International Conference

Neuroprotection and Neurorepair

Cellular and molecular mechanisms involved in stroke, ischemia and trauma

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Organisers:
Georg Reiser (1) Klaus G. Reymann (2)
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Advisory board: Barry Festoff (Kansas City, USA), Arne Schousboe (Copenhagen, Denmark), Bernd Seilheimer (Berlin, Germany), Klaus Unsicker (Heidelberg, Germany).

Topics:
- Ischemia: basic mechanisms
- In vitro-models for studies on neurodegeneration as substitute for animal experiments
- Ion transporters in physiology and pathology
- Molecular neuropathology and signal transduction in neuronal damage and protection
- Growth factors, neurorepair and neurogenesis
- Glutamate receptors as target for neuroprotection
- Plasticity after neuronal injury and neuronal restitution

Abstracts invited speakers

Ischemia: basic mechanisms

Ischemic tolerance of the brain

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Noxious stimuli, applied at doses close to but below the threshold of cell injury induce adaptive responses which protect the tissue against further stress (induced tolerance) from the same or other stimuli (cross tolerance). In the brain, among other stimuli, hypoxia, ischemia, seizures, spreading depression, heat, metabolic inhibition, and inflammation induce tolerance to subsequent focal or global cerebral ischemia. Via largely unknown but different signalling cascades, tolerance can occur within minutes ('early preconditioning'), and after an unprotected interval reoccurs in a protein sysnthesis dependent manner within many hours or days ('late preconditioning') after stimulus application. We have demonstrated that metabolic

inhibition, hyperbaric hyperoxygenation, hypoxia, erythropoeitin, and desferoxamin all can induce delayed tolerance to focal cerebral ischemia in mice and/or rats (some relevant species and strain differences exist!). Interestingly, we were able to show that all of the above stimuli also induce tolerance to oxygen/glucose deprivation in cultured purfied (primary) cortical neurons, demonstrating that tolerance is an intrinsic property of neurons. However, we also find that medium conditioned by hypoxic/aglycemic astrocytes induces tolerance in untreated primary cortical neurons, implying that humoral astrocytic - neuronal interactions also contribute to tolerance induction in vivo. The plethora of known inducers of tolerance to ischemia/hypoxia in the CNS makes likely the contribution of numerous signals and mediators of protection. We found evidence for involvement as signals or executors of HIF-1, oxygen free radicals, and glycolytic enzymes. Although induction of tolerance to cerebral ischemia in experimentalconditions is a robust event, the question arises whether the phenomen exists in humans. To this end we have shown that patients who have experienced a transitory ischemic attack before ischemic stroke present with a smaller neurological deficit and better outcome than patients without prior TIA. We speculate that TIAs serve as preconditioning stimulus in some stroke patients. In conclusion, preconditioning provides a unique opportunity to study endogenous neuroprotective mechanisms. Whether preconditioning may be used to induce protection in humans under conditions when a cerebral ischemic event can be anticipated remains to be elucidated.

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Upregualtion of TGF-ß1 expression in rat hippocampal neurons caused by ischemia and ß2-adrenoceptor stimulation.

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In previous work we have demonstrated that NGF released from cultured neurons leads to pronounced neuroprotection. However, under in vivo conditions not only NGF but various growth factors and cytokines are increasingly expressed after ischemia which could contribute to neuroprotection or neurodegeneration. Intraventricularly administered TGF-ß1 clearly reduced the infarct volume after focal cerebral ischemia in mice. In the present study we made, therefore, an attempt to find out whether endogenous TGF-ß1 was expressed in brain tissue after transient forebrain ischemia in the rat and whether this growth factor expression could be enhanced by ß2-adrenoceptor stimulation.

In a first approach it was shown by immunostaining that bioactive TGF-ß1 was expressed in CA1 pyramidal neurons of non-ischemic rats. TGF-ß1 immunoreactivity (ir) was further upregulated 3h and 6h after 10 min of forebrain ischemia without detectable activation of microglial cells and astrocytes. When neuronal damage proceeded from 2d to 4d after ischemia, TGF-ß1 ir disappeared in damaged but not in viable neurons. Double staining of the hippocampal slices clearly revealed that TGF-ß1 ir was no longer present in TUNEL-positive, but it was still expressed in TUNEL-negative, viable neurons. Interestingly, when the ß2-adrenergic clenbuterol was administered to the rats, the TGF-ß1 expression was still more pronounced and accelerated compared to the untreated ischemic rats and the percentage of damaged neurons in the CA1 hippocampal subfield was significantly reduced. The results suggest that the expression of endogenous TGF-ß1 can be increased by ß2-adrenoceptor stimulation and the enhanced TGF-ß1 level can contribute to neuroprotection.

Differentially expressed mRNA in ischemia tolerant rat brain detected by new differential display technique, RFD-PCR

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Previous work has shown that ischemic preconditioning (3 min) induces a state of tolerance to subsequent ischemic insults (7 min) in the CA1 region of the hippocampus in rat brain. The mechanism whereby ischemic tolerance is induced is unknown, but transcriptional regulations are suspected to be involved.

In the present work we have applied a new differential display technique called Restriction Fragment Differential Display - PCR (RFDD-PCR) to the CA1 region from rats made tolerant by two-vessel occlusion. RFDD-PCR allows selective amplification of adaptor-ligated cDNA fragments preferentially from the coding region of mRNA, and is thus ideal for studying transcriptional regulations. RFDD-PCR analysis of RNA from naïve and tolerant animals, one, two and three days after induction of tolerance showed that 81 cDNA fragments were significantly differentially expressed during the time course of tolerance development. A bioinformatical analysis predicted the identity of app. 50% of the fragments. After grouping of the fragments according to their function, it was found that extracellular matrix proteins constituted a majority of the differentially expressed fragments. For two of the fragments, namely GluR2-flop and p68 RNA Helicase, competitive quantitative PCR was performed, resulting in a verification of the degree of regulation forund by RFDD-PCR analysis.

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Global ischemia and status epilepticus: Novel neuroprotectice strategies

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Brief global ischemia causes delayed neurodegeneration in hippocampal CA1. Kainate induced status epilepticus causes delayed neurodegeneration in CA3 and often in CA1 as well. Cells restore their ionic gradients and recover excitability after these insults, but then GluR2, the AMPA receptor subunit that limits Ca^{2+} entry, is downregulated. GluR2 downregulation is expected to lead to formation of Ca^{2+} permeable AMPA receptors and increased toxicity of endogenous glutamate due to excessive influx of Ca^{2+} (the GluR2 hypothesis of delayed neurodegeneration). In these paradigms, cell death apparently requires an early translocation of Zn^{2+} from presynaptic fibers into the vulnerable postsynaptic neurons (Choi & Koh, Annu Rev Neurosci 21: 347, 98). To examine molecular mechanisms underlying Zn^{2+} toxicity, we administered CaEDTA (which chelates Zn^{2+} and releases equal amounts of Ca^{2+}) to adult male gerbils and induced global ischemia by 5 min bilateral carotid occlusion. Pretreatment with CaEDTA significantly protected against ischemia-induced damage and reduced downregulation of GluR2 mRNA and protein expression. These findings suggest a mechanism whereby Zn^{2+} entering during ischemia acts at the level of transcription to decrease GluR2 expression. CaEDTA is also protective against CA3 degeneration following kainate induced status epilepticus (Choi & Koh, ibid.), when the Zn^{2+} is likely to come from mossy fiber terminals. The source of the Zn^{2+} that enters CA1 neurons is less clear.

Neuroprotection by pretreatment with aurintricarboxylic acid, a molecule binding to nucleic acids, prevents downregulation of GluR2, presumably by interfering with some step in the causative transcriptional and/or translational events (Aronica et al., PNAS, 95: 7115, 98). Neuroprotection by the AMPA receptor antagonist, NBQX, does not prevent GluR2 downregulation, and may act directly by blocking $Ca2^{2+}$ permeable AMPA receptors (Pellegrini-Giampietro et al., J Neurochem 62: 1067, 94). It remains to be determined whether GluR2 recovers after NBQX protection or whether its expression remains down regulated. Survival of GluR2 (-/-) mice indicates that hippocampal neurons can adjust to permanent lack of GluR2.

Neuroprotection against anticipated ischemia (as in open heart surgery) and rescue after ischemia or seizures are therapeutic goals. Block at any of the steps leading to GluR2 downregulation, at the level of Ca^{2+} permeable AMPA receptors, or at steps in the subsequent cell death cascade appear possible and may markedly extend the therapeutic window.

<u>In vitro-models for studies on neurodegeneration as substitute for animal experiments</u>

Organotypic hippocampal slice cultures in the study of ischaemic neurodegeneration and in the development of neuroprotective strategies.

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Organotypic hippocampal slice cultures (HSC's) are rapidly evolving to fill a role in the profiling of new pharmaceutical leads for the treatment of ischaemic neurodegenration. Several groups have developed models of ischaemic neurodegeneration in HSC's based upon either oxygen/glucose deprivation or inhibition of metabolism. Due to the relative preservation of hippocampal organisation and to the relative maintenance of cellular stoichiometry HSC's have so far been found to be highly predicitve of in-vivo efficacy of neuroprotective compounds.

We have examined a number of potential conventional neuroprotective strategies including temperature modulation, neurotrophins, glutamate receptor antagonism, calcium channel antagonism etc...In addition to testing compounds which act at known targets or work through known mechanisms it is also possible to use HSC's to discover neuroprotective compounds which act through unknown mechanisms and at unknown targets. This approach becomes very powerfull when combined with new methods of combinatorial chemistry, since by synthesizing many variants of a compound it becomes possible to identify optimally effective molecules in a relatively short time frame.

We have recently developed this approach and used it to discover a new class of neuroprotective compounds through a process of functional screening. Beginning with a seed molecule, structural variations are synthesised by combinatorial chemistry to identify key features of the molecule which confer neuroprotective properties. These compounds are then tested for their ability to prevent uptake of the fluorescent exclusion dye propidium iodide, 24 hours after a 1 hour period of oxygen and glucose deprivation (ischaemia) or a 3 hour period of oxygen deprivation in the presence of glucose (hypoxia). Through systematically varying the structure of the molecule, it becomes possible to identify the optimal phamacophore of

the molecule. This molecule can then be tested for it's neuroprotective ability in animal models of ischaemia, thus reducing the time required for drug development and reducing the amount of in-vivo testing that is required.
We suggest that this model may be very useful in profiling compounds for pre-clinical testing and that it may be a useful interface between high throughput screening and in-vivo testing.

