimulation of posttraumatic regeneration of peripheral nerves in the area of diastasis with the material LitAr in the keratin sheath.

Materials and methods: In experiments on rats in the area of diastasis (0,5–1,0 cm) of the sciatic nerve the device (heterograft, design) was placed. It consisted of the cytoactive composite material LitAr (it had in its composition nanodimensional crystals of hydroxy- or hydroxyfluorapatite) placed in a biodegrading cylindrical tube (Fig. 1). The ends of the transected nerve were fixed to the tube with the help of surgical glue. Also clinical applications ($n = 5$) for the elimination of diastasis of the human median nerve were conducted.

Results: Within the 15 days after the reconstruction of the diastasis zone with the help of heterograft we could observe the separate implant biodegradation and the formation of the cell regenerate. By the 60th day the heterograft was almost completely biodegraded and on the place of diastasis a fragment of the sciatic nerve was formed, which consists of amyelinic ($\sim 71\%$) and myelin fibers ($\sim 68\%$) of their number in the intact sciatic nerve (Fig. 2. Histostructure of the restored fragment of the operated sciatic nerve. 1) nerve fasciles; 2) cribriform connective tissue; col.: van Gieson, 400 x mag.)

Conclusions: The keratin heterograft filled with LitAr has the advantage to the available material conductors. The regenerative axons are placed along the biopolymer fibers of the cytoactive material LitAr. The recovery of sensory and motor functions of the operated limb of rats by the 60-th day is reliably confirmed by the fact of the normalization of the morphological and physiological status of its nerve fibers.

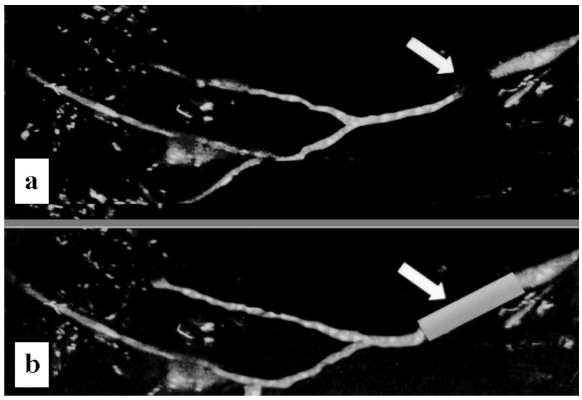

Fig. 1.

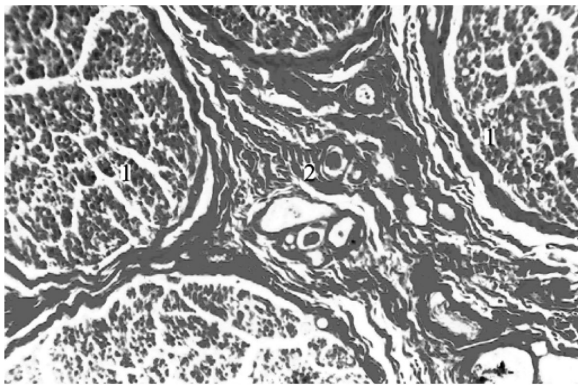

Fig. 2.

PP I-12

Transplantation of umbilical cord blood cells after perinatal hypoxic-ischemic brain injury – positive effects on the damaged tissue

K. Rosenkranz[a], S. Kumbruch[b], K. Marschner[c], R. Dermietzel[b], K. Markus[a] and C. Meier[d]
[a]*Ruhr-Universitaet Bochum, Functional Proteomics, Medical Proteome Center, Bochum, Germany*
[b]*Ruhr-Universitaet Bochum, Neuroanatomy and Molecular Brain Research, Bochum, Germany*
[c]*St. Elisabeth Hospital, Gynecology and Obstetrics, Bochum, Germany*
[d]*Universitaet des Saarlandes, Anatomy and Cell Biology, Homburg/Saar, Germany*

Objective: 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 neonatal rats

(Rosenkranz et al., 2010). Motor deficits resulting from the lesion were alleviated upon transplantation (Meier et al., 2006). However, the cellular and molecular mechanisms underlying these improvements of umbilical cells are still unknown. HUCB cells are capable of secreting substantial amounts of interleukins, growth factors, and chemokines in vitro (Neuhoff et al., 2007). As many of these proteins are renowned for their anti-apoptotic, angiogenic, and neuroprotective properties, it is possible that the release of these cytokines in vivo might mediate beneficial effects on the damaged host tissue.

Methods: The effects of transplanted umbilical cells on apoptosis, angiogenesis and neurogenesis after experimentally induced perinatal hypoxic-ischemic brain injury were analyzed by immunohistochemical staining, immunoblot and quantitative Realtime PCR analysis.

Results: Transplantation of hUCB cells led to a short-term increase of relevant growth factors at the lesion site. Furthermore transplanted hUCB cells caused a reduction of apoptotic cells and an increase of vital neurons. In addition an enhancement of angiogenesis could be observed after hUCB cell application.

Conclusions: In summary, the increase of neuronal growth factors in the lesioned brain and the observed positive effects on apoptosis, angiogenesis and neuroprotection after hUCB cell transplantation render the cytokine secretion of hUCB cells likely to mediate therapeutic effects in vivo.

PP I-13

Modeling of psychiatric disorders following ischemic brain injuries in mice

T. Stankovich and T. Deierborg
Lund University, Experimental Brain Research, Lund, Sweden

Objectives: Cerebrovascular injuries, such as ischemic stroke and cardiac arrest/cardiopulmonary resuscitation can lead to several neuropsychiatric disorders. Post-stroke depression, anxiety and fatigue are the most common psychiatric disorders following ischemic brain injuries. Our objectives are to use permanent focal brain ischemia (pMCAO) to the left or right hemisphere and global brain ischemia to study behavioral changes related depression and anxiety following ischemia.

Material and methods: To induce brain damage specifically in left and right primary motor/somatosensory cortex, the respective distal middle cerebral artery was occluded permanently by electro-coagulation after frontoparietal craniotomy. To induce global brain ischemia, affecting primarily striatum and hippocampus, we clamped the carotid arteries for 12 min. The behavioral test we used were: open field test to study motor activity and anxiety; the elevated plus maze for studying anxiety; forced swim test and tail suspension test to study depressive behavior in terms of immobility and the sucrose preference test to study anhedonia.

Result: We were able to detect a difference in behavior after left and right cortical injury. Right cortical injury resulted in significantly less immobility in the tail suspension test compared to injury in the left hemisphere. This difference in immobility was not found when the same animals were tested for immobility in the forced swim test. Mice with right cortical injury showed a significant higher motor activity in the open field test.

Mice subjected to global brain ischemia showed no difference in the open field test in terms of the time spent in the centre or close to the walls. Also there were no difference in, over all, motor activity. When the same mice were subjected to the elevated plus maze we detected a significant increased time that ischemic mice spent in the closed arms, compared to sham mice, suggesting anxious phenotype similar to what has been observed in patients that have suffered from global brain ischemia.

Conclusion: Stroke injury to the right cortical area resulted in less immobility in the tail suspension test compared to ischemic injury to the left hemisphere. This behavioral difference could possibly not be connected to a depressive phenotype but to an overall increase in motor activity.

Global brain ischemia in mice induced by bilateral artery occlusion reproduced an anxiety behavior that is known to affect patients that has successfully been resuscitated after heart arrest.

Topic: Regulatory Molecules and Drugs

PP IV-5

Toward neuroprotective mechanisms of hypobaric preconditioning: modification of group I mGluRs sistem

A. Belyakov[a], D. Semenov[a], M. Kislin[a], E. Tjulkova[a], T. Gluschenko[a], E. Salinska[b], J. Lazarewicz[b] and M. Samoilov[a]
[a]*Pavlov Institute of Phisiology of RAS, Laboratory of Regulation of brain neurons function, St.-Petersburg, Russian Federation*
[b]*Mossakowski Medical Research Centre of PAS, Laboratory of neurochemistry, Warsaw, Poland*

Objective: This study tested a working hypothesis that preconditioning hypobaric hypoxia modifies activity of group I metabotropic glutamate receptors (GI mGluRs) system, comprising their Ca^{2+} signaling, the receptors' protein level and spatial distribution.

Material and methods: We used adult male Wistar rats submitted *in vivo* either to severe hypobaric hypoxia (SHH, 3 hours, 180 torr), or to moderate hypobaric hypoxia (MHH, 3 times for 2 hours every 24 hours, 360 torr). The *ex vivo* methods include measurements of changes in the intracellular Ca^{2+} monitored with CTC and Fura-2 fluorescent probes evoked by the agonist of GI mGluRs in perfused slices of the piriform cortex, immuno(cyto)chemical analysis of the GI mGluRs proteins in the cortex and hippocampus and complex biochemical analysis of cellular and mitochondrial lipid peroxydation (LPO).

Results: Is was demonstrated that in the cortical slices of rats submitted 24 h earlier to SHH, stimulation with GI mGluRs agonist DHPG causes very significant increase in the level of bound $\tilde{N}a^{2+}$. In turn MHH resulted in significant decrease in bound $\tilde{N}a^{2+}$ level after DHPG administration, while the increases in free Ca^{2+} were enhanced. In case of SHH deep activation of LPO and accumulation of Schiff bases were detected, this can lead to structural and functional membrane damage.

Western blotting followed by immunocytochemical examination detected in MHH-preconditioned animals prevalence of expression of mGluR5 over mGluR1 in both brain areas studied; however in the cortex a general slight decrease in the immunoreactivity of GI mGluRs was noted, while in the hippocampus an increase in the level of mGluR5 predominated with a considerable decrease in the expression of mGluR1 in the ÑÀ1, ÑÀ3, ÑÀ4 areas. Immunocytochemical analysis of the cell body layers demonstrate increased expression of both GI mGluRs subtypes in the perinuclear area in the cortex and of mGluR5 in the hippocampus of MHH preconditioned rats. Besides western blotting revealed increased level of PLCβ1 in the nuclear fraction.

Conclusions. Injurious SHH induces massive Ca^{2+} imbalance including its influx to neurons and intracellular accumulation, as well as long-term changes in LPO and mitochondrial dysfunction associated with it. On the other hand preconditioning MHH induces adaptive changes including the increase in GI mGluRs-mediated release of $\tilde{N}a^{2+}$ from intracellular stores, prevalence of immunoreactivity of mGluR5 over mGluR1, and enhanced expression of these receptors in the perinuclear neuronal regions, most probably associated with intensification of transcriptional activity.

PP IV-6

Small rhogtpases in cerebral ischemia

S. Duque Santos[a], F. Neto[a], A.C. Fonseca[a], K. Lykke Lambertsen[b], T.S. Ribeiro[c] and J. Bettencourt Relvas[a]
[a]*Institute for Molecular and Cell Biology, Glial Cell Biology, Porto, Portugal*
[b]*Institute of Molecular Medicine, Neurobiology Research, Odense, Denmark*
[c]*Institute for Molecular and Cell Biology, Animal Facility, Porto, Portugal*

When the brain does not receive enough blood to maintain cellular homeostasis due to a cerebrovascular accident, an ischemic pathological cascade of events is initiated culminating in cellular death. Cerebral ischemia is the leading cause of death in Portugal (Correia et al., 2004) and remains a serious concern for public health as it affects cognition, behaviour and motor capacity resulting frequently in partial or total permanent incapacity.

The Rho-family of small guanosine triphosphatases (Rho GTPases) are a family of signalling molecules whose function is broad – cytoskeletal organization, cell cycle progression, cell adhesion, cell migration, transcriptional regulation and membrane transport pathways (Etienne-Manneville and Hall, 2002) and crucial for normal processing of extracellular stimuli.

In the central nervous system (CNS), Rho GTPases are essential regulators of neuronal morphogenesis, axonal growth and guidance, axonal ensheathment and myelination, and synapse formation (Luo, 2000). Under pathophysiologic conditions, such as in cerebral ischemia, activation of Rho GTPases is thought to inhibit adult CNS regeneration. In this study, we investigate the function of Rho GTPase signaling in brain ischemia and characterize the molecular players involved in this pathway.

Experimentally, cerebral ischemia was induced in mice by permanent occlusion of the middle cerebral artery (pMCAO) and the Rho GTPases pathway investigated by real-time PCR. Candidate Rho GTPases and their downstream effectors were eliminated in neuronal cell cultures by RNAi approaches and the effect of gene silencing evaluated after in vitro ischemia.