Dopaminergic neurodegeneration in mesencephalic organotypic cultures: an in-vitro model for Parkinson's disease.

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Parkinson's disease (PD) is a neurodegenerative disorder affecting selectively dopaminergic neurons of the substantia nigra. Although PD etiology is still unknown, there are some evidences for different pathogenic mechanisms involving oxidative stress and energetic metabolism impairment. We propose to develop an experimental model using rat mesencephalic organotypic cultures as a new approach for PD pathogenesis. This in vitro model allows to conserve most of the in vivo cytoarchitectural and functional characteristics of the tissue. Organotypic mesencephalic cultures will be maintained for short and long term periods for immunocytochemical characterization of dopaminergic neurons (using antibodies directed against tyrosine hydroxilase (TH), neurofilaments, calcium binding proteins, glutathione peroxidase), and astrocytes (anti-glial fibrillary acidic protein antibody). Survival of dopaminergic neurons within the substantia nigra and the ventral tegmental area will be assessed by evaluating TH positivity cells, and by electrophysiological recordings. Thus, continuous recording of spontaneous and evoked responses will be assessed to monitor dopaminergic neurons viability, using a microelectrode array enclosed in a microperfusion chamber. Glucose metabolism through the pentose phosphate shunt, that produces NADPH (necessary to regenerate reduced glutathione) and is therefore tightly connected to the redox state of cells, will be evaluated by a gas chromatography coupled to spectroscopy analysis. All the above analyses will be performed in basal culture conditions, after the induction of metabolic stress, and after the induction of neurodegeneration using 1-methyl-4-phenylpyridium ion (MPP+), dopamine, levodopa and hydrogen peroxide (H2O2). The occurrence of apoptosis after inducing neuronal cell death will be looked for, using the TUNEL technique. The potential potentiation by additional metabolic stress (induced by glucose deprivation) of the toxicity of MPP+, dopamine, levodopa and H2O2, which are all directly relevant to PD pathogenesis, will be studied.

Excitotoxic injury profiles of ATPA and the classical glutamate receptor agonists AMPA, KA and NMDA in organotypic hippocampal slice cultures

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The glutamate receptor agonist (RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid (ATPA) was originally introduced as an AMPA receptor agonist, but is now known at low concentrations, also to be a selective agonist of the glutamate receptor subunit GluR5, which is confined to kainic acid (KA) receptors. In this study we compared the excitotoxic effectcs of ATPA and the classical glutamate receptor agonists 2-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainic acid (KA) and N-methyl-D-aspartate (NMDA) on hippocampal slice cultures. We also studied the possible protective effects on CA1 pyramidal cells by low concentrations of ATPA supposedly acting on inhibitory interneurons expressing GluR5 receptors. From dose response measurements of propidium iodide (PI) uptake in entire hippocampal cultures the rank order of EC50 values were: AMPA(3.7 microM) > NMDA(10.8 microM) > KA(12.6 microM) > ATPA(32.7 microM). The relative vulnerability of CA1 and CA3 pyramidal cells was similar for 30 microM ATPA 3 microM AMPA and 10 microM NMDA with CA1 as the most susceptible of the two subfields, while CA3 was most susceptible to 8 microM KA. In 100 microM concentrations all four agonists induced the same, maximal PI uptake in all hippocampal subfields which was confirmed to correspond to total neuronal degeneration as visualized by degenerative changes in neuron specific protein (NeuN) immunostaining and by loss of immunostaining for microtubule associated protein 2 (MAP2). Using glutamate receptor antagonists, inhibition curves revealed that KA mediated most of its excitotoxicity via KA receptors, that AMPA excitotoxicity was mediated via AMPA receptors and that ATPA excitotoxicity more resembled that induced by AMPA than by KA not excluding, however, that ATPA might have a KA-receptor component. The possible neuroprotective effect of ATPA by activating CA1 stratum oriens interneurons was investigated by cotreatment with 1-3 microM of ATPA and 10 microM NMDA, but ATPA did not reduce PI uptake induced by NMDA. The study was supported by the Danish MRC, the Aslaug and Carl Friis's

Foundation, the Hede-Nielsen Foundation and the EU-Biotech program (BIO4-CT97-2307).

The basis of differential vulnerability of striatal neurons

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Medium spiny neurons in the striatum, comprising 95% of the neuronal population exhibit a marked vulnerability to energy deprivation which leads, in conjunction with overexcitation, to degeneration, e.g. in Huntingtons disease. They receive glutamatergic input from several cortical areas and express AMPA-, NMDA- and metabotropic receptors. Their AMPA-receptors are Ca-impermeable through the expression of GluRB, but they lack GluRD (flop) and thus display long lasting inward currents (slow desensitization) which allow recruitment of Ca-permeable NMDA-receptors and high threshold Ca-currents. We report here a combined electrophysiological and molecular study using patch-clamp recording from isolated neurons and single cell RT-PCR analysis of AMPA receptors. Furthermore we have observed a long lasting (seconds) G-protein dependent EPSP in neostriatal slices, likely mediated by metabotropic glutamate receptors which supports plateau-depolarizations upon repetitive input. The cholinergic giant aspiny interneurons also receive excitatory input from cortex and thalamus but are much more resistant to degeneration. Some of them are Ca-permeable but they all express GluRD (flop) conferring fast desensitization kinetics which restrict Ca-influx. In addition, they may be protected by inhibition through endogenous adenosine and taurine.

Mechanisms of seizure-induced cell death in rat hippocampal slice cultures

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Organotypic hippocampal slice cultures develop after exposure to low Mg prolonged seizure-like events (SLE) characterized by initial burst discharges, a subsequent tonic-like and then clonic-like electrographic activity recurring once every five to ten minutes. During the seizure-like events [Ca]o drops by about 0.6 mM and [K]o rises to near 12 mM as observed for seizure-like events in intact animals. Two hours after recurring seizure propidium iodide staining indicates augmented cell loss. Imaging techniques show that the extracellular calcium loss is associated with accumulation of Ca in the cytoplasma, and in the mitochondria. Mitochondrial membrane potential rises after onset of intramitochondrial calcium accumulation. Early during SLE NADH autofluorescence declines but then strongly increases. The rise in NADH autofluorescence is accompanied by prolonged and augmented production of free radicals as indicated by HET fluorescence. During recurrent seizure NADH overshoots and mitochondrial depolarizations decline in amplitude indicating increasing impairment of mitochondria. In the presence of α-tocopherol the alternations of mitochondrial functions are prevented and recurrent seizure-induced cell death is strongly diminished. Our findings suggested that calcium-dependent augmented production of free radicals in status epilepticus cell death.

Permeability Regulation at the blood-brain-barrier in vitro

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High amounts of new compounds of pharmaceutical interest are nowadays available by combinatorial chemistry. In order to develop their effectiveness in the central nervous system the substances have to cross the barriers between blood and brain. Thus, beside their binding to a specific receptor as the basis for their involvement in neuronal processes a key event is their permeation through the blood-brain-barriers. Since thousands of new therapeutic compounds will have to be tested in the near future alternatives to *in vivo* test systems have to be developed not only because of increasing ethical reasons but also simply by the extremely difficult and costly handling of huge amounts of laboratory animals. This simple consideration already shows that *in vitro* models are highly demanded that closely mimic the *in vivo* system at least with respect to barrier properties.

Two barrier systems are of main interest with respect to the passage of compounds into the brain. The blood-brain-barrier in its original is formed by a complex system of endothelial cells, astroglia and pericytes as well as the basal lamina interconnecting the cellular systems. The structural basis of this barrier are the endothelial cells with *tight junctions* as special features that seal the intercellular cleft. Astrocytes, pericytes and the extracellular matrix component are believed to control the integrity of this barrier.

The second system that prevents the free passage of substrates between blood and brain is the blood-cerebrospinal fluid-barrier built up the epithelium of the choroid plexus. This epithelial barrier again sealed by *tight junctions* becomes indispensable since the endothelium of choroid plexus capillaries is leaky and highly permeable to hydrophilic substrates.

We have developed two blood-brain-barrier models using either capillary endothelial or choroid plexus epithelial

cells. Both cell types need serum in the growth medium to proliferate, however, in the presence of serum the barrier properties were weakened. Withdrawal of serum reinforced the barrier properties drastically and the degree of cellular polarity became more marked. Brain Capillary Endothelial Cells (BCEC) exhibit leck permeabilities as low as 10^{-6} cm/s corresponding to electrical resistances around 300-400 $\Omega \times cm^2$. Hydrocortison was found to further strengthen the barrier increasing the electrical resistance up to 1200 $\Omega \times cm^2$ and decreasing the permeability for sucrose down to 10^{-7} cm/s in a physiological range. Evidence will be presented, that the intracellular glucocorticoid receptor is involved in the signalling and that gene expression is modulated drastically by addition of hydrocortison. Furthermore extracellular matrix components will be shown to have a strong impact on the barrier formation. A model for the regulation of the junctional permeability will be presented.
Very similar results have been found in the epithelial barrier system. Withdrawal of serum from the culture medium again increased the barrier properties. Due to the highly expressed Na^+K^+ATPase and the functional H^+Na^+exchanger the cells to build up a proton gradient across the cell monolayer. As a consequence this tightening the choroid plexus epithelial cells allows the culture to secrete cerebrospinal like fluid *in vitro*. The culture system is now in use to study transport across the blood-CSF-barrier.

Ion transporters in physiology and pathology

2P domain background K+ channels are important targets for neuroprotective drugs and volatile anaesthetics

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K+ channel openers are potent neuroprotectors. This was first shown using drugs such as cromakalim or pinacidil that activate ATP-sensitive K+ (KATP) channels. The protective effect of these drugs is suppressed by the KATP channel blocker glibenclamide. KATP channels also play a key role in ischemic preconditioning via activation of adenosine A1 receptors. Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death has also been observed. Adenosine A1 receptors and KATP channel, again, play an important role in this cross-preconditioning processes.
We have recently cloned and expressed a new family of K+ channels comprising 4 transmembrane domains and 2P regions. These channels are non voltage-dependent background channels and are expressed in different tissues but are usually very abundant in the brain. Two types of channels have a particular interest with respect to CNS function. The first one is TASK, a channel that strictly follows the Goldman equation and which is highly regulated by external pH (active at a physiological pH of 7.3, inactive at pH < 6.9-7). Changes of this channel activity probably play an important role in situations such as ischemia. The second type is TREK-1, a K+ channel that is activated by arachidonic acid and polyunsaturated fatty acids. This channel is also highly mechano-sensitive and inhibited by intracellular increases of cAMP and by PKC. The molecular mechanisms by which the regulations take place will be discussed.
This class of channels is the target of volatile anaesthetics and of drugs that provide potent neuroprotection.

Ion exchangers in hypoxia/ischemia

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The hippocampus is highly susceptible to neuronal injury by hypoxia and ischemia. The excessive release of glutamate during such events leads to sustained influx of $Ca^{2+}$and Na^+ and eventually to pronounced neuronal damage, especially in the CA1 region. While the pathways involved in $(Ca^{2+})_i$ increase have been thoroughly studied, the role of the entry ports involved in $(Na^+)_i$ elevation is less clear. Besides entering through Na^+-permeable ion channels Na^+ is also taken up via Na^+-dependent transport systems, such as glutamate transporters, Na^+/H^+ exchangers and Na^+/Ca^{2+} exchangers. During the last few years it became increasingly clear that Na^+/H^+ exchangers and Na^+/Ca^{2+} exchangers exist in multiple isoforms and splice variants, some of which appear to be brain specific. Na^+/Ca^{2+} exchangers couple the Na^+ to the Ca^{2+} gradient by electrogenically exchanging 3 Na^+ for 1 Ca^{2+}, causing Ca^{2+} extrusion (forward mode) or Ca^{2+} accumulation (reverse mode). Operating in parallell with Ca^{2+} pumps and Ca^{2+}-binding proteins, the Na^+/Ca^{2+} exchangers are critically involved in maintaining the Ca^{2+} homeostasis under normoxic conditions. During ischemia they can be expected to operate in reverse mode, because the decrease in membrane polarity, caused in part by the high $(K^+)_e$ and the large increase in $(Na^+)_i$ would favour the entry of Ca^{2+}. Until recently the contribution of the reverse mode to ischemic neuronal damage could not be assessed due to the lack of specific inhibitors. We show that the novel specific inhibitor KB-R7943 decreases ischemia-induced neuronal cell death in organotypic hippocampal slice cultures and improves population spike recovery after an insult in acutely isolated

hippocampal slices from adult rats. Both models may constitute a valuable interface between high throughput screening and in-vivo testing. Na^+/H^+ exchangers play an important role in pH- and volume regulation. Although specific inhibitors for some of the most frequent isoforms are still missing we can show that broad spectrum inhibitors like harmaline can reduce cell death in slice cultures even when applied after the insult, indicating that these exchangers play an important role during reperfusion. Our data suggest that besides the NMDA receptor and Na^+ channels the Na^+/Ca^{2+} exchanger in reverse mode and the Na^+/H^+ exchanger also contribute significantly to neuronal damage during ischemia in vitro. *This study is supported by LSA grant 2507A-0086H.*

Molecular neuropathology and signal transduction in neuronal damage and protection

Receptor dysfunction and the molecular basis of motor disorders

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Disorders of motility excede most neurological symptoms, comprizing both, increases in and reduction of neuronal activity. Spinal and brain stem neurons are particularly affected by disturbances in the equilibrium of excitatory and inhibitory impulses.

Glycine receptor (GlyR) mutant alleles in murine and human disorders: GlyR dysfunction is associated with an increased muscle tone. In the spastic mouse, insertion of a LINE-1 element into the Glrb gene affects splicing of beta transcripts, reducing GlyR number. The phenotype is modulated by a Mendelian background gene. Spasmodic mice carry the allele Glra1(A52S), diminishing a subunit agonist affinity. In oscillator mice, a Glra1 microdeletion causes lethality by a complete loss of GlyRs. Human hyperekplexia (startle disease, stiff baby syndrome) is caused by GLRA1 mutations affecting GlyR affinity and ion conductance. The substitution P250T diminished chloride conductances and enhanced desensitization, defining an intracellular determinant of channel gating. In the rare case of recessive hyperekplexia, we found a GLRA1(null) allele, consistent with a complete loss of receptor function.

Critical illness Polyeuropathy (CIP) - Excitotoxicity by endogenous compounds: The concept of excitotoxic cell death predicts that calcium channels, including NMDA receptors, are excessively activated due to an accumulation of agonistic factors. CIP is a frequent cause of weakness in intensive care patients, characterized as an axonal degeneration of motor nerves. Subjected to a sensitive bioassay, CIP sera proved toxic to spinal neurons. Seize fractionation revealed a low molecular weight neurotoxic activity other than glutamate, which was completely antagonized by the NMDA receptor antagonist MK 801. Cytotoxicity coincided with the formation of reactive oxygen species. These observations suggest that an humoral factor contributes to the pathogenesis of CIP.

Protease signalling, transglutaminase-mediated neuronal protein aggregation and synapoptosis: Strategies for neuroprotective paradigms

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It is now certain that components of hemostasis/ and thrombolysis cascades, including proteases and transglutaminases (TGases), also participate in neural plasticity. Neurons undergo apoptosis in culture when exposed to active thrombin by activating the protease-activated receptor 1 (PAR-1), which is localized to hillocks and long neurites. Its activation triggers retraction, cell rounding and finally death and involves both heterotrimeric and monomeric G-proteins with prominent [Ca2+]i mobilization. A proapoptotic molecule, essential for apoptotic body formation, is tissue TGase (tTG), the most prominent member in a gene family of dual function enzymes, involved in programmed cell life as well as death. As with thrombin, a critical coagulation factor (F. IIa) outside the brain, a prominent TGase is F.XIIIa that is activated by thrombin to cross-link fibrin. As with other TGases, tTG's best known function is the [Ca2+]-dependent cross-linking of substrate proteins via g glutaminyl-e-lysine isodipeptide bonds. Cytokines, such as IL-1b and TNF-a are important in regulation and the recent demonstration that tTG effects GTP hydrolysis (as a GTPase), established it also as a G-protein (Gah) the designation tTG/Gah indicates this duality of function. At what level of regulation this switch from life to death of this tTG/Gah occurs is unknown but clues to its basis came from the influence of either IL-1b or TNF-a on cultured astrocytes where long (L) and short (L) isoforms were found. We were interested in whether a) the forms related to development; b) they are altered by disease and/or injury in neural cells; c) they result from alternative mRNA splicing; and d) which form is associated with programmed life vs. death. To approach this we studied mouse spinal cord using primers for L and S tTG/Gah transcripts and found progressive evidence for switch from L to S in the neonatal period. In a rat injury model, rapid appearance of

S form appeared (<8 h), preceding apoptotic cell death. In brains of Parkinson (PD) and Alzheiner's disease (AD) victims, increased S form was found compared with controls. Evidence for isodipeptide bonds were also found in situ, co-localizing with inclusion bodies (neurofibrillary tangles and Lewy bodies). In immunopurified cultures of rat embryo spinal motor neurons, phosphorylated MAPK was most intense 1 h after thrombin addition and Rho GTPases A and B were distributed predominantly within axon branches. C. botulinum C3 exoenzyme, blocking RhoA activation, prevented thrombin-induced apoptosis, in contrast to trophic factor-withdrawal (a JNK1/p38-mediated event). Monoclonal antibody to L isoform (containing the GTP binding domain) stained hillocks and neurites of control cells, while antibody to S isoform (without GTP binding domain) only stained thrombin-treated cells undergoing apoptosis. Isodipeptide bonds formed in these cells and not control cells. Thus, cross linking of substrates, antedating inclusion formation and apoptosis, is first associated with alternative transcription to an S form of tTG/Gah, and is under the influence of thrombin's death signal. Supported by Medical Research, DVA, Christopher Reeves Paralysis Foundation,the Missouri Alzheimer's Fund and the KUMC Alzheimer Disease Center

Thrombin Signaling in Brain

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A serine protease thrombin has diverse action on brain cells and implicated in various pathological conditions. Thrombin functions are attributed to the activation of protease activated receptor-1 (PAR-1). At low concentrations, thrombin protects the neurons and astrocytes against hypoglycemia and oxidative injury; while high amounts of thrombin found in the brain during trauma, stroke and head injury, kill the brain cells that are detached from the matrix by apoptosis.Thrombin also induces morphological changes in neurons and astrocytes and these cytoskeletal changes precede thrombin-mediated cell killing and protection.Thrombin-induced morphological changes, killing and protection involve activation of small GTP binding protein RhoA. In addition, thrombin-mediated apoptosis also involves activation of certain casapases. How PAR-1 regulates these diverse thrombin signals is not known.

In order to identify the molecules that regulate thrombin signaling via PAR-1, we used yeast two-hybrid system to identify the molecules that directly interact with PAR-1 intracelluar regions. Human and rat brain c-DNA libraries were used for the screen. We have identified couple of molecules that may be involved in regulating thrombin signals intracellularly. We are currently carrying out experiments to determine if these molecules are necessary for thrombin- induced morphological changes, cell killing and protection and the results will be discussed at the meeting.

The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations

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We have considered the extracellular serine protease thrombin and its receptor as endogenous mediators of neuronal protection against brain ischemia. Exposure of gerbils to prior mild ischemic insults, here two relatively short-lasting occlusions (2 min) of both common carotid arteries applied at one day intervals two days before a severe occlusion (6 min), caused a robust ischemic tolerance of hippocampal CA1 neurons. This resistance was impaired if the specific thrombin inhibitor hirudin was injected intracerebroventricularly before each short-lasting insult. Thus, efficient native neuroprotective mechanisms exist and endogenous thrombin seems to be involved therein. *In vitro* experiments using organotypic slice cultures of rat hippocampus revealed that thrombin can have protective but also deleterious effects on hippocampal CA1 neurons. Low concentrations of thrombin (50 pM, 0.01 U/ml) or of a synthetic thrombin receptor agonist (10 μM) induced significant neuroprotection against experimental ischemia. In contrast, 50 nM (10 U/ml) thrombin decreased further the reduced neuronal survival that follows the deprivation of oxygen and glucose, and 500 nM even caused neuronal cell death by itself. Degenerative thrombin actions might be also relevant *in vivo*, since hirudin increased the number of surviving neurons when applied before a 6 min occlusion. Among the thrombin concentrations tested, 50 pM induced intracellular Ca^{2+} spikes in fura-2 loaded CA1 neurons whereas higher concentrations caused a sustained Ca^{2+} elevation. Thus, distinct Ca^{2+} signals may define whether thrombin initiates protection or not. Taken together, *in vivo* and *in vitro* data suggest that thrombin can determine neuronal cell death or survival following brain ischemia.