Such information is crucial to design better therapies for cerebral ischemia and to understand if the permanent and irreversible neuronal deficit caused by ischemia is affected by eliminating Rho GTPases expression and activity. This knowledge will be essential to interpret their functional roles in ischemia and whether rescuing neuronal cell death by interfering with the Rho GTPases pathway is possible.

PP IV-7

Nucleotides as neuroprotective drugs with activity as antioxidants and P2Y receptor ligands

D. Förster[a], B. Fischer[b] and G. Reiser[a]
[a]*Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany*
[b]*Bar-Ilan University, Department of Chemistry, Ramat-Gan, Israel*

Objectives: Reactive oxygen species (ROS) are becoming a growing object of research due to their versatile association with neurodegenerative disease and aging. Oxidative stress is involved in many neurodegenerative disorders and pathological conditions, like stroke or traumatic brain injury. The main sources of ROS are complexes I and III of the respiratory chain in mitochondria. Under normal conditions, ROS are scavenged by the antioxidant system, which might be overstressed by excessive ROS formation. Due to their high reactivity, ROS damage lipids, DNA and proteins, eventually leading to cell death. The focus of this study is to identify nucleotides which can act as potent antioxidant drugs and mediate neuroprotection via P2Y-R activation in brain or may act on mitochondria. This study is expected to enhance the understanding of the underlying mechanisms of neuronal protection.

Methods: In the first approach, reactive oxygen species were measured in isolated rat brain mitochondria (RBM) from 2-3 month old rats challenged with Antimycin A, a complex III inhibitor. ROS were detected by addition of superoxide dismutase (SOD) which converts superoxide radicals to hydrogen peroxide. In presence of hydrogen peroxide and horseradish peroxidase, Amplex Red is converted to fluorescent resorufin. Initial slopes of resorufin fluorescence graphs were determined in order to quantify the amount of ROS development.

Slice cultures are an established method to demonstrate protection or degeneration. Then, 12 day old organotypic rat hippocampal slice cultures (OHC) were deprived of oxygen and glucose (OGD) for 40 min and pre-incubated with ATP 24 h before and during OGD. Neuroprotective effects were investigated via propidium iodide (PI) fluorescence, which is increased in cell death.

Results and Conclusions: The presence of ATP, GTP, UTP, AP_3A, and AP4A attenuated ROS production in RBM to different degrees. The ATP-preincubated slice group showed lower PI fluorescence in comparison to control indicating a protection exerted by ATP. We conclude that nucleotides are potential drug candidates and sufficient to provide neuroprotection. Further results indicate antioxidant and even antiapoptotic action of these nucleotides. Involvement of P2Y receptors is suggested but the detailed mechanism still needs to be elucidated.

PP IV-8

Evaluation of neuroprotective properties of a continuously administered A_{2A} receptor antagonist in a rodent stroke model

U. Fronz[a,b], S. Weidlich[a,b], A. Kranz[a], K. Nieber[b], J. Boltze[a,c] and D.-C. Wagner[1]
[a]*Fraunhofer Institute for Cell Therapy and Immunology, Ischemia Research Unit, Leipzig, Germany*
[b]*University of Leipzig, Institute of Pharmacy, Leipzig, Germany*
[c]*Translational Centre for Regenerative Medicine, Leipzig, Germany*

Objective: The selective A_{2A} antagonist CSC (8-(3-chlorostyryl)-caffeine) has been shown to be neuroprotective when administered pre-ischemically in rodent models of transient cerebral ischemia, potentially due to reduction of excitotoxicity. However, the STAIR (Stroke Therapy Academic Industry Round Table) quality assurance criteria were not satisfactorily implemented in previous studies. Moreover, data on pharmacokinetics were not evaluated.

The current study investigated the neuroprotective effect of continuous administration of CSC under conditions simulating a clinically relevant scenario. Treatment was initiated after a two-hour time window and rigorous implementation of STAIR criteria, especially randomization, power controlled study design and blinded data analysis, assured the significance of the results.

Material and methods: Spontaneously hypertensive rats were randomly assigned to two groups and underwent permanent middle cerebral artery occlusion (MCAO). CSC (5 mg/kg/d) or the vehicle was continuously applied for three days using subcutaneously implanted osmotic minipumps. Correct pump function was controlled by residual volume measurement and determination of the serum CSC concentration by an appropriate HPLC method. Treatment efficacy was monitored by magnetic resonance imaging. Additionally, MAP-2/Fluoro-Jade C double staining, NeuN/Fluoro-Jade B double staining as well as MAP-2/GFAP/Iba-1 triple staining were carried out to show neurodegeneration and to characterize the infarct border zone.

Results: The results of this study imply that continuous application of CSC under STAIR conditions does not have an effect on lesion volume. This was also confirmed by the immunohistochemical examination. The HPLC analysis revealed a shift of the CSC peak in serum samples of treated animals. Mass spectrometric analysis found that a metabolite of CSC is responsible for the shorter retention time.

Conclusion: The chromatographic findings allow us to assume that extensive hepatic biotransformation of CSC leads to metabolites which may probably have lost their A_{2A} antagonistic character, resulting in decreased effectiveness. Furthermore, the study results suggest that A_{2A} antagonists in general seem to have a very narrow time window of less than two hours, depending on the early incidence of glutamate-mediated excitotoxicity after stroke. This could considerably limit their clinical benefit.

PP IV-9

Morphologic changes after intrastriatal botulinum toxin a injection for the purpose of treatment of parkinson's disease symptoms

A. Hawlitschka[a], O. Schmitt[a], V. Antipova[a], R. Benecke[b], M. Witt[a] and A. Wree[a]
[a]*University of Rostock, Institute of Anatomy, Rostock, Germany*
[b]*University of Rostock, Department of neurology, Rostock, Germany*

Objectives: Parkinson's disease is one of the most frequent neurodegenerative diseases. In Germany 300000–400000 people are affected. Systemic application of anticholinergic drugs ameliorates symptoms of Parkinson's disease (PD), but is hampered by adverse side-effects. Since botulinum toxin A (BoNT) blocks cholinergic transmissions locally we investigated in the 6-hydroxydopamine (6-OHDA) animal model of PD the hypothesis whether intrastriatal application of BoNT may contribute to an improved therapeutic concept of PD without systemic side effects.

Material and methods: In the first test series different doses of BoNT (100 pg, 1 ng and 2 ng) were applicated stereotacticaly in the right striatum of young adult rats and the brains were investigated 2 weeks, 1, 3, 6 and 12 month post injection. In a second test series animals were treated with BoNT 4 weeks after application of 6-hydroxydopamine for lesion of dopaminergic neurons of the substantia nigra (hemiparkinson model) and afterwards brains were examined. In addition to Nissl staining, immunohistochemical visualisation of cholinergic neurons via choline acetyle transferase (ChAT) and dopaminergic axons and terminals via tyrosine hydroxylase (TH) and stainings for synaptic proteins and cytoskelet proteins were performed. Furthermore we performed stereological investigations on BoNT treated brains and counted cholinergic cells to verify, whether there is a cytotoxic effect of BoNT.

Results: 1. It is possible to inject BoNT in the striatum in a narrow dose range. 2. The application of BoNT leads to dose dependent measurable morphologic changes in the striatum. 3. In the striata of treated animals are – in contrast to controls – many small round structures detectable, which are immunoreactive for ChAT or TH, which we called botulinum toxin induced varicosities (BiVs) at the moment. 4. The number of ChAT-immunoreactive neurons in the left and in

the right striatum remains unchanged after right-side striatal BoNT-injection.

Conclusion: It can be supposed, that the observed conspicuous structures are formed by a BoNT-induced retention of the synaptic vesicles in the boutons of the treated striata. Till now we could prove, that the cholinergic system and also the dopaminergic system are affected after local intrastriatal BoNT-injections. Further systems are probably also involved. Parallel performed behaviour studies confirm this thesis.

PP IV-10

The branched-chain fatty acids phytanic acid and pristanic acid can mediate intracellular Ca^{2+} signalling through activation of the free fatty acid receptor GPR40

N. Kruska and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objective: In diseases with peroxisomal impairment like Refsum disease, Zellwegers syndrome and a-methylacyl-CoA racemase deficiency the two branched-chain fatty acids phytanic acid (2, 6, 10, 14-tetramethylpentadecanoic acid) and pristanic acid (3, 7, 11, 15-tetramethyl-hexadecanoic acid) play an important role. The accumulation of phytanic acid and/or pristanic acid in plasma and tissue of patients suffering from these diseases with defects in the peroxisomal α- and/or ß-oxidation is a hallmark and seems to lead to manifold neurological symptoms and neurodegeneration. Although previous studies analyzing the toxicity of the two branched-chain fatty acids discovered that the toxic activity of phytanic acid and pristanic acid is mediated by multiple mitochondrial dysfunctions, the exact signalling mechanism through which toxicity is mediated for both fatty acids is still unknown. Therefore, we recently studied the effect of phytanic acid and pristanic acid on hippocampal astrocytes, neurons and oligodendrocytes and revealed that a cellular overload of phytanic and pristanic acid leads, in addition to mitochondrial dysfunctions, to severe Ca^{2+} deregulation via the intracellular $InsP_3$-Ca^{2+} signalling pathway in glial cells [1]. This observation suggests the involvement of a membrane receptor coupled to intracellular Ca^{2+} release. Here, we analyzed the ability of phytanic acid and pristanic acid to activate the free fatty acid receptor GPR40. GPR40 is a G protein-coupled receptor, which is activated by medium to long chain saturated and unsaturated fatty acids.

Materials and methods: To elucidate the potency of fatty acids, we investigated the changes in intracellular Ca^{2+} concentration in HEK293 cells transfected with the human GPR40 receptor by Fura-2 fluorescence analysis. Details were described in [2].

Results and conclusions: We demonstrated for the first time that the free fatty acid receptor GPR40 could be activated by the two branched-chain fatty acids phytanic acid and pristanic acid. Moreover, treatment of HEK-GPR40 cells with methyl derivates of these fatty acids causes no significant increase in intracellular Ca^{2+} concentration, which suggests that the GPR40 activation is due to an interaction of the carboxylate moiety of the fatty acids with the receptor. Our results indicate that the phytanic acid- and pristanic acid-mediated Ca^{2+} deregulation via the intracellular $InsP_3$-Ca^{2+} signalling pathway can participate through GPR40 activation. We suppose that the activation of the free fatty acid receptor GPR40 might be involved in the signalling cascade of fatty acid-mediated toxicity.

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PP IV-11

Very long chain fatty acids induce detrimental changes in cellular functions like loss of myelin cells in primary neural cells

N. Kruska, S. Rönicke and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: A major characteristic in diseases with peroxisomal disorders, like the severe hereditary neurodegenerative disease X-linked adrenoleukodystrophy (X-ALD) is the accumulation of very long chain fatty acids (VLCFA) in plasma and tissue of patients suffering from these diseases. X-ALD is caused by mutations in the ABCD1 gene, coding for the peroxisomal membrane transporter adrenoleukodystrophy protein (ALDP). The impaired function of ALDP leads to a failure in the degradation of VLCFA in peroxisomal ß-oxidation which is responsible for VLCFA accumulation. However, it is still unknown how elevated levels of VLCFA in patients are linked to the clinical symptoms of de-myelination, adrenal insufficiency and inflammation. Previously, we found that VLCFA exert a strong toxic activity leading to Ca^{2+} deregulation, mitochondrial dysfunction and cell death in mixed cultures of neural cells (astrocytes, neurons and oligodendrocytes). Strongest impairment was seen in the myelin-producing oligodendrocytes [1].

Material and methods: We used established cell cultures of primary rat oligodendrocytes and astrocytes, as well as primary astrocytes from control and ALDP-deficient mouse. Fluorescence-stained proteins were detected by confocal microscopy. Analysis of protein levels were performed by protein-biochemical methods. Specific fluorescent dyes allowed monitoring of cell physiological processes (mitochondrial membrane potential, ROS generation) in high temporal and spatial resolution by video fluorescence microscopy [2].

Results and conclusions: Here, we demonstrate that long-term application (up to 14 days) of VLCFA in mature oligodendrocytes leads to detrimental changes in the expression and localization of the myelin basic protein (MBP), which is essential for the cells to form the myelin sheath around neuronal axons. This result is consistent with the observed demyelination in X-ALD disease. Additionally, we studied the capability of VLCFA to induce the release of cytokines. In these experiments, we found that in primary astrocytes the release of GRO/CINC-1 and IL-6 was increased after stimulation with VLCFA. These findings indicate a clear connection between the accumulation of VLCFA and inflammation in X-ALD. To further investigate the changes in neural cells after VLCFA accumulation, we are studying the consequences of VLCFA exposure on brain cells derived from the ALDP-deficient mouse model. In this context, our results indicate that astrocytes from ALDP-deficient mice are much more vulnerable to VLCFA exposure than cells from control mice. Therefore, the ALDP-deficient cells are a suitable model for studying the human disease.

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PP IV-12

The neuroprotective effects of bilobalide in stroke are dose- and time-dependent

D. Lang and J. Klein
School of Pharmacy, Department of Pharmacology, Frankfurt am Main, Germany

Introduction: Bilobalide is one of the active components of the Ginkgo biloba leaf extract EGb761. It has been shown to exhibit neuroprotective effects in various cell culture models, a finding we could extend to hippocampal slice preparations: oxygen-glucose deprivation caused swelling of the slices and increased slice water contents with both events being completely prevented by bilobalide (1–10 μM).

Objective: The aim of this study was, firstly, to assess the neuroprotective effects of bilobalide in an in vivo model and, secondly, to explore the drug's mechanism of action.

Methods: To this end, we used the middle cerebral artery occlusion (MCAO) as a permanent stroke model. Female CD-1 mice (28–32g) received either vehicle or bilobalide (1–10 mg/kg i.p) 1h before, or 1h or 3hrs after stroke. Sham-operated mice underwent the same procedure as the control group, but the filament was not pushed to the origin of the middle cerebral artery. Motor coordination and balance, as assessed by chimney, corner and rotarod tests, were controlled before and 24hrs after the occlusion of the middle cerebral artery.

Results: Control animals suffered a large stroke extending from the forebrain to the cerebellum, with a major damage in striatum and hippocampus. In these mice sensorimotor deficits manifested themselves in postural asymmetries in the corner test and a decrease in their performance between presurgical and postoperative assessment while sham-operated animals did not show any significant impairment. In mice treated with bilobalide 10 mg/kg i.p. 1h before stroke the striatal area

was prominently protected, while only the hippocampal region showed an ischemic insult. In contrast, lower doses (0.1–3 mg/kg) or application of 10 mg/kg 1h or 3hrs after stroke had limited or no significant protective effects.

To test the bioavailability of the drug in the brain, we implanted microdialysis probes into the mouse striatum and collected microdialysis and blood samples during a 6 h period after the administration of bilobalide (10 mg/kg i.p.). In the microdialysates, maximum levels were reached 40 min after application and bilobalide remained detectable for up to 4 hrs. In animals undergoing MCAO, bilobalide was detectable when given prior to MCAO but bilobalide levels were close to the detection limit when given after MCAO. In experiments investigating the drug‘s mechanism of protection, we found out so far that bilobalide does not inhibit the glutaminase that generates glutamate from glutamine.

Conclusion: Our findings indicate a neuroprotective effect of bilobalide in higher concentrations, especially when given early in the stroke event.

PP IV-13

Protection of astrocytes is mediated by Protease-activated receptor 2 and α-crystallin

R. Li, T. Hanck and G. Reiser
Medizinische Fakultät der Otto-von-Guericke Universität Magdeburg, Institut für Neurobiochemie, Magdeburg, Germany

Objectives: Accumulating evidence suggested that a-crystallin (comprising aA-crystallin and aB-crystallin) and protease-activated receptor 2 (PAR-2) participate in neurodegeneration and -protection. Our previous report demonstrated that PAR-2 activation and high expression of a-crystallin rescue astrocytes from C2-ceramide and staurosporine treatment [1]. In the current study, we determined the influence of downregulation of a-crystallin on astrocytes' death. To understand the mechanism of cytoprotection of astrocytes by PAR-2 and a-crystallin, we investigated the role of p38, ERK and JNK in cytoprotection of astrocytes. Moreover, we measured the expression pattern of a-crystallin under PAR-2 activation. Finally, experiments of mimicking dephosphorylation/pseudophosphorylation of a-crystallin were set up to understand for requirement of the phosphorylation sites for cytoprotection.

Materials and methods: Staurosporine, C2-ceramide, PAR-2 activating peptide, SB203580, PD98059 and SP600125; Lactate dehydrogenase assay, siRNAs of aA-crystallin and aB-crystallin, Real-time polymerase chain reaction and western blot.

Results and conclusion: Our data indicate that after down-regulation of a-crystallin, cell death caused by C2-ceramide and staurosporine is higher than in normal astrocytes. Activation of PAR-2 increases the protective effect in astrocytes over-expressing aA-crystallin, but does not affect the protective effect in astrocytes over-expressing aB-crystallin. Application of specific inhibitors of p38 and ERK inhibits the protection of astrocytes by PAR-2 activation and high expression of a-crystallin. These results suggest that p38 and ERK mediate the protection of astrocytes by PAR-2 and a-crystallin. In addition, we found that PAR-2 activation increases the expression level of aA-crystallin and phosphorylation level of aB-crystallin at Ser59. Further experiments by mimicking phosphorylation or unphosphorylation of a-crystallin demonstrated that the phosphorylation of aA-crystallin at Ser122 and Ser148 and aB-crystallin at Ser45 and Ser59 is required for the protection. These data suggest that PAR-2 and α-crystallin are involved in survival of astrocytes by regulating the expression level and the phosphorylation status of a-crystallin, which is mediated by p38 and ERK. Our results reveal that p38 and ERK pathways are important for modulation of survival of astrocytes. The current findings help in uncovering the molecular mechanism of neurodegeneration and provide a new possible way to regulate astrocyte death.

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PP IV-14

Ethanol withdrawal-induced hypersensitivity to excitotoxic challenges – Neuroprotective effects of cannabinoids

M. Rubio[a], B. Roussel[a], J. Ramos[b], F. Docagne[a], D. Vivien[a], J. Fernandez-Ruiz[b] and C. Ali[a]

[a]*INSERM U919, UMR CNRS 6232, Caen, France*
[b]*Departamento de Bioquímica y Biología Molecular III y "Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas* (*CIBERNED*)*, Facultad de Medicina, Universidad Complutense, Madrid, Spain*

Excessive alcohol consumption, as well as alcohol withdrawal, are both associated to neurological syndromes. Chronic alcohol exposure leads to important neurochemical modifications, profoundly affecting GABAergic and glutamatergic neurotransmissions. As a result of these neuroadaptative mechanisms, a neuronal hyperexcitability develops, with strong repercussions when alcohol is stopped. Accordingly, we hypothesized that chronic alcohol consumption and withdrawal could lead to an increased sensitivity to excitotoxic challenges, as occurs in several conditions including ischemic stroke.

We thus developed a model of chronic alcohol exposure followed by alcohol withdrawal in pure murine neuronal cultures. Ethanol (100 mM) was administered once a day for 3 days and then removed by transferring cells to a basal culture medium for 48 hours. We first observed that ethanol withdrawal led to a 25% increase in NMDA (10 μM)-induced neuronal death. We then investigated the effect of the endocannabinoid system on this ethanol withdrawal-mediated potentiation of excitotoxic neuronal loss. The administration of the non-selective cannabinoid agonist HU-210 (1 μM) decreased NMDA-stimulated calcium influx and consequently decreased NMDA-induced neuronal death in alcohol exposed and withdrawn neurons. By contrast, the inhibition of the cannabinoid system with the CB_1 receptor antagonist rimonabant (SR141716) provoked an increase in NMDA-stimulated calcium influx and consequently an increase of neuronal death in alcohol exposed and withdrawn neurons. Our findings suggest that alcohol deprived patients could be more sensitive to excitotoxic damages, as occurs in pathological conditions such as stroke. Stimulation of the endocannabinoid system could represent an interesting neuroprotective strategy for these patients at risk.

PP IV-15

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

U.H. Schröder[a], M. Straßburger[a], J. Breder[a], C.F. Sabelhaus[a], S. Kahlert[b], G. Reiser[b] and K.G. Reymann[a]

[a]*Leibniz-Institut for Neurobiology, 1Project Group Neuropharmacology, Magdeburg, Germany*
[b]*Otto-von-Guericke University, Institute for Neurobiochemistry, 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 as yet.

In the present study we assessed the effects of inhibition of Na^+/H^+ exchangers on ischemic damage in organotypic hippocampal slice cultures (OSCs) from 10-day-old rats by propidium iodide staining and in acutely isolated hippocampal slices from adult animals by extracellular electrophysiology.

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 5'-(N-ethyl-N-isopropyl)-amiloride (EIPA) and 3-[2-(3-guanidino-2-methyl-3-oxopropenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride (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 Ca^{2+} accumulation via the Na^+/Ca^{2+} exchanger.

PP IV-16

Neuroprotective role of Let7f miRNA in post-stroke pathogenesis in female rats.

F. Sohrabji, A. Selvamani, R. Miranda and P. Sathyan
Texas A&M HSC, Neuroscience and Exp Therapeutics, College Station, United States

Question: MicroRNAs (miRNAs) are small, non-coding RNA molecules of 18-25 nucleotides that control cellular function by inhibiting translation or by degrading mRNA. Cerebral miRNA profiles are significantly altered during pathological conditions such as stroke and identification of neuroprotective miRNA are actively sought as therapeutic targets. Previous work from our lab indicated that estrogen increases cortical infarct in acyclic older females) while decreasing cortical infarct in younger females (Selvamani and Sohrabji 2008). Microarray analysis using ischemic cortical tissue from these two groups revealed that Let 7f was differentially regulated by the severity of stroke ($>$ 20 fold) and this was confirmed by real-time PCR.

Methods: To directly test the role of this miRNA, animals were subject to MCA occlusion and received 4h later ICV infusions of either Let 7f knockdown oligos, or scrambled (contol) oligos. All animals were tested on several behavioral assays pre and post stoke. Five days post stoke all animals were terminated and their brains analyzed for infarct volume. Cortical tissue was further analyzed for the expression of Let7f target genes by quantitative PCR, and normalized to cyclophilin expression.

Results: Post stroke infusion of anti-Let7f significantly reduced cortical infarct volume by 70% and striatal infarct volume by 78% as compared to animals infused with scrambled oligos. Furthermore, this reduced infarct volume was accompanied by improved function on the rotarod task as well as the cross midline, vibrissae-evoked forelimb placement task. To dissect the role of Let 7f in post-stroke pathogenesis as well as confirm the efficacy of the anti-Let7f treatment, we determined the expression of 18 target genes of Let7f that are routinely associated with survival and neuronal function. At day 5 post stroke, 10 of the 18 genes were significantly upregulated in the anti-Let7f group, including BDNF, a neurotrophic and angiogenic growth factor, Aquaporin4, a water channel associated with astrocytes, prostaglandin E synthase, the enzyme that generates PGE2, the synaptic protein, synaptotagmin, the vesicular glutamate transporter SLC17A7, and the tumor suppressor gene PTEN.

Conclusions: These studies indicate that suppression of Let7f may be a viable therapeutic approach to reduce stroke-related cell death.

PP IV-17

Brain allopregnanolone levels and distribution in response to stroke

A. Tomkins, S. Chung, D. Pepperall, M. Calford and N. Spratt
University of Newcastle, School of Biomedical Sciences and Pharmacy, Callaghan, Australia

Allopregnanolone, a progesterone metabolite and neurosteroid, has previously been reported to be neuroprotective in models of brain injury including in stroke. Allopregnanolone acts at the $GABA_A$ receptor to increase inhibition. It is by this action, limiting excitotoxicity, that allopregnanolone is believed to be neuroprotective. However, a previous study by our group failed to show neuroprotection in our stroke model. To determine possible reasons, and because allopregnanolone has previously been shown to be rapidly upregulated in response to stress, we sought to determine whether allopregnanolone was also upregulated in response to stroke, whether this occurred in penumbra, and whether further increases in penumbral allopregnanolone concentrations occurred following exogenous allopregnanolone administration.