Role of desensitization and Ca2+ homeostasis in AMPA/KA receptor mediated neurotoxicity.

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Cultures of mouse neocortical neurons undergo a dramatic morphological and functional development during two weeks in culture. Such cultures grown for an increasing number of days were used to assess if developmentally related changes in sensitivity to AMPA((RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate) and kainate (KA) induced cytotoxicity could be correlated with changes in the desensitization of AMPA/KA receptors, the Ca2+ permeability of these receptors and the relative expression of the AMPA-GluR2 subunit controlling the Ca2+ permeability. Neurons in which the desensitization was blocked exhibited a pronounced sensitivity to both AMPA and KA induced toxicity particularly at later stages of development. Also the Ca2+ permeability of the receptors under these conditions increased during development. However, the relative expression of the GluR2 subunit only changed marginally during development in culture. These results suggest that the toxic action of AMPA and KA in these neurons is better correlated with regulation of the receptor desensitization process than with changes in the Ca2+ permeability as regulated by the presence of the GluR2 subunit in the AMPA receptor complex.

The molecular biology of stress responses in brain and blood and antisense technology

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Delayed, long-lasting deleterious changes of a similar nature occur in the mammalian brain in response to acute psychological stress, head trauma or exposure to acetylcholinesterase (AChE) inhibitors. We are exploring the molecular and neurophysiological mechanisms underlying these convergent consequences in mice and in perfused brain slices. *In vivo,* disruption of the blood-brain barrier under stress leads to efficient brain penetrance of anti-AChEs[1]. This induces a cascade of transcriptional responses suppressing synthesis and vesicle packaging of acetylcholine. In parallel, enhanced production of the stress-associated "Readthrough" AChE isoform AChE-R potentiates acetylcholine hydrolysis[2]. In brain slices, the reduced acetylcholine bioavailability suppresses the neurophysiological excitation induced by anti-AChEs. Such suppression can protect the brain from increased susceptibility to seizure activity and neuronal toxicity. However, prolonged accumulation of AChE causes, in anti-AChE treated mice, delayed hypersensitivity to AChE inhibitors and excessive glutamatergic excitation. In AChE transgenic mice, inherited AChE excess causes progressive deterioration of cognitive[3] and neuromotor[4] faculties and creates an extreme sensitivity to head trauma, itself an important risk for Alzheimer's disease. To prevent the deleterious morphogenic activities of AChE that are independent of its catalytic capacity, AChE overexpression can be suppressed using antisense oligonucleotides (AS-ODNs)[5]. AS-ODN treatment prevents the AChE induction that follows head injury. In head injured AChE transgenic mice, AS-ODNs further minimized the morbidity, facilitated neurological recovery, improved survival of CA3 hippocampal neurons and limited excessive dendritic growth. These findings suggest antisense prevention of AChE overproduction for early intervention with the cascade of events leading from variable stress insults to long-term neurological consequences. In the blood, stress responses involve rapid increases in white blood cell (WBC) and platelet counts, implying the existence of stress-responsive factors that modulate blood cell proliferation and augment immunological and blood clotting responses. In acutely stressed mice, we observed hematopoietic overproduction of AChE-R, and accumulation of the cleaved, variant-specific C-terminal AChE Readthrough Peptide ARP. Antisense suppression of AChE-R mRNA prevented, and injected synthetic ARP augmented *in vivo* the stress-induced WBC expansion. Moreover, transgenic mice overexpressing AChE-R[6] presented elevations in hematopoietic progenitors, WBC and platelet counts. In cell cultures, synthetic ARP improved the survival of cultured human $CD34^+$ progenitors and enhanced myeloid and megakaryocyte expansion in a manner similar to that of cortisol[7]. Our findings present AChE-R as a stress response element in both brain and blood, and its C-terminal peptide ARP as a promoter of hematopoietic expansion characteristic of acute and chronic stress responses.

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Neuroprotection or neurorepair in the adult brain: a preclinical approach

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The traditional view of the mammalian brain is that neurons are not added in adulthood. Thus, cell loss as a result of injury, disease or aging is permanent. The recent identification of stem cells in the adult mammalian nervous system and reports of adult neurogenesis in the primate brain, however, suggest that naturally occurring regenerative mechanisms may exist in the human brain. These observations raise the possibility that intrinsic stem cells and mechanisms of neurogenesis may be manipulated for purposes of repair. Several neuroendocrine and experiential factors that regulate neurogenesis in the adult dentate gyrus have been identified. Among others, adrenal steroid hormones have been shown to inhibit the production of new granule neurons whereas removal of circulating glucocorticoids results in a clear increase in neurogenesis. The biological relevance of adult neurogenesis is suggested by the observation that cell proliferation in the dentate gyrus can be modulated by experience. Stressful experiences are known to activate the hypothalamic-pituitary adrenal axis and increase levels of circulating adrenal steroids. Several different types of stressful experiences have been shown to inhibit granule cell production in the hippocampal dentate gyrus of rats, tree shrews and primates. In a recent study, rats were exposed to chronic social stress (resident-intruder paradigm) for 18 days. A second group of animals was also stressed on a daily basis. However, these animals received a daily treatment with transcranial magnetic stimulation (TMS) according to a standard protocol. This newly developed neurophysiological approach allows a painless and non-invasive stimulation of the intact brain and is used therapeutically in treating e.g. depressed patients. Compared to stressed rats, stressed+TMS treated animals had a significantly lower level of stress hormones. Currently we are investigating whether TMS treatment normalizes the proliferation rate in the dentate gyrus of chronically stressed animals.

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Growth factors, neurorepair and neurogenesis

Molecular mechanisms of motoneuron degeneration: Cell biology and clinical application

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In higher vertebrates, motoneurons are generated in excess during embryonic development, and a significant proportion of the newly generated cells that have made functional contact with their target field are eliminated. Embryonic motoneurons thus serve as a model for studying processes underlying the pathological changes in various forms of human motoneuron disease. During development and postnatal life, motoneurons are maintained by neurotrophic factors which are produced by skeletal muscle and glial cells. A variety of such factors which are members of various gene families have been identified. They support motoneuron survival in cell culture, and gene inactivation by homologous recombination has shown that these factors play together in supporting survival of these cells, both during embryonic and postnatal development.

Factors of the ciliary neurotrophic factor/leukemia inhibitory factor/cardiotrophin-1 family act on motoneurons through complex receptors involving the transmembrane molecules leukemia inhibitory factor receptor-β (LIFR) and gp-130. Inactivation of the genes for these factors has shown that double gene inactivation of CNTF and LIF leads to a loss of more than 35% of motoneurons. This corresponds to clinically apparent muscle weakness. In contrast inactivation of the CNTF gene only results in a mild phenotype which is characterized by 10% loss of muscle strength in comparison to wild-type animals. Screening of patients with amyotrophic lateral sclerosis has shown that 4 out of 104 patients show point mutations (G to A) at position 3400 of the *LIF* gene, which leads to amino acid exchange of valine to methionine at position 64 of mature LIF protein. This mutation has not been found in control patients. Moreover, homozygous gene inactivation of CNTF, which is found in about 2-3% of the population, results in earlier onset of ALS in comparison to patients which carry an intact CNTF gene. These data indicate that mutations in the genes for CNTF and LIF might be relevant and act as modifier genes, which, in combination with other genetic predispositions, might lead to motoneuron disease.

Investigations with isolated motoneurons, sensory and sympathetic neurons have shown that exposure of these isolated neurons to neurotrophic factors (nerve growth factor or CNTF) leads to a more than 25fold upregulation of members of the IAP/ITA family. The avian ITA is homologous to the baculoviral and mammalian inhibitor of apoptosis (IAP proteins), which can prevent apoptosis by inhibition of specific caspases. Overexpression of ITA in primary neurons supports survival of these cells in the absence of neurotrophic factors, and *ita* antisense constructs inhibited NGF-mediated survival. These data indicate that the upregulations of members of the IAP/ITA family is an essential signaling event for survival of primary neurons in response to neurotrophic factors.

Immunophilin Ligands and Nerve Regeneration

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Neuroimmunophilin ligands, FK506 (tacrolimus) being the prototype of this class, increase nerve regeneration in the peripheral and central nervous systems by accelerating its rate. The mechanism by which neuroimmunophilin ligands accelerate nerve regeneration is distinct from that underlying immunosuppression as shown by the retention of this property by nonimmunosuppressant derivatives of FK506. Furthermore, the 12-kD FK506-binding-protein (FKBP-12) immunophilin mediating immunosuppression is not required for FK506 to promote neurite outgrowth as demonstrated by the ability of FK506 to maintain its neurotrophic activity in primary hippocampal cell cultures from FKBP-12 knockout mice. Additional studies using human neuroblastoma SH-SY5Y cells revealed that the neurotrophic action of FK506 is completely prevented by addition of a monoclonal antibody to the immunophilin FKBP-52 (also known as FKBP-59 or Hsp-56); FKBP-52 together with Hsp-90 and p23 constitute mature steroid receptor complexes. Moreover, Hsp-90 binding compounds geldanamycin (a benzoquinone ansamycin) and radicicol (an antifungal compound), which disrupt mature steroid receptors by preventing the association between Hsp-90 and p23, are also neurotrophic. The neurotrophic activity of these compounds is presumably mediated via a yet identified "gain-of-function" resulting from the disruption of the mature steroid receptor complex. One possible downstream mediator is suggested from the known interaction between Hsp-90 and mitogen-associated protein (MAP) kinase/extracellular signal-regulated kinase (ERK2). This hypothesis is supported by the ability of the MAP kinase kinase (MEK) inhibitor PD 098059 to completely inhibit the neurotrophic activity of neuroimmunophilin ligands, geldanamycin and radicicol. Components of steroid receptor complexes thereby represent novel targets for the design of neuroregenerative drugs.