Male spontaneously hypertensive rats (SHR) were subjected to 90 min middle cerebral artery occlusion (MCAo) with a silicone-tipped intraluminal filament and sacrificed either 3 or 6 h post-occlusion. Allopregnanolone distribution was assessed by diaminobenzadine (DAB) immunohistochemistry. Allopregnanolone-positive cells were stained with DAB and delineated using a thresholding approach from high resolution scans obtained using an Aperio slide scanner and software. In a separate group of animals brain allopregnanolone was quantified by gas chromatography mass spectrometry (GCMS) analysis on samples of striatum and sensory cortex obtained by micropunch.. Allopregnanolone 8mg/kg in cyclodextrin was administered 20 min post-reperfusion and animals were sacrificed 1 h post-injection.

Immunohistochemical analysis of brain slices demonstrated greater allopregnanolone immunoreactivity in

the sensory, insular, and piriform cortices compared with the striatum and motor cortex at 6 h. Immunoreactivity increased significantly between 3 and 6 h in the former regions. GCMS quantification revealed 2- to 3-fold higher levels of brain allopregnanolone in animals administered allopregnanolone compared to non-injection controls. This finding was consistent in striatum (infarct core) and cortex (penumbra), and in both stroke and contralateral hemispheres.

A dramatic increase in allopregnanolone immunoreactivity was seen between 3 and 6 h in penumbral stroke regions, whereas little change was seen in either unsalvageable stroke core (striatum) or anterior cerebral artery supplied motor cortex. This suggests a gradual upregulation of allopregnanolone within areas of excitotoxicity. Moreover GCMS analysis demonstrates significant increases in brain allopregnanolone in all regions following exogenous administration of the neurosteroid. These results indicate that there is upregulation of allopregnanolone within stroke regions. However, this can be significantly augmented by exogenous administration at a standard dose. These results do not explain our previous results showing a lack of neuroprotection by allopregnanolone following stroke.

PP IV-18

New targets for stroke therapy-an in vitro approach

B. Wielsch[a], F. Nitzsche[a], J. Boltze[1], M. Müller[a,b]
[a]*Fraunhofer IZI, Ischämiforschung, Leipzig, Germany*
[b]*Vita 34 AG, Leipzig, Germany*

Objective: Stroke is a leading cause of chronic disability in humans for which no effective treatment is available beyond a 4.5 hour time window, leaving more then 90% of patients untreated. The aim of our study was to find new therapeutically relevant molecules, which play a crucial role in the regulation of (patho)-physiological processes after stroke beyond the 4.5 hour time window.

Material and methods: Neurons and Glia were harvested from fetal mouse brain at Embryonic day 18.5. The cells were cultured under normoxic (21% O_2, 4,5% glucose) or ischemic (1% O_2, without glucose) conditions for a defined period of time and were analysed after 0h, 24h, 48h and 72h reoxygenation.

Results and conclusion: The in vitro system allows the analysis of new endogenous key factors, which are regulated or activated after oxygen-glucose-deprivation. We focussed on two protein families, SUMOs / SENPs (small ubiquitin like modifiers/ SUMO proteases) and MMP / ADAMS (matrix metalloproteinase / a disintegrin and metalloprotease domain), as extremely promising and interesting candidates. Both have been implicated in the regulation of a broad range of cellular processes and have been shown to be critically upregulated during cerebral ischemia. These proteins have an important function in stroke regeneration and/or decrease inflammatory processes, shown to be a major cause for cell loss following cerebral ischemia. We have analyzed the expression patterns and were able to show, that our candidate proteins are expressed in neural tissue under normoxic and ischemic conditions. Subsequently we will study the proposed protective function during the regeneration process after oxygen glucose deprivation using loss of function and overexpression studies.

We hope that these and our following studies, will help to elucidate the molecular signalling pathways activated after oxygen-glucose-deprivation, in order to enhance the therapeutic intervention during cerebral ischemia.

Topic: Stem Cells and Neurogenesis

PP III-1

Differential tropism of immortalized and non-immortalized human neural stem cell lines in a focal demyelination model.

L. De Filippis, D. Ferrari, C. Zalfa, L. Rota Nodari, M. Gelati and A.L. Vescovi
University Milan Bicocca, Biotechnology and Biosciences, Milan, Italy

Human neural stem cell (hNSC) therapy in the CNS is reaching the stage of clinical application, but a critical element is represented by the heterogeneity of paradigms used to establish comparable hNSC. In order to assess the therapeutic potential of hNSC lines endowed of intrinsically different proliferation and differentiation potentials, but deriving from the same source and cultured under GMP standard conditions, we transplanted into a focal demyelination model induced by lysophosphatydilcholine (LPC) a non immortalized hNSC line and its derivatives immortalized re-

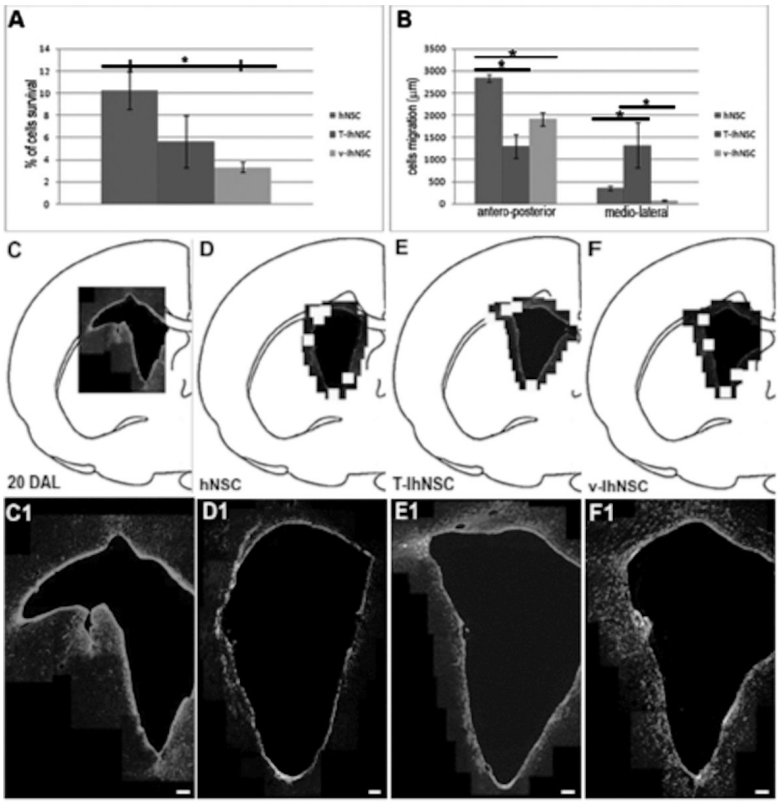

Fig. 1.

spectively with v-myc (IhNSC) and c-myc T58A (T-IhNSC). We previously showed that, compared with hNSC and v-IhNSC, T-IhNSC rise high percentages of oligodendrocytes soon after removal of mitogens and are prone to a precocious differentiation. Given the differential in vitro oligodendrogenic potential we analyzed the progeny of hNSC, T-IhNSC and v-IhNSC after transplantation into the brain of demyelinated rats, in order to elucidate if a different proliferation potential could also determine a different tendency to integration and differentiation in vivo. The three lines were all able to survive after transplantation in the subventricular zone (SVZ) and to migrate along the ventricular wall (Fig. 1). In particular, after 15 days, hNSC and T-IhNSC were able to reach the lesioned striatum and the corpus callosum differentiating into β-tubulinIII+ neuronal cells and O4+ and MBP+ oligodendrocytes, whereas v-IhNSC remained mainly confined in the SVZ and originated only sporadic β-tubulinIII+ neuronal cells (Fig. 2). A significant reduction of Iba1+ microglia activation was also observed in transplanted animals with respect to controls, suggesting an immunomodulatory effect of hNSC on the acute inflammatory reaction. These results show a differential tropism in vivo of hNSC depending on their intrinsic proliferation potential and point to hNSC as a reliable source of cells for transplantation in patients affected by neurodegenerative diseases.

PP III-2

Constitutive expression of neural markers by mesenchymal stem cells isolated from human umbilical cord

K. Drela[a], H. Kozlowska[a], M. Jurga[b], K. Domanska-Janik[a], N. Forraz[b], C. McGuckin[b] and B. Lukomska[a]
[a]*Mossakowski Medical Research Centre, NeuroRepair Department, Warsaw, Poland*
[b]*Cell Therapy Research Institute, Lyon, France*

Mesenchymal stem cells (MSC) are of clinical interest because of their potential use in autologous transplantation. The ability of MSC to differentiate into multiple different cells of mesodermal origin has of-

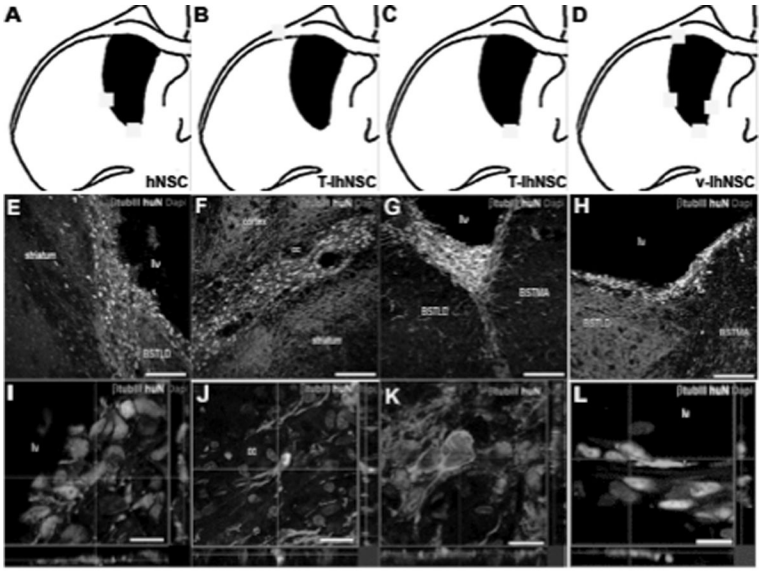

Fig. 2.

fered therapeutic tool for the treatment of hematopoietic malignancies and graft versus host disease. Recently, MSC have been shown to ameliorate a variety of neurological dysfunction. This effect is believed to be mediated to their paracrine functions since it is known that MSC produce bioactive substances that promote endogenous neurogenesis. However, the critical question that remains unanswered is whether MSC can transdifferentiate into neural cells. To be "primed" toward a neuronal fate, MSC have to express neural antigens. In an attempt to clarify this issue we explored the constitutive expression of different markers by mesenchymal cells isolated from human umbilical cord Wharton jelly (HUC-MSC) and compare their expression with neural stem cell line derived from human umbilical cord blood (HUCB-NSC) established in our laboratory.

Materials and methods: Gene expression pattern in HUC-MSC (passage 5 of cells cultured in MSCGM hMSC Lonza medium) and HUCB-NSC (cultured in DMEM/F12 medium+2% FBS) was performed by PCR reaction. Total RNA was extracted using TRIzol (Invitrogen). Then cDNA was synthesized from total RNA, using High Capacity RNA-to-cDNA kit (Applied Biosystems). PCR reactions were carried out using template cDNA in the presence of specific primers. Concomitantly immunocytochemical analysis of gene-related proteins was employed.

The results: of our studies have demonstrated that HUC-MSC, in addition to pluripotent (Oct3/4,Nanog1), mesenchymal (CD73, CD90, CD105, CD166) and extracellular matrix (Fibronectin, Vimentin, Collagen1) genes, spontaneously express neural genes i.e. Nestin, NF200, βIIITubulin, MAP2 and GFAP. The initially expressed neuroectodermal genes were comparable with mRNA expression of the same neural genes in HUCB-NSC; however for quantitative analysis RT-PCR technique is needed (the study in progress). In addition to neural genes non-induced expression of neural proteins was found. Subsets of HUC-MSC were positive for several markers, including: Nestin, NF200, βIIITubulin and GFAP.

Summary and conclusion: We have demonstrated that MSC derived from human umbilical cord Wharton jelly acquire neural progenitor-like properties by expressing neuronal and astrocytic specific markers. However, it is of clinical interest whether transplanted MSC respond with an appropriate neural pattern of differentiation when exposed to the environment of the host brain.

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PP III-3

Adenoviral cell transduction in the subventricular zone

S. Hildebrandt, S. Haas and A. Wree
Medical Faculty/University of Rostock, Institute of Anatomy, Rostock, Germany

Objectives: The subventricular zone (SVZ) contains a pool of neural progenitor cells (NPCs) possessing the capability of ongoing neurogenesis in the adult mammalian brain. The putative role of these cells for endogenous cell restauration therapies needs further investigation. The goal of this study was to investigate the fate of adenovirus-transduced GFP-expressing SVZ-cells in intact control mice and in 6-OHDA-lesioned hemiparkinsonian mice. We hereby applied a new rapid and easily to perform method to clean and concentrate adenoviruses by the use of membrane chromatography columns.

Material and methods: We injected adult male C57BL/6-mice with a virus-containing solution into the right SVZ by stereotaxic microinjection. For this injection we used an atraumatic glass capillary microinjection technique. The animals were sacrificed after three, seven, thirty or ninety days after injection of viruses and brain slices containing the GFP-expressing transduced cells were examined by fluorescence microscopy. Finally brain slices were immunohistochemically stained against the stem cell markers nestin or glial fibrillary acidic protein (GFAP) and neurofilaments (M+H). Primary antibody-detection was performed with red fluorescent Cy3- or with blue fluorescent AMCA-conjugated antibodies, because transfected cells expressed GFP.

Results: Detection of GFP-containing cells at the injection site revealed that the SVZ had been correctly targeted in all animals. Three and seven days after virus injection no GFP-expressing cells were found in the core of the striatum, rostral migratory stream or the olfactory bulb. One and three months after virus injection GFP-containing cells were detectable in various regions of the forebrain and their fate was characterized by immunohistochemistry. Transduced cells in the SVZ were counterstained with nestin. After different time periods we found GFAP (blue fluorescence) and NF (M+H) (red fluorescence) immunoreactive cells.

Conclusion: We demonstrate that NPCs in the SVZ in naïve animals migrate into the olfactory bulb and differentiate into neurons only in this region. Labeling neural progenitor cells, with an adenovirus, is a good method to mark these cells over long time periods. Systemic injections (Brdu) or a complicate immunohistochemically staining can be omitted. These findings will be compared in further studies with the results obtained in hemiparkinsonian mice lesioned one week after virus injection.

PP III-4

Canonical wnt signaling is part of the de novo neurogenesis within the adult brain parenchyma following focal cerebral ischemia and promotes neurogenesis

I. Iordanova[a,b,c], M. Anderson[a,c], G.H. Sun[a,b,c], T. Palmer[a,c], R. Nusse[d] and G. Steinberg[a,b,c]
[a]*Stanford University, Neurosurgery, Stanford, United States*
[b]*Stanford Stroke Center, Stanford, United States*
[c]*Stanford Institute for Neuro-innovation and Translational Neurosciences, Stanford, United States*
[d]*Beckman Center, Developmental Biology, Stanford, United States*

Objectives: Canonical Wnt signaling has been identified as an essential component of adult neurogenesis within the hippocampus. Its role within the adult Subventricular zone, SVZ, is less well understood, as is its role in the context of post-stroke neurogenesis. With this work we aim to 1) determine the presence of active Canonical Wnt signaling following experimental focal cerebral ischemia 2) identify the cell types that it is upregulated by 3) establish its effects on neurogenesis, regeneration and recovery following stroke through administering a liposomal Wnt-3a protein preparation directly into the brain parenchyma.

Materials and methods: 12-week-old (adult) male Axin 2 Wnt signaling reporter mice [1], were subjected to Middle Cerebral Artery Occlusion, MCAO, for 35 min, followed by reperfusion. BrdU was injected intra-peritoneally daily for 7 days, after which the animals were sacrificed, the tissue fixed in 4% PFA, cryosectioned and analyzed using immunohistochemistry. 3 ul of freshly made Wnt-3a liposomal preparation [2], active *in vitro*, was injected intraparenchymally (in two deposits) using a stereotactic frame, into the lesioned hemisphere, 1 day, 3 days, 7 days and 14 days after MCAO. Animals were sacrificed 1 month later and the number of Doublecortin positive cells estimated using stereology.

Results:

1. Canonical Wnt signaling is upregulated by Nestin positive cells at the adult murine SVZ.

2. Following stroke, Canonical Wnt signaling is detected at high levels within 85% of all neuronal progenitors expressing Doublecortin, DCX, outside the SVZ, and by mature astrocytes, positive for s-100beta and GFAP, located at the infarct border.

3. Intra-parenchymal injection of a recombinant Wnt-3a protein-liposomal preparation activates Canonical Wnt signaling *in vivo* (in Axin 2 reporter mice) and significantly increases the number of Nestin and Doublecortin positive cells. Higher numbers of Doublecortin positive cells were found in the Wnt-3a liposome treated groups for all 4 time points following focal cerebral ischemia.

Conclusion: Canonical Wnt signaling is part of both the endogenous neurogenesis at the SVZ within the naïve adult brain and the *de novo* neurogenesis following stroke. While it is still unclear how essential is its part in the process in those two contexts, activating the pathway after stroke augments neurogenesis. This is the first time, to our knowledge, that a liposomal preparation of the recombinant Wnt-3a protein has been tested *in vivo* within the brain parenchyma and its activity confirmed.

Reference

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[2] N.T. Morrell et al., Liposomal packaging generates Wnt protein with in vivo biological activity, *PLoS One* **3** (8) (2008), e2930.

PP III-5

Enhanced cell proliferation is not an endogenous neuronal repair mechanism in the Asphyxial Cardiac Arrest model in rats

G. Keilhoff, R. John, H. Schweizer, C. Langnaese and U. Ebmeyer
University of Magdeburg, Inst. of. Biochemistry and Cell Biology, Magdeburg, Germany

We analyzed the long-term consequences of asphyxial cardiac arrest for hippocampal cell proliferation in rats to evaluate the idea that the ischaemia-induced degenerated CA1 region may be repopulated by endogenous (stem) cells. Studies were performed in an asphyxial cardiac arrest model with 5 minutes of asphyxiation and three different survival times: 7, 21, and 90 days. Sham-operated non-asphyxiated rats served as control. Cell proliferation was studied by labelling dividing cells with BrdU. Neurodegenerative/regenerative pattern at single cell levels was monitored by immunohistochemistry. Alterations of gene expression were analyzed by real-time quantitative RT-PCR. Analysis of BrdU-incorporation demonstrated an increase 7, 21 as well as 90 days after global ischaemia in the hippocampal CA1 pyramidal cell layer. Adequate results were found in the dentate gyrus. Differentiation of BrdU-positive cells, investigated by cell phenotype-specific double fluorescent labelling, showed increased neurogenesis only in the dentate gyrus of animals surviving the cardiac arrest for 7 days. The majority of newcomers, especially in the damaged CA1 region, consisted of glial cells. Moreover, asphyxia was able to induce the migration of microglia and astroglia from close-by areas into the damaged area and/or the activation of resident cells. Further we show microglia proliferation/activation even 90 days after cardiac arrest. This morphological finding was confirmed by PCR analysis. The results indicate that asphyxia triggers cell proliferation in general and gliogenesis in particular. Thus, changes occur, perhaps providing a framework for glial scarring in the hippocampus. As neurogenesis is not the prominent feature, our results argue against a functional recovery. Further, from the finding of microglia proliferation up to 90 days after insult we conclude that delayed cell death processes take place which should be considered for further therapy strategies.

PP III-6

Overexpression of cxcr4 enhances human mesenchymal stem cells migration and functional recovery after focal cerebral ischemia

G.-M. Kim[a], M. Hwang[b], H.Y. Kang[b], K.S. Jin[b], B.J. Kim[a], W.Y. Lee[a], S.J. Lee[c], O.Y. Bang[a] and K.W. Sung[d]
[a]*Samsung Medical Center, Sungkyunkwan University, Department of Neurology, Seoul, Korea, Republic of*
[b]*Samsung Biomedical Research Institute, Seoul, Korea, Republic of*
[c]*National Cancer Center, Koyang, Korea, Republic of*
[d]*Samsung Medical Center, Sungkwunkwan university, Department of Pediatrics, Seoul, Korea, Republic of*

Background and objectives: Intravenous delivery of mesenchymal stem cells (MSCs) prepared from adult bone marrow reduces infarction size and ameliorates functional deficits in rat cerebral ischemia models. However, in vivo reparative capability is limited due

to lack of their survival in the infracted brain. To test the hypothesis that SDF-1alpha/CXCR4 contributes to the therapeutic benefits of MSC delivery in cerebral ischemia, we compared the efficacy of systemic delivery of human MSCs (hMSCs) transfected with a CXCR4 gene.

Methods: MSCs were isolated from human bone marrow and cultured. Adenovirus vector was used to produce hMSCs over-expressing CXCR4 (Ad-CXCR4-hMSCs) for transplantation. In vitro migration of hMSCs was investigated in the presence of SDF-1alpha. A transient middle cerebral artery occlusion (MCAO) was induced by intraluminal vascular occlusion with a microfilament. Rats were assigned to one of three groups to receive hMSCs via tail vein injection 24 hours after MCAO: untransduced hMSCs, Ad-CXCR4-MSCs or control medium only. Neurological function was evaluated using modified neurological severity score.

Results: In vitro migration assay revealed increased migration of Ad-CXCR4-hMSCs than that of hMSCs, which was ameliorated by the administration of CXCR4 receptor antagonist AMD3100. When compared with untransduced hMSCs, Ad-CXCR4-hMSCs homed in toward the infarct region in greater numbers. In addition, enhanced interaction of SDF-1alpha/CXCR4 in peri-infarct area was observed in Ad-CXCR4-hMSCs. Behavioral test revealed that the functional improvement was greater in Ad-CXCR4-hMSCs than untransduced hMSCs ($p < 0.05$).

Conclusion: These data support the hypothesis that SDF-1alpha/CXCR4 interaction contributes to facilitating hMSCs migration in cerebral ischemia. The present study shows that combined therapy is more therapeutically efficient than hMSC cell therapy alone, and it may enhance functional recovery after acute ischemic stroke.

PP III-7

Systemic transplantation of human umbilical cord blood mononuclear cells enhances endogenous repair processes in rats with focal brain ischemia

H. Kozlowska, E. Pawlak, A. Habich, M. Janowski, A. Jablonska, E. Wanacka, B. Lukomska and K. Domanska-Janik

Mossakowski Medical Research Centre, Polish Academy of Sciences, NeuroRepair Department, Warsaw, Poland

Stem cell transplantation offers an exciting new therapeutic avenue for stroke not only to prevent damage, which has been the focus of conventional therapeutic strategies, but also to actually repair the injured brain. Indeed, exogenous stem cell grafting in animal models of CNS damage improves function by replacing the lost neurons. However, therapeutic mechanism different from the expected contribution of cell replacement have been also postulated. Many studies applying systemic delivery of cells in ischemic stroke disorders have shown significant functional recovery with very few or frequently no cells entering brain. It seems that transplanted cells could propel local micro-environmental signals to sustain active endeavors for damaged neurons substitution. *The question arises* if systemic infusion of cells enhances endogenous neurogenesis previously activated by focal ischemic brain injury.

Materials and methods: Experimental model of focal ischemic brain injury was performed by local application of Na/K ATP-ase pump inhibitor – ouabain (OUA) (1μl/50nmol) into the striatum of CsA-immunosuppressed adult Wistar rats. Three days later 10^7 human umbilical cord blood CD34 negative mononuclear cells (HUCB-MNC) were infused into internal carotid artery (i.a.). At 30 day thereafter rat brains were removed and the neurogenic regions and tissue around the damaged areas were analyzed immunohistochemically.

Results: Analysis of brain tissue in OUA injured rats transplanted i.a. with HUCB-MNC revealed augmentation of proliferative cells (Ki-67$^+$) in subventricular zone (SVZ) of ipsilateral hemisphere and at the border of the lesion area as well as higher number of DCX$^+$ cells in SVZ. Moreover, the extensive neuroblast migration and their survival/accumulation in the periinfarct striatum were observed in comparison to non-transplanted rats after OUA injury onset. HUCB-MNC i.a. injection into rats with brain infarct showed also a significant increase of cells with immature (Nestin$^+$) or more mature (NF-200$^+$) neuronal phenotypes alongside OUA lesion. The intensive staining of GFAP at the border of injured area in HUCB-MNC transplanted and non-transplanted rats reflected gliosis however, the increased expression of GFAP in brain tissue of the former ones may point to the possible expansion of endogenous progenitors. *In conclusions*, HUCB-MNCs transplanted i.a. into rat model of OUA lesioned brain activates the endogenous stem cell compartment where the newly arisen cells adopt a neuronal or astrocytic fate. This effect may prove applicable for future clinical therapy.