Immunophilins in the CNS – potential targets for neuroprotective processes in the brain after cerebral ischemia.

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Immunophilins are cis-trans peptidyl-prolyl isomerases (PPIases) which are involved in protein folding processes. The immunophilins, cyclophilin A and FK506 binding protein 12 (FKBP12), were discovered by their ability to bind and mediate the immunosuppressive effects of cyclosporin A, FK506 (Tacrolimus) and rapamycin. Immunophilins are enriched in the brain than in the immunosystem and are important in the regulation of neuronal functions. For example, FK506 is able to bind to FKBP12 in the brain and this complex specifically binds to serine/threonine protein phosphatase 2B (calcineurin) thereby inhibiting its phosphatase activity.

In the present paper we studied the temporal pattern and the cellular distribution of FKBP12 in the hippocampal formation after global cerebral ischemia in gerbils. Moreover, we investigated the neuroprotective effect of FK506 on ischemia-induced neurodegeneration in the hippocampus. Two experimental models were used: (I) transient global ischemia in gerbils and (II) transient exposure of organotypic hippocampal slice cultures prepared from rats to oxygen/glucose deprivation.

From our data we conclude that FKBP12 may be involved in the pathophysiology of neuronal degeneration following global ischemia. Four to six hours after the insult and parallel to the degeneration of neurons FKBP12 is upregulated in perikarya of CA1 pyramidal cells whereas days a few later it is localized in micro- and astroglial cells. FK506 (10 mg/kg) administered intraperitoneally both prior to global ischemia and after reperfusion was highly neuroprotective to CA1 pyramidal cells. In contrast, we did not observe any protection by this drug when organotypic hippocampal slices in cultures were exposed to oxygen/glucose deprivation. These results suggest, that FK506 and possible inhibitors of immunophilins have clinical potential for the treatment of stroke and ischemia.

TGF-ßs: Master molecules and switches in the regulation of neuronal survival and differentiation

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TGF-ßs are multifunctional, contextually acting cytokines with key roles in the control of cell fate, proliferation and differentiation. Major members of the superfamily include the TGF-ßs, the activins, the bone morphogenetic proteins (BMPs), and the GDNF family. TGF-ßs initiate their responses via membrane receptors,

heteromeric complexes of type I and type II serine/threonine kinases. GDNF and its congeners signal through a receptor complex consisting of the receptor tyrosine kinase c-ret and associated GFRa receptors. Evidence has accumulated during the past decade suggesting that TGF-ßs and their receptors are widely expressed in the developing and adult nervous system. This presentation will focus on the roles of TGF-ß in the control of neuron survival, differentiation, and death. Although TGF-ßs are not neurotrophic factors per se, they crucially control the actions and efficacies of other growth and neurotrophic factors. Examples will be presented showing that TGF-ß can synergize with other non-neurotrophic proteins to induce neurotrophic functions. Other examples will document that TGF-ß can induce responsiveness of neurons to neurotrophic factors, which in the absence of TGF-ß do not elicit detectable responses. The molecular bases of these synergisms will be discussed. Finally, the talk will feature a novel member of the TGF-ß superfamily, GDF-15/ MIC-1. GDF-15 is strongly expressed by macrophages/microglia and choroid plexus, from where the protein is secreted into the CSF. Documented activities of GDF-15 include promotion of midbrain dopaminergic and raphe serotonergic neurons. Most notably, GDF-15 protects lesioned nigrostriatal dopaminergic neurons in vivo. Together, these data underscore the complexity of the TGF-ß/neuro- trophic cytokine network in the nervous system and add to further disassembling the classic neuro- trophic factor concept. Supported by SFB 530 Homburg/Saar, SFB 488 Heidelberg, DFG Forschergruppe "Zentrale aminerge Systeme und Mechanismen", and BMBF/KfA/BEO 0310951 7

Glutamate receptors as target for neuroprotection

Zinc Entry and Neuronal Death In Vitro: Involvement of Glutamate Receptors, Calcium Channels and NAD+ Depletion.

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Zinc toxicity may participate in the pathogenesis of neuronal death following global ischemia, focal ischemia, head trauma, and spinal cord injury. We modeled this Zn2+-mediated death in cortical cell cultures, utilizing both a chronic non-depolarizing exposure (e.g. 40 mM Zn2+ for 24 hr), and a fast depolarizing exposure (e.g., 400 mM Zn2+ for 5 min in the presence of 60 mM K+). Fast Zn2+ entry and death can also be triggered by applying Zn2+ together with kainate, with Zn2+ entry occurring prominently through Ca2+ (and Zn2+) permeable AMPA receptors in some neurons, and through voltage- gated calcium channels in most neurons. We showed that zinc exposure under non-depolarizing or depolarizing conditions caused an early reduction in culture ATP levels and an increase in glycolytic intermediates preceding glyceraldehyde- 3-phosphate dehydrogenase (GAPDH). Recently, we showed that pyruvate reversed the latter effect suggesting that zinc inhibits GAPDH indirectly. We went on to demonstrate that zinc exposure caused an early reduction in NAD+ levels without an increase in NADH levels, and this reduction can be reversed by addition of pyruvate, or niacinamide. In addition, pyruvate induced an increase in lactate levels after zinc exposure, and the protective effect of pyruvate was attenuated by the competitive lactate dehydrogenase inhibitor, oxamate. Zinc toxicity was also attenuated by inhibitors of NAD+ catabolizing enzymes. We hypothesize that zinc may directly or indirectly activate an NAD+ catabolizing enzyme which reduces the NAD/NADH ratio, thereby inhibiting GAPDH, and that pyruvate restores NAD+ levels at the expense of NADH levels via its conversion to lactate.

Role of group-I metabotropic glutamate receptors in neurodegeneration/neuroprotection

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Activation of group-I metabotropic glutamate receptors (mGlu1 and -5 receptors) may either enhance or attenuate excitotoxic neuronal death depending on the experimental conditions. We examined the possibility that these receptors may switch between opposite functional modes in regulating excitotoxicity. In mixed cultures of cortical cells, the selective nGlu1/5 agonist, DHPG, amplified NMDA toxicity when applied only one time either in combination with NMDA or shortly before the NMDA pulse. In contrast, two consecutive applications of DHPG were neuroprotective. This switch in the response to DHPG was time-dependent and required an initial activation of protein kinase C. Similar results were obtained in pure cultures of cortical neurons, except that a single application of group-I agonists was less effective in potentiating NMDA toxicity. In cultures of hippocampal pyramidal neurons, DHPG was instead neuroprotective even after a single application, suggesting that group-I mGlu receptors were endogenously switched into a neuroprotective mode. These data suggest that group-I mGlu receptors are subjected to an activity-dependent

switch in regulating excitotoxic neuronal death, and therefore the recent story of these receptors is critical for the response to agonists or antagonists. However, the spontaneous trend of group-I agonists to be neuroprotective in pure neuronal cultures suggested that a glial mechanism might also be involved in the regulation of excitotoxicity. To examine this possibility, we studied NMDA toxicity in cultured cerebellar granule cells on which a coverslip of confluent astrocytes had been flipped. Activation of group-I mGlu receptors amplified NMDA toxicity only if astrocytes were present, being otherwise neuroprotective. An enhanced production of nitric oxide (NO) might contribute to this form of glial-dependent potentiation, because astrocytes expressed high levels of mGlu5 receptors and brain NOS under our conditions. We conclude that multiple factors affect the modulation of excitotoxic death by group-I mGlu receptors, and this should be taken into account when these receptors are viewed as targets for neuroprotective drugs.

Systemically active antagonists of Group I metabotropic glutamate receptors.

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Activation of Group I metabotropic glutamate receptors has immediate excitatory effects which can lead to epileptic activity and/or excitotoxic brain damage. This is seen for example after focal intracerebral administration of 3,5-dihydroxyphenylglycine, which acts equipotently on mGlu1 and mGlu5 receptors. The relative contribution of mGlu1 and mGlu5 receptors to excitatory effects varies according to the brain region (mGlu5 being significant in the hippocampus, mGlu1 in the cerebellum).

Group I antagonists can protect against excitotoxic or ischemic brain damage. Compounds initially studied either acted at both mGlu1 and mGlu5 or with some preferential effect on mGlu1. We have now studied the systemic effects of two novel antagonists highly selective for mGlu5 (SIB-1893 , (E)-6-methyl-2-styryl-pyridine and MPEP, 2-methyl-6-(phenylethynyl)-pyridine) in DBA/2 mice. Clonic convulsions induced by the intracerebroventricular administration of the selective mGlu5 agonist (R,S)-2-chloro-5-hydroxyphenylglycine are potently suppressed by both mGlu5 antagonists (SIB 1893 ED_{50} = 0.19 mg/kg. i.p.; MPEP ED_{50} = 0.42 mg/kg i.p.). Clonic convulsions induced by 3,5-dihydroxyphenylglycine are less potently suppressed (SIB 1893 ED_{50} 31 mg/kg, i.p.; MPEP ED_{50} 22 mg/kg, i.p.). These compounds are clearly powerful tools in evaluating the contribution of ativation of mGlu5 to excitotoxic damage.

Optic nerve axon injury: Intraaxonal signaling, retinal ganglion cell death and the expression of glutamate receptor genes

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Diffuse axonal injury is supposed to be responsible for secondary neurodegeneration after closed head neurotrauma. Mechanical injury leads to axon shear, stretch, and rupture, resulting either in secondary axotomy or internal axon damage. But not much is known about molecules that transfer signals from the injured axonal compartment to the cell soma and which regulate processes resulting in cellular degeneration, survival, regeneration or repair of axonal damage. It is therefore an unresolved question how intraaxonal signaling affects transcriptional regulation in response to injury. With a screen for differentialley expressed genes in the inner retina we identified a variety of molecules which are associated with the axonal cytoskeleton and are putatively involved in intraaxonal signaling. Some of these molecules could be involved in transcriptional regulation of genes that are involved in the activation of target genes which control cell death or survival. Most interestingly, axon injury leads to an altered expression of glutamate receptor genes. Data will be presented which suggest a link between intraaxonal signaling processes and the control of glutamate receptor gene expression in the inner retina.