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PP III-8

Conditionally immortalised neural stem cells improve behavioural recovery after transient model of ischaemia in mice

S. Patkar[a], R. Tate[a], M. Modo[b], R. Plevin[a] and H. Carswell[a]
[a]*University of Strathclyde, SIPBS, Glasgow, United Kingdom*
[b]*Kings College London, Centre for the Cellular Basis of Behaviour, London, United Kingdom*

The present study investigated effects of the conditionally immortalised Maudsley hippocampal murine stem cell line clone 36 (MHP36) cells on sensorimotor and histological outcome in mice subjected to transient middle cerebral artery occlusion (tMCAO). MHP36 cells were used because they proliferate only at low temperatures (33°C) *in vitro*, develop into mature neurons and glia on transplantation into the higher temperature brain (37°C) and cease dividing once matured reducing the chance of producing tumours.

Adult male C57BL/6 mice underwent tMCAO for 45 mins or sham surgery. MHP36 cells (25,000cells/μl) or vehicle were transplanted into ipsilateral cortex and caudate 48 hrs post-tMCAO, producing 4 groups ($n = 8$): sham/vehicle; sham/MHP36; MCAO/vehicle; MCAO/MHP36. The Clark's deficit score was evaluated on 1, 3 and 4 days post-tMCAO to confirm a significant deficit when compared to sham surgery. In addition, cylinder and ladder rung tests were used to assess sensorimotor function pre- and 2, 7, 14 and 28 days post-tMCAO. These tests monitor forelimb use during vertical exploration in a cylindrical enclosure and footfaults, respectively. Mice were perfusion fixed at 28 days post-tMCAO for measurement of infarct volumes. Data expressed mean $\pm$ S.E.M., $^{**}p < 0.05$, $^{***}p < 0.001$, Bonferroni's post test.

Using the Clark's deficit score, animals undergoing tMCAO were significantly impaired when compared to sham surgery. Injection of either vehicle or MHP36 cells did not affect recovery at 3 and 4 days post tMCAO. In the cylinder test, impairment of forelimb use was significantly improved by 4 weeks in MCAO/MHP36 mice (-0.300 ± 0.16, $n = 8$) when compared to MCAO/vehicle mice (0.460 ± 0.10, $n = 8$)(Fig. 1). In the ladder rung test, stepping errors were observed in the contralateral fore- and hind-limbs in both MCAO groups when compared to pre-MCAO. At 4 weeks post-MCAO there was no significant difference observed in use of contralateral forelimb between MCAO/MHP36 mice (4.95 ± 0.12, $n = 8$) and MCAO/vehicle mice (4.51 ± 0.10, $n = 8$) despite a trend for better recovery in MCAO/MHP36 mice. Infarct volumes did not differ between the experimental groups.

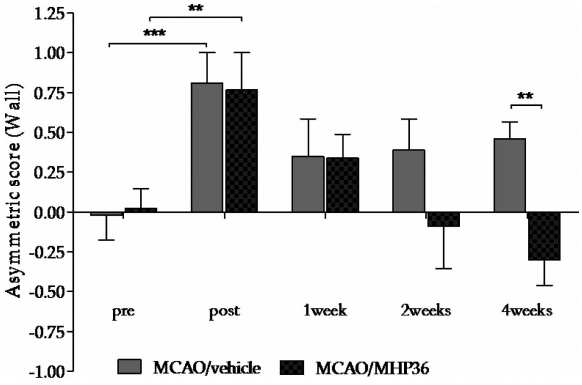

Fig. 1.

The present study suggests that MHP36 cells improve functional recovery in MCAO mice after intracerebral administration. This is the first study showing a potential benefit of MHP36 cells after ischaemic/reperfusion injury in mice, further highlighting the possibilities of stem cell transplantation for different types of neurodegenerative disease.

PP III-10

New sub-population of bone marrow stem cells expressing embryonic and neural markers

S. Sauerzweig[a], M. Schumann[a], K.G. Reymann[a,b] and H. Braun[b]
[a]*Leibniz Institute for Neurobiology, Projectgroup Neuropharmacology, Magdeburg, Germany*
[b]*Institute Applied Neurosciences, Magdeburg, Germany*

Bone marrow stem cells (BMSC) represent an autologous stem cells resource for treatment of neurodegenerative diseases. BMSC have been referred to as homogenous population of cells. However in the last time a growing number of facts have been established indi-

cating the existence of specific sub-populations. Here we investigated the influence of a complete serum-deprivation on the composition of BMSC under *in vitro* conditions.

For this frozen aliquots of BMSC from young rats were seeded in culture dishes with alpha-medium containing 20% of fetal bovine serum (basal conditions). After few days the serum was completely removed for 3 days. Serum deprived BMSC, called SD-BMSC and BMSC under basal conditions were investigated by immunohistochemistry, qRT-PCR and time-lapse microscopy. We found a sub-population of small nestin+ BMSC we called S-BMSC. After complete serum-deprivation these S-BMSC selectively started proliferation and generated a progeny of small round or spindle shaped cells lying on top of fibroblastic BMSC. We called them SD-BMSC since they had been emerged after complete serum deprivation. SD-BMSC expressed the early neural stem cell marker nestin, GFAP and S100β and were also positive for Oct4 and Sox2 both typical marker for embryonic stem cells. By qRT-PCR we defined that SD-BMSC up-regulated expression of Oct4, Sox2, GFAP and KLf4, genes essential for the generation of induced pluripotent stem cells.

In conclusion we demonstrate that BMSC contain sub-populations of small cells expressing nestin and reacting on a serum-depletion by proliferation which obviously comes along with some kind of genetic reprogramming characterized by up-regulation of marker typical for neural and embryonic stem cells.

PP III-11

CXCL12 in the cns of eae mice and its relevance to neuro/oligo genesis

R.I. Zilkha-Falb and A. Ben-Nun
Weizmann Institute of Science, Immunology, Rehovot, Israel

Background: CXCL12, a potent chemoattractant for different immune cells, which is also crucial for neuronal guidance in developing brain is constitutively expressed primarily in astrocytes and microglia, and in neurons in discrete neuroanatomical regions of adult brain. However, its functions in the adult central nervous system (CNS) is unclear.

Methods and results: In experimental autoimmune encephalomyelitis (EAE), a strong upregulation of CXCL12 was observed, particularly by astrocytes in areas of CNS inflammation. However, we noted that while CXCL12 expression in the CNS is increased with the development of severe pathological and clinical EAE, the levels of CXCL12 remained relatively high also after the reduction of CNS inflammation associated with spontaneous clinical recovery. In this study we show that not only astrocytes, but also newly generated neuronal and oligodendrocyte progenitors express CXCL12 in the CNS of mice with EAE. Although co-localization analysis strongly suggested that neuronal and oligodendrocyte progenitor cells express CXCL12, a further confirmation for possible de novo expression of CXCL12 by neuronal and oligodendrocyte progenitors, was obtained from analysis of in-vitro differentiated adult neural stem/progenitor cells (aNSCs), where CXCL12 staining could be observed in cell bodies as well as along processes of NG2 and DCX cells.

Furthermore, addition of exogenous CXCL12 enhanced the in-vitro differentiation of aNSCs towards DCX neuronal and NG2 progenitor cells after 8 days of differentiation. Moreover, the addition of AMD3100, a CXCR4 antagonist, markedly reduced the effect of the exogenous CXCL12 in promoting the differentiation of neuronal (DCX) and oligo (NG2) progenitors, and their differentiation to beta-Tubulin III and MBP mature neural and oligodendrocyte cells, respectively.

Conclusions: These results altogether show that CXCL12 plays a role in directing the differentiation of aNSCs into mature neuronal and oligodendrocyte cells, and link the CXCL12 in the CNS of EAE mice with neural repair, suggesting a dual function of CXCL12 in CNS insult.

Topic: Translational Studies and Clincical Research

PP II-17

Immunotherapy for stroke

R. Macrez, D. Vivien and C. Ali
Cyceron, Inserm U919, Caen, France

Tissue-type plasminogen activator (tPA, endogenous and exogenous) is a molecule with two faces during stroke, with a clear beneficial vascular fibrinolytic activity and at the same time, several potentially damaging effects at the levels of the neurovascular unit (NVU) and brain parenchyma. Accordingly, significant im-

provements of the overall benefit of thrombolysis can be expected, provided one can limit side effects of tPA, including promotion of blood-brain barrier (BBB) alterations and/or N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity. Based on our knowledge of the molecular events sustaining the endangerment of the NVU and brain parenchyma, we developed here an immunotherapy strategy for stroke, relying on antibodies specifically targeting the pro-neurotoxic effects of tPA. Through a relevant model of thrombo-embolic stroke in mice, we provide a complete set of pre-clinical data, demonstrating the efficiency of this strategy. A single administration of these antibodies (alone or in association with late tPA injection) confers a dramatic reduction of excitotoxic/ischaemic brain injuries and in parallel, of a set of critical events of the ischaemic cascade including BBB leakage, activation of MMP-3, MMP-9 and of the PDGF-CC pathway. As a consequence, this immunotherapy offers an improved long term neurological outcome and extends the therapeutic window for thrombolysis.

PP II-18

Perfusion ct infarction thresholds in an animal mca occlusion-reperfusion model are not affected by time within 2 hours of stroke onset

D. McLeod[a,b], M. Parsons[b,c,d], C. Levi[b,c,d], B. Hiles[a,b], S. Chung[a,b], D. Pepperall[a,b], D. Buxton[e], S. Beautement[e] and N. Spratt[a,b,c]

[a]*University of Newcastle, School of Biomedical Sciences, Callaghan, Australia*

[b]*Hunter Medical Research Institute (HMRI), John Hunter Hospital, New Lambton, Australia*

[c]*John Hunter Hospital, Neurology, New Lambton, Australia*

[d]*University of Newcastle, School of Medicine, Callaghan, Australia*

[e]*John Hunter Hospital, Hunter Health Imaging, New Lambton, Australia*

Objectives: Following ischaemic stroke it is not known whether CT perfusion (CTP) thresholds for infarction change over time. Serial scanning is not feasible in humans due to cumulative radiation and intravenous iodinated contrast dosage. However this knowledge would strengthen the use of CTP for penumbral imaging in patient selection for thrombolysis. In our established rat CTP stroke model we aimed to determine infarction thresholds at serial timepoints following middle cerebral artery occlusion (MCAo) by coregistration of serial CTP scans with histological maps of infarction at 24 hours.

Material and methods: Male outbred Wistar rats (N = 4; weight 360–400 g) underwent isoflurane anaesthesia and CTP scanning prior to, immediately after, and every 30 min following intraluminal MCAo and again following reperfusion at 2 hours. A Receiver-Operating Characteristic (ROC) analysis was performed for absolute and relative thresholds for cerebral blood flow (CBF, ml.100g.min^{-1}), and volume (CBV, ml.100g^{-1}), mean transit time (MTT, sec) and delay time (DT, sec).

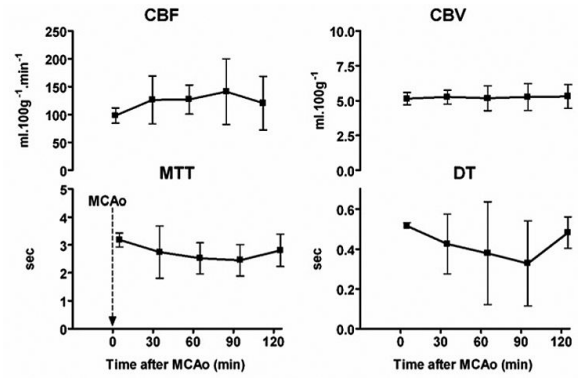

Fig. 1.

Results: The 'normal' deconvolved perfusion absolute values showed little variation over time (Fig. 1) and only modest differences between animals. The ROC analysis using both absolute and relative thresholds followed by two-way ANOVA showed that time had no significant effect overall on infarction thresholds ($P > 0.05$). Absolute and relative thresholds for predicting infarction showed little variation over time for each experimental animal, but some variation between animals, particularly for the DT measure.

Conclusion: This preliminary data indicates that both the 'normal' deconvolved perfusion absolute values and CTP thresholds for infarct prediction change little over time within the first 2 hours after stroke onset. This provides some reassurance that when imaging patients at varying times after onset of acute stroke, the duration of symptoms is unlikely to alter the reliability of the infarct core maps. Ongoing studies will expand on this dataset and also determine thresholds over time in animals with permanent MCAo for generation of penumbra prediction maps.

PP II-19

99 acute stroke treatments in combination

V. O'Collins, M. Macleod, G. Donnan and D. Howells
[a]*Florey Neuroscience Institutes, Stroke, Melbourne, Australia*
[b]*University of Edinburgh, Centre for Clinical Brain Sciences, Edinburgh, United Kingdom*

Objective: Combination therapy has been identified as a promising strategy to improve acute stroke management. This strategy might raise efficacy by targeting multiple sites within the ischaemic cascade, extending time-windows for treatment and reducing side-effects. We sought to review the evidence from animal models of ischemic stroke to determine whether combining treatments improved efficacy, and to investigate whether the mechanism of action, time of treatment and mode of delivery of the combination were related to outcome.

Materials and methods: Systematic review and meta-analysis (Macleod et al. 2004) were used to capture and explore combination therapy data. Multiple databases were searched (Web of Science, Current Contents, Biosis Previews, Pubmed, CAB abstract and Society for Neuroscience) and data was extracted where experiments were conducted in focal ischemia models contrasting groups receiving no treatment, a single treatment and a dual treatment with infarct size as an outcome. The random effects model of DerSimonian and Laird (DerSimonian, 1986) was used to obtain overall effect sizes. Data was partitioned to facilitate ranking of individual treatments and combinations, and to investigate the relationship between outcome and treatment characteristics.

Results: We found that 99 treatments had been tested in combination in 287 experiments. These experiments entailed a comparison of control, single and dual drug conditions utilizing 5,831 animals. On average, one treatment reduced infarct volume by 20.5% compared to control, and the inclusion of a second intervention improved efficacy by an additional 17.7%. However, the overall effect size of combination therapy (38.3%) was substantially reduced after adjusting for possible publication bias. Greater benefits were obtained where two treatments had complementary – rather than identical – mechanisms of action and modes of delivery. The greatest additional gains were elicited by the combination of neuroprotection and reperfusion, and the greatest protection overall was from the combination of reperfusion and hypothermia. Combination therapy suggests a ceiling to the amount of brain which can be rescued after stroke in animals: as the level of protection offered by the first treatment increases, the marginal utility gained by adding treatments decreases suggesting a law of diminishing returns for combination neuroprotection.

Conclusions: There is cause for optimism in the development of treatments of acute ischaemic stroke. Combining two complementary treatments brings additional benefits. Nevertheless, the literature must be read in light of potential publication bias. Further, there may be limits to salvageability.

PP II-20

Deleterious effect of granulocyte-colony stimulating factor combined with high-frequency repetitive magnetic stimulation on angiogenesis and functional recovery in the early subacute phase of stroke in rats

B.-M. Oh, J. Beom and T.-R. Han
Seoul national university hospital, Dept. of Rehabilitation Medicine, Seoul, Korea, Republic of

Question: Does granulocyte-colony stimulating factor (G-CSF) combined with repetitive transcranial magnetic stimulation (rTMS) have an additive effect on the angiogenic mechanism and functional outcome in the early subacute phase of stroke?

Methods: Thirty-five seven-week old male Sprague-Dawley rats were subjected to middle cerebral artery occlusion and divided into four groups as follows: normal saline administration with sham rTMS (group 1, $n = 8$), G-CSF administration with sham rTMS (group 2, $n = 10$), G-CSF with low-frequency (1 Hz) rTMS (group 3, $n = 7$), and G-CSF with high-frequency (20 Hz) rTMS (group 4, $n = 10$). Animals were subsequently received G-CSF or normal saline for 5 days and treated with 20-minute rTMS on their lesioned hemisphere for 2 weeks concurrently. Neurological function was evaluated on day 1, 7, 15, and 25. Expression of angiogenic factors, that is, Akt, phospho-Akt, endothelial nitric oxide synthase (eNOS), phospho-eNOS were measured by western blot ($n = 6$ in each group). Brain tissue was harvested in the ischemic core, ischemic border zone, and contralateral homologous cortex on day 25.

Results: Neurological functional score and the modified foot fault test result were significantly poor in group 4 as compared to group 2 on day 25 ($p = 0.009$ and 0.014, respectively). The results of rotarod performance test were comparable between four groups. Expression of Akt in the ischemic core was significantly lower in group 3 and 4 than in group 1, and lower in group 4 than in group 2 ($p = 0.003$). Expression of eNOS in ischemic core was lower in group 3 than in group 2 ($p = 0.047$). Expression of phospho-Akt in ischemic core was lower in group 4 than in group 2 ($p = 0.047$), whereas lower in group 2, 3 and 4 than in group 1 in ischemic border zone ($p = 0.043$). Also, expression of phospho-eNOS in ischemic core was lower in group 4 than in group 1 and 2 ($p = 0.015$), whereas lower in group 3 and 4 than in group 1 in ischemic border zone ($p = 0.010$).

Conclusions: The G-CSF combined with high-frequency rTMS, administered in the early subacute phase of stroke, may exert an adverse effect on angiogenic mechanism and functional recovery. This study highlights a potential hazardous effect of high-frequency rTMS especially in the early subacute phase after ischemic stroke.

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PP II-21

Effective assessment of a free radical scavenger, edaravone, on motor palsy of acute lacunar infarction

Y. Omote[a], Y. Oota[a], T. Fukushima[b], H. Matsushima[b], H. Fujii[b], K. Takamatsu[b], K. Goto[b], T. Ota[b] and K. Abe[a]
[a]*Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University, Neurology, Okayama, Japan*
[b]*Brain Attack Center Ota Memorial Hospital, Hiroshima, Japan*

Background: Free radicals are considered to be important in the occurrence of neural damage during cerebral infarction. Though there is a randomized, placebo-controlled, double blind study at multiple centers in Japan has been showing the effectiveness of a free radical scavenger, edaravone, on acute cerebral infarction, clinical studies are scarce yet. This study investigated the effect of edaravone on the outcome of patients with acute lacunar infarction.

Methods: We retrospectively evaluated 168 consecutive patients with first-ever acute lacunar infarction who were admitted to our hospital within 24 hours after the onset between January 2004 and June 2008. Of these, 79 patients underwent both edaravone and conventional therapy (edaravone group), and other 89 patients underwent conventional therapy only (non-edaravone group). The clinical outcome was assessed by the National Institutes of Health Stroke Scale (NIHSS) scale.

Results: There was no significant difference in patients' baseline characteristics and the incidence of administration of conventional therapy except for aspirin, between the two groups. The reduction of NIHSS scale during hospitalization (1.6 ± 1.2 vs. 1.0 ± 1.2; $p = 0.003$), especially about motor palsy (1.1 ± 1.1 vs. 0.5 ± 1.0; $p = 0.001$), and the percentage of patients with favorable outcome (NIHSS at discharge $\leqslant 1$) (88.6% vs. 75.3%; p=0.026) was significantly larger in edaravone group than non-edaravone group.

In the results of multiple regression analysis, the administration of edaravon (standardized partial coefficient; sb $= -0.145$, $P = 0.026$) and NIHSS on admission (sb $= -0.553$, $P = 0.001$) were significantly related to the reduction of NIHSS.

Conclusions: This study proved that edaravone improves the outcomes of patients with acute lacunar infarction, especially about motor palsy, irrelevant to the different conventional therapy concomitantly performed.

PP II-22

Syngeneic transplantation of adipose tissue-derived cells for experimental stroke

J. Taubert, S. Safdari, C. Pösel, A. Kranz, J. Scheibe, J. Boltze and D.-C. Wagner
Fraunhofer Institut, Ischemia Reserach Unit, Preclinical Trials, Leipzig, Germany

Introduction: Stroke is one of the most common medical emergencies in the United States and Europe. Established therapeutic approaches for stroke are time-restricted and often associated with serious adverse effects. Cell therapies might be effective however several cell sources are confounded with limited availabilities and ethical problems. Adipose tissue derived regenerative cells (ADRC) provide a promising alternative

since they allow an autologous transplantation within a short time window. Prior studies with ADRC showed beneficial effects in animal models of myocardial infarction probably due to antiapoptotic, immunomodulating and angiogenic effects. The aim of this study was to determine if these cells show similar effects on cerebral ischemia.

Methods: Seventy-two spontaneously hypertensive rats (SHR) received middle cerebral artery occlusion (MCAO) and were randomly distributed to one of the following treatment groups: (a) 8×10^6 cells per kg bodyweight ($n = 18$) and a commensurate control; (b) 16×10^6 cells per kg bodyweight ($n = 18$) and a commensurate control. The ADRC were isolated from SHR of the same inbred strain like the recipients and transplanted 24h following MCAO. Assessment of functional recovery was monitored regularly over 84 days by three behavioral testes: the beam walk test, the modified neurological severity score and the ladder rung test. The infarct volume was investigated in vivo using magnetic resonance imaging (MRI) at days 1, 8, 29 and 84.

Results: All animals showed significant neurological deficits following experimental brain ischemia accompanied by a circumscribed cortical infarction. Subjects treated with ADRC both in a low and a high dose showed no statistically significant differences to the according control groups in any of the used endpoints for therapeutic efficacy. Delayed and intravenous administration of ADRC failed to produce beneficial effects after stroke in SHR rats.

Topic: Traumatic CNS Injuries

PP IV-19

Xenon neuroprotection against hypoxic-ischemic injury is mediated by the NMDA-receptor glycine site

P. Banks, N. Franks and R. Dickinson
Imperial College London, Anaesthetics, Pain Medicine and Intensive Care, London, United Kingdom

Background: The inert anaesthetic gas xenon is neuroprotective and is about to begin clinical trials as a treatment for ischemic brain-injury. We have previously shown that xenon inhibits NMDA-receptors [1, 2]. NMDA-receptor antagonism is plausible as a mechanism underlying xenon neuroprotection. Nevertheless, other molecular targets for xenon have been identified [3,4], the two-pore-domain potassium channel TREK-1 and the ATP-sensitive potassium channel K_{ATP}. Whether any of these putative targets are relevant to acute xenon neuroprotection is not known. We recently showed that xenon inhibits NMDA-receptors by competing with the co-agonist glycine at the glycine-binding site [5]. Here we test the hypothesis that inhibition of the NMDA-receptor at the glycine site underlies xenon neuroprotection against hypoxia/ischemia. If this hypothesis is correct xenon neuroprotection should be attenuated at elevated glycine concentrations.

Methods: We use an *in-vitro* model of hypoxia/ischemia using organotypic hippocampal brain-slices from mice, subjected to oxygen-glucose deprivation (OGD). Neuronal injury is quantified by propidium-iodide (PI) fluorescence. PI is a membrane impermeable dye that only enters cells with compromised cell membranes where it binds to DNA and RNA and emits a characteristic red fluorescence.

Results: We show that 50% atm xenon is neuroprotective against hypoxia/ischemia when applied immediately after injury, or even after a delay of 3 hours following injury. To validate our method, we show that neuroprotection by gavestinel is abolished when glycine is added, confirming that NMDA-receptor glycine-site antagonism underlies gavestinel neuroprotection. We then show that adding glycine abolishes the neuroprotective effect of xenon, consistent with competitive inhibition at the NMDA-receptor glycine-site mediating xenon neuroprotection.

Conclusion: We have shown that xenon neuroprotection against hypoxia/ischemia can be reversed by elevating the glycine concentration. This is consistent with competitive inhibition by xenon at the NMDA-receptor glycine-site mediating xenon neuroprotection. Not only does this provide a molecular mechanism (competitive inhibition), but it also, for the first time, clearly identifies the NMDA-receptor as playing a major role in xenon neuroprotection. This finding may have important implications for xenon's clinical use as an anaesthetic and neuroprotectant.

Reference

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PP IV-20

Multipotential pericytes in the rat spinal cord react to spinal cord compression injury

U. Graumann[a], M.-F. Ritz[b], B. Gutierrez[a], T. Janner[a] and O. Hausmann[a,c]
[a]*Swiss Paraplegic Research, Spinal Injury Research, Nottwil, Switzerland*
[b]*Department of Biomedicine, Brain Tumor Biology, Basel, Switzerland*
[c]*Hirslanden Clinic St. Anna, Neurocenter, Lucerne, Switzerland*

Objectives: Adult bone marrow (BM)-derived stem cells may contribute to vascular healing following spinal cord trauma. However, the reports from experimental transplantation studies are controversial in regard to the fate of these cells since there is indication that the stem cells give rise to pericytes but not to vascular endothelial cells.