Molecular mechanisms underlying ischemia-induced neuronal death: role of Ca^{2+} permeable AMPA receptors

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Considerable evidence suggests that Ca^{2+}-permeable AMPA receptors are critical mediators of the delayed neuronal death associated with transient global ischemia and sustained seizures. Global ischemia suppresses GluR2 mRNA, as indicated by *in situ* hybridization, and GluR2 subunit expression, as indicated by quantitative Western analysis and double immunolabelling, in vulnerable neurons of the hippocampal CA1 prior to the onset of neuronal death. This change in AMPAR subunit composition would be expected to result in increased expression of Ca^{2+} permeable AMPA receptors and enhanced glutamate pathogenicity in vulnerable neurons. Global ischemia increases AMPA receptor-mediated Ca^{2+} influx into CA1 pyramidal neurons prior to cell death and electrical recording from individual pyramidal neurons in fresh hippocampal slices. In slices from post-ischemic animals, CA1 neurons with robust action potentials exhibit

greatly enhanced AMPA-elicited rises in intracellular Ca^{2+}, as revealed by Ca^{2+} imaging. Basal Ca^{2+} concentrations and amplitudes of AMPA currents were unchanged. These observations provide evidence for Ca^{2+} influx through AMPA receptors in neurons destined to die and implicate Ca^{2+}-permeable AMPA receptors in the pathogenesis of ischemia-induced delayed neurodegeneration.
To examine whether acute down regulation of the GluR2 subunit, even in the absence of a neurological insult, can cause neuronal cell death, we performed GluR2 "knockdown" experiments. Antisense oligodeoxy-nucleotides targeted to GluR2 mRNA induced delayed death of pyramidal neurons in the hippocampal CA1 and CA3. Antisense-induced neurodegeneration was preceded by a reduction in GluR2 mRNA, as indicated by *in situ* hybridization analysis, and in GluR2 protein, as indicated by Western blot analysis. GluR2 antisense suppressed GluR2 mRNA in the dentate gyrus, but did not cause cell death. Administration of the AMPA receptor antagonist CNQX or of the Ca^{2+}-permeable AMPA receptor channel blocker, 1-naphthyl acetyl spermine, afforded protection against antisense-induced cell death, indicating that antisense-induced cell death is mediated by Ca^{2+}-permeable AMPA receptors. GluR2 antisense and brief sublethal global ischemia acted synergistically to cause degeneration of pyramidal neurons. These findings demonstrate that down regulation of GluR2 is sufficient to induce delayed death of specific neuronal populations.

Neuroprotective Actions of Novel and Potent Agonists of Group II Metabotropic Glutamate Receptors

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Previous studies have shown that activation of group II mGlu receptors may contribute to neuroprotective mechanisms in vitro and in vivo. We have examined the neuroprotective potential of novel potent group II mGlu agonists: LY354740, LY379268 and LY389795. The compounds were neuroprotective against toxicity induced by N-methyl-D-aspartic acid (NMDA), kainic acid and staurosporine as measured by release of lactate dehydrogenase (LDH) activity into culture supernatants and DNA fragmentation by oligonucleosome formation. Further evaluation of the neuroprotective effects of LY354740 in vivo showed that in a gerbil model of transient global cerebral ischaemia induced by a 3 min occlusion of the common carotid arteries, the compound dosed intraperitoneally (ip), prevented both CA1 hippocampal cell loss and apoptosis measured by TdT fragment end labelling of DNA. Treatment of gerbils with LY379268 (10mg/kg ip) provided a sustained neuroprotection against a 5 min occlusion at time points from 6hr to 28 days after surgery. The neuroprotective effects of LY379268 were blocked by administration of the mGlu receptor antagonist LY341495. Parallel studies to examine neurotrophic factor expression in the hippocampal regions of the brains taken from gerbils sacrificed at 6, 24 , 72 and 120 hours post-injection indicated that the mechanism of mGlu2 agonist-mediated neuroprotection did not involve induction of neurotrophic factor expression. The neuroprotective effects of LY379268 were also studied in a rat model of kainic acid - induced neurotoxicity. Administration of LY379268 (3-30mg/kg ip) to rats produced a delay in the onset and decreased the severity of KA-induced limbic seizures. The anticonvulsant effects were accompanied by a dose -dependent decrease in the severity of KA-induced neuronal necrosis in the hippocampus, entorhinal cortex and other regions of the brain. Taken together, these results support the view that agonists of mGlu receptors 2/3 may be useful agents in the therapeutic treatment of neurodegenerative diseases.

Reversal of neurodegenerative processes by selective modulation of group-I or group-III metabotropic glutamate receptor subtypes

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Group-I (mGluR1 and -5) and group-III (mGluR4, -6, -7, and -8) metabotropic glutamate receptors are known to modulate neurotoxicity of excitatory amino acids and beta-amyloid-peptide (b-AP). In order to specifically address which individual mGluR subtypes exert neuroprotective effects, we have used (+)-PPG (phosphonophenylglycine), as selective group-III agonist in combination with mGluR subtype-deficient mice and MPEP (methyl-phenylethynyl-pyridine), a highly selective mGluR5 antagonist. In cortical cultures exposed to a toxic pulse of NMDA, (+)-PPG reversed excitotoxicity with an IC50 of 4 microM, while its enantiomer (-)-PPG was found inactive. This correlates most closely with activity of (+)- and (-)-PPG at recombinant mGluR4a. In cortical neurons prepared from mGluR4-deficient mice, (+)-PPG showed no protection against the NMDA-insult, while Group-I antagonists retained protective activity. Thus, in vitro neuroprotection via group-III mGluRs is mediated by mGluR4. In addition, MPEP reversed toxicity of NMDA and b-AP, providing evidence for mGluR5 as attractive target for neuroprotective therapy.

Growth factors, neurorepair and neurogenesis

Intracellular mechanisms underlying the neural response to the myelin-associated neurite growth inhibitor NI35/250/Nogo-A

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Injuries to the mature mammalian brain and spinal cord are followed by permanent deficits due to a lack of regrowth of severed fiber tracts. One of the main impediments to axonal growth appears to be the non-conducive environment of central white matter. Besides neurite growth impairing properties of the glial scar tissue which forms around the lesion site, the presence of neurite growth inhibitory molecules, such as Nogo-A, MAG and certain chondroitin-proteoglycans that are associated with oligodendrocyte membranes and myelin sheaths are thought to play an important role for the lack of fiber regeneration in the adult CNS. Moreover, in vivo studies have shown that the neutralization of Nogo-A can lead to axonal regrowth over long distances and increased structural sprouting. Meanwhile, important advances have been made towards our understanding about the molecular and cellular mechanisms evoked by Nogo-A that lead to growth arrest and collapse of growth cones. Intracellular calcium measurements have revealed that growth cone collapse is mediated by the transient rise of intracellular calcium due to the release from intracellular stores. Subsequent studies demonstrated that RhoA, a member of the Rho family of small GTPases plays a critical role in the regulation of the growth cone cytoskeleton during Nogo-A-induced collapse and retraction. Furthermore, the neuronal intrinsic state, in particular the intracellular levels in cyclic nucleotide, seem to modulate the neuronal responsiveness that determines inhibition or advance of fibers in the adult mammalian CNS.

Neurotrophin receptor signaling in neuronal survival, differentiation and plasticity

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Neurotrophins signal through Trk receptors to regulate neuronal survival, differentiation and function. TrkB receptors are activated by two ligands, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4). To elucidate the intracellular signaling pathways that mediate the diverse effects of BDNF and NT4, we have generated in the mouse germline signaling mutants of TrkB, which uncouple adapter proteins from binding to TrkB. One of these mutations impaired the ability of NT4 to promote the survival of developing sensory neurons, but did not interfere in a major way with BDNF responses suggesting differential activation of TrkB by these two ligands. Moreover, the differentiation of CNS neurons was not affected suggesting that signaling via the Shc site on TrkB may be redundant with other pathways (Minichiello et al. Neuron 21, 335-345, 1998).
BDNF and its high-affinity receptor TrkB also regulate both short-term synaptic functions and long-term potentiation (LTP) of brain synapses. Since LTP is currently viewed as the best available cellular model of learning and memory, BDNF/TrkB signaling may be involved in higher cognitive functions. We have generated conditionally gene targeted mice in which TrkB receptors are specifically removed from the forebrain only during postnatal development. Western analysis revealed nearly complete loss of TrkB protein in dissected hippocampus and anterior forebrain samples of mutant compared to control mice. Mutant mice grow into adulthood without gross morphological abnormalities. Adult mutant mice are behaviorally hyperreactive to stress and exhibit a severe impairment in spatial learning tasks like the Morris water maze, but succeed in nonspatial learning tests, such as active and passive avoidance that do not require complex behavioral responses. Homozygous mutants show impaired LTP at CA1 hippocampal synapses. Interestingly, heterozygotes show a partial but substantial reduction of LTP although appear behaviorally normal. Thus, modest impairments in CA1 LTP are not necessarily paralleled by impaired learning. Rather, LTP may need to be reduced below a certain threshold before behavioral defects become apparent. This study establishes an essential role for TrkB neurotrophin receptor signaling in hippocampus-mediated complex learning and memory processes (Minichiello et al. Neuron 24, 401-414, 1999). Financial support from DFG, VW, HFSPO, EU.

Neurogenesis and gliogenesis in ischemic Brain

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To examine cell proliferation following ischemia, animals were administered bromodeoxyuridine systemically at various times following global ischemia. BrdU incorporation was quite high in the region of the hippocmapus in the first 1 to 4 days following ischemia,

being observed mainly in miroglia. Beginning at about 7-8 days there was increased BrdU incorporation into cells in the subgranular zone that peaked at 9-11 days and decreased to baseline by 21 days. Though 2 minutes of global ischemia did not stimulate cell proliferation, 3 minutes did with a peak at 5 and 10 minutes of global ischemia. Using neuronal markers, it was possible to show that the newborn cells began to assume a neuronal phenotype (NeuN and MAP2 immunostained in BrdU positive cells) around 20 days following ischemia. At 40 days following ischemia about 60% of the cells migrated into the granule cell layer, and about half of these cells were neurons. Of the remaining 30-40% cells, these migrated into the dentate hilus where only about a quarter were identified as GFAP stained astrocytes.

Plasticity after neuronal injury and neuronal restitution

Postlesional plasticity

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Brain Development and Neural Stem Cells

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During development of the central nervous system, most neurons are generated from undifferentiated, multipotent cells, often referred to as "neural stem cells". Within the last decade it became evident that neural stem cells continue to function in the brain of adult mammals, including rodents, primates, and humans. Proliferation, migration and differentiation of newly generated neuronal cells can be observed in two areas of the adult brain: the dentate gyrus and the subependymal zone of the lateral ventricles. This phenomenon opens new possibilities for the development of brain repair strategies. In particular, neural transplantation studies using stem cells or their progeny have already provided encouraging results in animal models of Parkinson's disease and Chorea Huntington. Another possible strategy makes use of the endogenous stem cell population of the adult brain. In this case, enhanced proliferation of stem cells, the proper guidance of neural progenitor cells to a target area, differentiation into specific neuronal lineages, as well as functional synaptic connectivity are needed to allow for neuronal replacement in areas of neurodegeneration. The ambitious approach of finding molecules that could orchestrate such a "repair from within" can only be performed if research from multiple areas is combined: I. Many growth factors and neurotrophic factors are known to be essential for brain development and it is very likely that some of these molecules are "reusable" to stimulate neural stem cells in the adult brain. II. Cell culture experiments, employing adult neural stem cells, have already helped to define several candidate molecules for neural stem cell proliferation and differentiation. III. The analysis of neural stem cells in the adult brain can reveal signals that are present in neurogenic regions. In particular, the subventricular zone harbors a continuously dividing stem cell population that can be exogenously influenced to generate more progenitor cells. The rostral migratory stream provides the essential matrix for long-distance migration through the adult brain and within the olfactory bulb neuronal differentiation into two types of interneurons takes place throughout life. The molecular and systemic signals that coordinate the naturally-occurring neurogenesis could be potential tools for novel brain repair strategies.

Lesion-induced and use-dependent mechanisms of reorganization following neuronal injury in the visual system

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Topography and size of receptive fields change at the border of 2 months old lesions in the adult cat visual cortex (Eysel&Schweigart, Cereb. Cortex 9,101-109,1999). This reorganization is preceded by an early increase of spontaneous and stimulus evoked activity (Eysel et al., Restor. Neurol. Neurosci. 15,153-164,1999). In an attempt to differentiate lesion-induced and use-dependent mechanisms, we now have studied the spatio-temporal pattern of early lesion-induced changes in vivo and have characterized long-term potentiation (LTP) and depression (LTD) in the same post-lesion time-window in vitro. Experiments were performed in the anaesthetized, adult cats with focal excitotoxic visual cortex lesions (1.5-3.0mm) in vivo and in cortical slice preparations of rats (age>22d) with focal infrared laser lesions (1-1.5mm). In the adult cat we compared neurons at identical recording sites in vivo, pre-lesion and post lesion, with a 7 electrode array for 4-5 days. Increased excitability was observed 2-4 days after lesioning within 2 mm from the border of the lesion. RF field and subfield sizes were slightly increased in correlation with the changes in neuronal activity. After visual training of the post-lesion RFs for 1 hour there was a small but significant increase in RF size (0.4°-0.8°) in about half of the tested neurons. This RF increase was similar to that observed in unlesioned adult cats (Eysel et al., Neuroreport 9, 949-954, 1998). Hence, small RF size

changes on the order of 1° can be induced within minutes to hours in normal cells and in cells at the border of sub-acute cortical lesions in vivo. To test our hypothesis that plasticity is changed at the border of cortical lesions we investigated LTP and LTD in layers 2/3 of 350µm slices of rat visual cortex with 1-6 day old laser lesions. We observed significant increases of LTP (sub- and intracortical inputs) and LTD (intracortical inputs) in the surround of cortical lesions between day 1 and day 6 after lesioning. We conclude that increased excitability is an early sign of plasticity in regions that undergo long-term reorganization following lesions in the visual cortex, and that this is accompanied by an intrinsic upregulation of the potential for synaptic plasticity. Supported by the DFG, SFB 509, TP C4 and TP C7.

Potential usefulness of donor Schwann cells or ONECs in supporting directional host axonal regeneration in the adult rat CNS

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The transplantation of axon growth promoting substrates (cellular and acellular) into the CNS is of substantial interest due to the potential therapeutic benefits of supporting axonal regeneration across lesion sites. Although much encouraging data has been obtained, an optimal growth supportive substrate has yet to be developed. Ideally, such substrates should possess the capacity to integrate with the host tissues, where they may support and direct axonal regeneration across the lesion site. Using light- and electron microscopic techniques, we have been investigating the potential of donor glial cells to form such bridges. To obtain a detailed understanding of the interactions of donor and host elements in an easily accessible region of the CNS, we have transplanted glial cells into the adult rat thalamus. Suspensions of tissue cultured Schwann cells or olfactory nerve ensheathing cells (ONECs) were loaded into a glass micropipette and stereotaxically transplanted into the adult rat thalamus. The donor cells were grafted in the form of elongated columns which extended from the thalamus, across the choroid fissure and into the hippocampus. Within the first 24 hours post operation (p.o.), donor cells extended processes, in parallel with the long axis of the transplant. Microglial and astroglial cells rapidly responded by migrating into the transplant, where they adopted the orientation of the transplanted cells. The ensuing „mosaic" pathway, of intimately mixed donor (PNS) and host (CNS) glial profiles co-operated with each other in the support and direction of host axonal growth. Neurofilament (NF)-positive axons grew into the column of cells and immediately adopted an orientation, in parallel with the long axis of the graft. Regenerating thalamic axons extended across the choroid fissure (a natural barrier between the thalamus and hippocampus) and entered the hippocampus. Control experiments, using microtransplanted fibroblast, revealed a similar pattern of microglial migration. However, neither astrocytic nor NF-positive axonal profiles could be detected within the grafts. These experiments demonstrate that the beneficial axogenic effects of such glial cell transplants involves a highly regulated interaction between a number of (CNS and PNS) cellular elements, resulting in the formation of an organised pathway capable of supporting axonal regeneration across natural barriers within the brain.

Brain repair using an embryonic stem cell strategy

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Transplantation of neuroepithelial precursor cells represents a promising experimental strategy for cell replacement in the central nervous system. Embryonic stem (ES) cells may provide a solution to a key problem in neural transplantation, the limited availability of suitable donor tissue. ES cells are derived from the inner cell mass of early blastocysts and can be expanded to virtually unlimited numbers while retaining their potential to generate all tissues and cell types. We have developed in vitro protocols which permit the generation of precursors for neurons, oligodendrocytes and astrocytes from ES cells. Transplant experiments show that these ES cell-derived precursors can participate in brain development and replace neurons and glia in animal models of neurological diseases. ES cell-derived glial precursors might be particularly useful for the treatment of myelin disorders. Following transplantation into the spinal cord of myelin-deficient rats, they efficiently myelinate large numbers of host axons. Widespread cell delivery and myelin repair in multiple regions of the brain can be achieved by intraventricular transplantation in the embryonic and neonatal brain. Our findings demonstrate that it is possible to generate cell type-specific somatic precursors from ES cells and that these cells can be used for nervous system repair. Combined with recent progress in the generation of human ES cells and the possibility of cloning embryonic cells from adult tissue, this route may eventually permit the generation of large numbers of neural precursors from the same patient.

Abstracts Poster

Synaptic plasticity in the dentate gyrus is affected in glutathione-depleted rats

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Long-term potentiation (LTP) have been proposed as a cellular model for the underlying mechanisms of learning and memory. LTP consist of a long-lasting increase in synaptic efficacy, after stimulating afferent fibers with high frequency. These long-lasting changes insynaptic efficacy probably involve pre-synaptic and post-synaptic events. LTP like memory have phases, which are sustained by different molecular mechanisms. While the initial stage of LTP seem to depend on increases in intracellular Ca++ concentration through NMDA receptors, in the later phases, like memory, the synthesis of new proteins is crucial. Glutathione is a sulphured tripeptide that acts as a potent antioxidant in the nervous system. Recently some in vitro studies have suggested that the cellular redox status can affect the molecular mechanisms involved in synaptic plasticity. However, the effects of the disturbance of glutathione system on LTP have not been studied. In this work we have evaluated the input-output relationship, long-term potentiation and the paired-pulses interactions in rats depleted of glutathione by diethylmaleate injection. Our results indicate that the basal synaptic transmission at the dentate gyrus is not affect in depleted-glutathione rats, however is not possible to induce neither LTP in the hippocampal synaptic efficacy, nor paired-pulses facilitation. This data suggest that low glutathione-concentrations affect both, long-lasting and short-lasting synaptic plasticity. Two possible, non excluding mechanisms might be involved: a) The redox status affect the ion flux through NMDA receptor, which is implicated in both forms of plasticity, or b)The efficacy of transmission in GABAergig Further experiments should serve to clarify these issue. Our results stress the importance of redox systems in brain function.

Key words: glutathione-depleted, LTP, synaptic plasticity, paired pulses

Mechanical trauma induces delayed nerve cell death in rat entorhinal-hippocampal slice cultures

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The aim of the present study was to develop an in-vitro model that produces a delayed nerve cell death, since this type of cell death represents the major target for putative clinical drug interventions against acute brain injury. We used entorhinal-hippocampal slices from 12 days old rats. The slices were cultured for 14 days before experiments. At experimental day zero the cultures were incubated with propidium iodide (PI) 3 h prior to a mechanical crush in the middle layers of the entorhinal cortex. The cultures were digitally photographed daily for seven days. PI fluorescence intensity was measured in selected regions, as an indication of cell death.

Mechanical crush treatment induced a prominent cell death at the site of primary injury at day 1. This cell death decreased over the following days, with a slower decrease taking place at days 3-7. A transient cell death was observed in the dentate gyrus, CA1, and possible in CA3 at days 1 and 2. At days 4-7 a delayed cell death developed in dentate gyrus, CA1, and possibly in CA3.