CNS pericytes are described as multipotent stem cells of mesenchymal origin. The mural cells are in close conjunction with endothelial cells and have therefore an important impact in vessel stabilization. Further, they induce or maintain the blood-neural barrier by synthesis of most basal lamina elements. In order to elucidate the contribution of infiltrating putative endogenous BM-derived stem cells in vessel remodeling after spinal cord injury (SCI), we investigated their incidence in injured rat spinal cord tissues. In addition, the expression of angiogenic and arteriogenic factors was analyzed in a time-dependent manner.

Material and methods: The temporal appearance of circulating stem cells following spinal cord compression injury in rats was analyzed by immunohistology and RT-qPCR. Therefore, we investigated the expression of CD34, CD133, CXCR4, and the mobilization factor SDF-1. Blood vessel structures were visualized by stainings with Reca-1 and alpha-SMA. The expression of angiogenic and arteriogenic factors, e.g. VEGF, PDGF-BB, Ang-1, and PlGF was examined by RT-qPCR.

Results: The number of CD133 positive cells was markedly reduced 2 days post-SCI. The cells reappeared in the spinal tissues after 2 and 4 weeks of the experimental period. CD133 expressing cells colocalized with alpha-SMA and were identified as pericytes surrounding arterioles. Increasing amounts of CD34 positive cells coexpressing the macrophage/microglia marker CD11b emerged after 2 weeks post-SCI. The expression of SDF-1 was stimulated only after 3 weeks, while its receptor CXCR4 was overexpressed over a long period starting at 2 days after injury. VEGF, PDGF-BB, Ang-1, and PlGF expression was decreased with the lowest level at day 2 and 3 postinjury.

Conclusions: Our results show that resident CD133 expressing pericytes react to SCI, whereas no markers could be attributed clearly to BM-derived cells. The delayed induction of the mobilization factor SDF-1 may impede efficient neovascularization, since cavity formation has already been started 2 weeks post-SCI. The expression of factors involved in stabilization of blood vessels are negatively modulated. Disturbance of vessel integrity is a possible consequence of these modulatory effects during the phase of secondary injury after SCI.

PP IV-21

Combination Therapy of Traumatic Brain Injury in rats with a Stem cell Mobilization by Granulocyte-Colony Stimulating Factor and Human umbilical cord matrix stem Cell(Wharton jelly stem cell) Intravenous injection enhances functional recovery.

M.T. Joghataei, M. Bakhtiary, M. Hadjighassem and M. Marzban
Faculty of Medicine. Iran University of Medical Sciences(IUMS)., Department of Anatomy., Tehran, Iran, Islamic Republic of

Objective: Emerging clinical studies of treating traumatic brain injury with autologous adult stem cells led us to analysis the effect of an combination therapy by intravenous injection of Human umbilical cord matrix stem Cell (Wharton jelly stem cell) with bone marrow cell mobilization induced by granulocyte colony stim-

ulating factor (G-CSF) in rats with cortical compact device

Methods: Adult male Wistar rats ($n = 50$) were injured with controlled cortical impact device and divided into five groups

Group 1 (10): TBI +PBS
Group 2 (10): TBI + Brdu (intraperitoneally)
Group 3 (10): TBI +G-CSF (subcutaneous)
Group 4 (10): TBI + 2×10^6 hUCMSC
Group 5 (10): TBI + 2×10^6 hUCMSC +GCSF

All injections were performed 1 day after injury into the tail veins of rats. All cells label with Brdu before injection into the tail veins of rats. Neurological functional evaluation of animals was performed before and after injury using Neurological Severity Scores (NSS) Animals were sacrificed 42 days after TBI and brain sections were stained by Brdu immunohistochemistry.

Results: Statistically significant improvement in functional outcome was observed in treatment groups when compared with control ($p < 0.01$). This benefit was visible 7 days after TBI and persisted until 42 days Neurological Severity Scores (NSS) showed no significant differences among the hUCMSC and G-CSF treated groups at any time point (end of trial). Rats with hUCMSC +G-CSF treatment had a significant improvement on NSS five and six week compared to other treatment group ($p < 0.01$). Histological analysis showed In G-CSF-hUCMSC treated traumatic rats exhibited significantly increased numbers of BrdU immunoreactive cells in their traumatic core compared with other labeled group.

Key word: Combine,G-CSF,Wharton jelly,TBI

PP IV-22

Food restriction suppresses inflammation and apoptosis in rat model of brain injury

N. Loncarevic-Vasiljkovic[a], J. Popic[a], K. Smiljanic[b], S. Pracer[a], V. Pesic[a], D. Milanovic[a], L. Rakic[a], S. Kanazir[a] and S. Ruzdijic[a]
[a]*Institute for Biological research, Neurobiology, Belgrade, Serbia*
[b]*Institute for Biological Research, Neurobiology, Belgrade, Serbia*

Objective: Neurons in the CNS are especially sensitive to inflammatory damage. After the CNS injury, inflammation spreads through surrounding tissue leading to neuronal damage and cell death. Numerous studies proved that food restriction (FR) can be beneficial and, to some extent neuroprotective, in different pathological conditions. The rationale of this study was to investigate whether FR lasting 3 months prior to cortical injury can modulate processes of inflammation and neuroapoptosis in the ipsylateral cortex after stab injury.

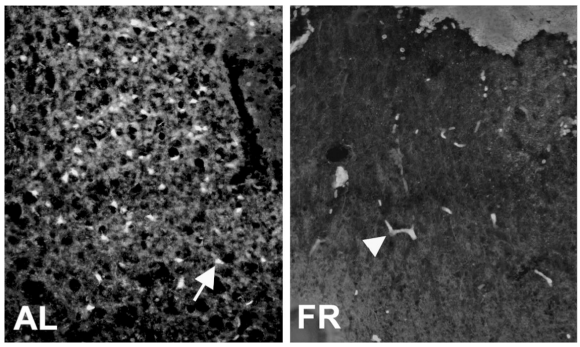

Fig. 1. Fluoro-Jade B staining of cortical tissue surrounding injured site 2nd day after injury. Numerous Fluoro-Jade B-positive cells were detected in AL animals (left panel). No positive cells were detected in FR animals at same time point (right panel). Degenerating neurons are marked with arrows, arrowheads point to blood vessels.

Material and methods: Two groups of young adult Wistar rats were used in the study: (i) animals fed ad libitum (AL) and (ii) animals fed 50% of normal daily food intake 3 months prior to injury (FR). Unilateral cortical stab injury to sensorimotor cortex served as a model of brain injury. Western blot analysis was used to detect expression of marker of inflammation – Tumor Necrosis Factor alpha (TNF-α) and marker of apoptosis – active fragment of caspase 3, while the Fluoro-Jade B staining was used to show degenerating neurons. The expression was examined at 2nd, 7th, 14th and 28th day post injury.

Results and conclusion: Interestingly, Western blot analysis showed TNF-α expression only in AL group at 2nd day after injury, while its expression was not detected in FR group at any time point. The identical expression profile was observed for death marker – active fragment of caspase 3. Fluoro-Jade B staining followed the pattern of changes observed for TNF-α and caspase 3. In the AL group injury induced neuronal degeneration in surrounding cortical tissue, and the Fluoro-Jade-labeled cells were detected at 2nd, 7th and 14th day after injury, with peak in the number of labeled cells on 2nd day after injury (figure below). Conversely, Fluoro-Jade-labeled cells were not detected in FR group at any time point after injury.

Our results suggest that FR as pretreatment can abolish inflammation in the injured CNS tissue, that way

preventing surrounding tissue from neurodegeneration and apoptosis.

PP IV-23

Reorganization plasticity in the visual cortex of the adult rat after optic nerve crush

T. Macharadze[a], R. Pielot[b], T. Wanger[c], H. Scheich[c], E. Gundelfinger[b], J. Goldschmidt[c] and M. Kreutz[a]
[a]*Leibniz Institute for Neurobiology, PG Neuroplasticity, Magdeburg, Germany*
[b]*Leibniz Institute for Neurobiology, Neurochemistry, Magdeburg, Germany*
[c]*Leibniz Institute for Neurobiology, Auditory Learning and Speech, Magdeburg, Germany*

Incomplete experimental crush of the adult rat optic nerve (ONC) has been suggested to serve as an experimental model of diffuse axonal injury. After incomplete ONC neuronal activity in the contralateral visual cortex is initially decreased, but then recovers during a period of several weeks. The details of how the patterns of neuronal activity in individual cortical columns, layers or cell types change during this recovery process have remained largely elusive.

We here investigated the patterns of neuronal activity in visual cortex after ONC in adult rats using thallium autometallography (TlAMG). TlAMG is a novel method for high-resolution mapping of neuronal activity [Goldschmidt et al. (2004) Neuroimage 23(2): 638–647]. It is based on the fact that in neurons the uptake rates of K^+ and K^+-analogs like the heavy metal ion Tl^+ increase with increasing activity. Compared to the ^{14}C-2-deoxyglucose method, which is similar in rationale, thallium autometallography offers the advantage that the tracer can be detected non-radioactively with cellular and subcellular resolution by means of a histochemical method, a modified Timm-technique for the detection of heavy metals in the brain.

The detailed analysis of the spatial distribution of the tracer includes the reconstruction of the 3D dataset by registration of the histological slices. We find that, after ONC, thallium uptake in the contralateral primary visual cortex is reduced in layers IV and V as compared to the ipsilateral side. Contralateral to the lesion we observed clustered groups of layer V pyramidal cells or clustered columns with high thallium uptake. Many of these cells could be retrogradely labeled by the intracollicular injection of the tracer biotinylated dextran amine. During recovery the number of stained layer V pyramidal cells steadily increases, and contra- to ipsilateral differences vanishes at around six weeks after the lesion. At ten weeks no differences between ipsi- and contralateral side could be observed.

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PP IV-24

Effects of Pigment Epithelium Derived factor on traumatic brain injury

A.L. Pina[a,b], P. Casalis[a], V. Lang[a], M.Y. Terzi[a], E. Bruendl[b], E.-M. Stoerr[b], A. Brawanski[b], U. Thomale[a] and P. Vajkoczy[a]
[a]*Charite-Berlin Medical University, Neurosurgery, Berlin, Germany*
[b]*Regensburg University Clinic, Neurosurgery, Regensburg, Germany*

Pigment epithelium-derived factor (PEDF) is a potent antiangiogenic and tumor-differentiating factor that can also protect and differentiate neurons. The neurotrophic and neuroprotective effects of PEDF on brain cells has been demonstrated mainly by in vitro experiments that showed the ability of the protein to support the survival of neurons in the presence of various neurotoxic stimuli and, in some cases, to induce neuronal differentiation. In this study we report the in vivo effects that PEDF exerts on neurogenesis, apoptosis and proliferation of microglia cells after traumatic brain injury (TBI).

Adult male rats were sham operated or subjected to unilateral controlled cortical impact injury (CCI). Animals were divided into two groups: one for expression studies, at 4hrs, 1, 4 and 7days after CCI. In the other group, animals received PEDF and/or aCSF (vehicle) into the lateral ventricle as well as intraperitoneal injections of Bromo-deoxy-Uridine (BrdU) over a period of 7 days. Brain tissues from these animals were prepared for real time PCR mRNA expression or immunohistological analysis. Detection of BrdU (for proliferating cells), single stranded DNA (for apoptotic cells) and ED1 antigen (for activated microglia), were performed using immunocytochemical methods. The number of labelled cells into the lesioned and neurogenic areas (Subventricular Zone, SVZ, and Dentate Gyrus, DG) was measured using a computer-assisted stereology system.

Our results show that PEDF mRNA expression after CCI increase with time. Intraventricular infusion of PEDF is associated with a reduced lesion volume, increased number of proliferating cells and diminished number of apoptotic and microglia cells around the lesioned area. Increased number of proliferating cells into the SVZ and no changes in the DG could also be detected in comparison to controls.

Our results indicate that in vivo PEDF may be a multifunctional neuroprotective agent, influencing neurogenesis, apoptosis and inflammatory processes in TBI.