The present data indicate that crush injury may induce two types of secondary cell death. The first, early, type of cell death is induced quickly and is maximal at days 1-2, after which the dead cells are gradually removed. The second, delayed, type of cell death becomes observable only from day 4. The relative contribution of apoptotic versus necrotic cell death following the crush is yet unknown, but being under current investigation. PI only labels permeated cells and therefore, in theory, only necrotic cells. However, it may be that the delayed cell death observed with PI reflects apoptotic cells that has not succeeded to be removed without plasma membrane leakage. The decrease in PI observed in the cortical region at day 2-7 and in the hippocampus both at day 3 and to some extent at day 7, indicates that PI-labeled cells are efficiently disintegrated, possibly by microglia.

These results may represent the first demonstration of trauma-induced delayed cell death using organotypic slices. Furthermore, this model may provide useful for future investigations of mechanisms underlying delayed nerve cell death, as a foundation to develop clinical treatment.

Leptin synthesis in bovine intramuscular preadipocytes (BIP) during growth phase

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Leptin, a hormone secreted from adipocytes, is thought to regulate food intake and energy expenditure. Previous reports have demonstrated that leptin has the ability of angiogenesis for the construction and repair of capillary in brain and other organs. We have established a clonal bovine intramuscular preadipocyte cell line (BIP) derived from Japanese Black cattle, which have the capacity to proliferate and differentiate into mature adipocytes. We investigated the expression of BIP cells. BIP cells proliferated rapidly to confluence until 3 days after seeding, and then to a low growth rate. BIP cells between 1st and 2nd culture day showed the highest growth rate. PCR products corresponding to leptin mRNA was present from 2 to 4 days in culture. The cytoplasm leptin concentration, detected by a western bolt analysis, was a peak on 2nd culture day (1.37 micro-g/mg protein) and decreased gradually afterward. These data reveal that leptin was synthesized in preadipocyte (BIP cells) during the growth phase. Most reports have demonstrated that leptin mRNA expression in rodents and humans does not occur in preadipocytes. This difference may be because a ruminant cell line was used or because the cells were intramuscular preadipocytes rather than cells from normal fat depots in the present experiment. Moreover, in common with our results, leptin mRNA levels were correlated with the number of preadipocytes in the culture.

Neuroprotective and cognition enhancing properties of melatonin, N-acetylserotonin and their synthetic analogs in experimental models of Alzheimer's Disease type neurodegeneration

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Recently it was shown that endogenous pineal hormone melatonin (Mel) as well as it precursor N-acetylserotinin (NAS) protect neurons against oxidative challenges such as glutamate and H2O2 [J. Pineal Res., 1998, 24:168]. In the present work we report date on NAS, melatonin and their newly synthesized derivatives neuroprotective activity against b-amyloid peptide (bAP) - specific hallmark of Alzheimer's Disease (AD) and on their cognition enhancing properties in toxicological animal model of AD. Exposure of cerebella granule cells (CGC) against bAP fragment 25-35 (bA), responsible for bAP neurotoxicity, caused reduction of cell viability in dose-dependent manner (IC50= 25mM). Joint incubation of 25mM bA with 25-100 mM of NAS or melatonin or some their synthetic analogs for 72-96 h increased the amount of surviving neurons up to the control level. For estimation of compounds cognition enhancing properties animal model of AD-type neurodegeneration induced by cholinergic neurotoxin - AF64A have been used. Two weeks after AF64 treating (3nm/3mcl, i.c.v.) animals cognition functions were tested in active avoidance performance and in Morris water-maze test. Chronic treatment with melatonin, NAS and their analogs CA-15 and CA-18 (0.3 - 3.0 mg/kg orally, daily for 12-14 days) reverses the negative effect of AF64A both in learning and in retention tests (in active avoidance), and in water-maze paradigm up to the level of control group of animals. It can be suggested that neuroprotective properties of NAS, melatonin and their derivatives might mediate their cognition enhancing action.

Cell type specific upregulation of vascular endothelial growth factor (VEGF), angiopoietins (Ang-1, Ang-2) and the tie-receptor-family (tie-1, tie-2) in a rat model of cerebral ischemia

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Background: VEGF (vascular endothelial growth factor) is an endothelial cell specific mitogen that has been implicated in hypoxia-mediated angiogenesis under physiological and pathological conditions. The angiopoietins (Ang1 and Ang2) constitute a novel family of endothelial growth factors that are ligands for the endothelium-specific receptor tyrosine kinase, tie2. Unlike VEGF, which functions during the early stages of vascular development, the angiopoietins, their common receptor tie2 and the associated receptor tie1 exert their function at later stages, i.e. during vascular remodeling and maturation. It is hypothesized that Ang2-induction leads to vessel proliferation or regression depending on the presence or absence of VEGF. The purpose of the present study was to investigate the participation of these factors in cerebral ischemia-induced angiogenesis. Methods: We used the middle cerebral artery occlusion model (MCAO) in the rat to investigate mRNA and protein localization of VEGF, angiopoietins, tie-1 and tie-2 by nonradioactive in-situ hybridization and immunohistochemistry. Results: We observed a maximum induction of VEGFmRNA 12h after MCAO in the periphery of the ischemic area with gradual downregulation during the following days. The predominant VEGF expressing cell types were microglial cells and macrophages as determined by double-immunohistochemistry with CD11b, ED1 and alba1-antibodies. Ang2mRNA was upregulated in a biphasic pattern. There was a first peak 12h to 24 h after MCAO, Ang2mRNA was observed in the infarct area, in the peri-

infarct area, to a lesser extent in the corresponding area of the contralateral hemisphere and in both hippocampal formations. The majority of Ang2mRNA expressing cell types were Ecs in the tips of endothelial cell cords, though also some astrocytes could be observed to express Ang2mRNA. 3d MCAO (2nd stage) the Ang2-mRNA expression in ECs had shifted from the limited induction in tips of EC cords to all ECs in an expressing vessel. Ang2-mRNA transcripts were now seen at high levels in small and also larger vessels surrounding the infarct area, while Ang2-mRNA expression in the contralateral hemisphere had disappeared. In order to show proliferation and remodeling of cerebral vessels around the infarct area we performed double immunohistochemistry using CD31- and Ki67-antibodies. 24h, 3d and 7d after MCAO we found a strong coexpression of both antigens on vessels immediately adjacent to the infarct area. To determine which cells undergo apoptosis in the contralateral hemispheres, we performed a double labeling study combining the TUNEL assay with immunohistochemistry for lectin. The single apoptotic cells in the contralateral hemisphere could be identified as Ecs. A marked tie1mRNA upregulation was detected three days after MCAO in the periinfarct zone. Tie1 protein could also be found in vessels immediately adjacent to the infarct zone at this point of time. 7days after MCAO the expression pattern was nearly unchanged. Tie2 protein was broadly expressed in the endothelium of the quiescent vasculature of the rat brains and no ischemia-induced upregulation could be detected. Three days after MCAO endothelial cells around the infarct zone showed a tie2mRNA-upregulation whether at 7d expression was no longer detectable. Ang1-mRNA was constitutively expressed in different cell types of glial, e.g. astrocytes and neuronal origin, e.g. Purkinje cells, throughout the cortex and cerebellum. There was no change in expression following MCAO. Conclusion: The failure of angiogenesis and insufficient growth of collateral vessels is a major problem in vascular diseases, such as cerebral stroke. It is of considerable importance for therapeutic purposes to elucidate the exact mechanisms by which the growth factor signaling cascade can again be recruited for an angiogenic response in adults. We observed a specific upregulation of the examined angiogenic factors and receptors in experimental induced cerebral ischemia. Their temporal and spatial expression pattern suggests different mechanisms of induction and different roles in the specific stages of angiogenesis.

Moderate hypoxia reduces pentylenetetrazol-induced seizures: A role of a calcium-sensor protein, visinin-like protein –1 ?

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It has been shown that a moderate hypoxia has a neuroprotective influence in that it reduces pentylenetetrazol (PTZ)-induced seizures in mice (1) and rats (2). The cellular mechanisms of this effect are still unknown. Previous investigations from our group had revealed that after hypoxia plus PTZ certain cellular markers (c-Fos, ornithine decarboxylase) were altered in their expression, which might partly explain the neuroprotective effect of hypoxia (1). In this study we used differently treated rats (hypoxic animals, active and passive controls with or without subsequent PTZ injection) to immunohistochemically localize two calcium sensor proteins (visinin-like proteins-1 and –3, VILIP-1 and VLILIP-3) in rat hippocampus. In control rats VILIP-1 was found to be localized in numerous (probably GABAergic, [3]) interneurons. A clear dorso-ventral gradient was found in the density of VILIP-1-immunoreactive neurons. Only a very few cells expressed VILIP-3. Hypoxia alone did not significantly alter the number of VILIP-1 containing hippocampal neurons in comparison to controls. PTZ-treatment increased the number of VILIP-1 expressing nerve cells (twice as much as in controls, $p<0.001$). In those hypoxic animals which showed drastically reduced seizures after PTZ (stage 1) we found only slightly increased amounts of VILIP-1 immunopositive neurons (about 115% of controls, difference to controls was not significant). The number of VILIP-3 –containing neurons was unchanged during the experiments. Our data show that moderate hypoxia prevents the PTZ-triggered overexpression of VILIP-1 in certain hippocampal neurons. Hence, calcium-mediated processes might contribute to the neuroprotective effect of hypoxia prior to PTZ.

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Mutually Protective Actions of Kainic Acid Epileptic Preconditioning and Sublethal Global Ischemia on Hippocampal Neuronal Death: Involvement of Adenosine A1 Receptors and KATP Channels

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Preconditioning with sublethal ischemia attenuates the detrimental effects of subsequent prolonged ischemic insults. The mechanisms underlying this neuroprotection remain largely unknown. The main objective of this

research was to elucidate potential *in vivo* cross-tolerance between different neuronal death-generating treatments such as kainate administration that induces seizures and global ischemia. This study also investigates the effects of a mild epileptic insult on neuronal death in rat hippocampus following a subsequent, lethal epileptic stress, using kainic acid (KA) as a model of epilepsy and characterize common endogenous mechanisms in these different models of tolerance. Three preconditioning groups were as follows. Group 1 was injected with 5 mg/kg KA prior to a 6-min global ischemia. Group 2 received a 3 min global ischemia prior to 7.5 mg/kg KA. Group 3 was injected with a 5 mg/kg dose of KA prior to a 7.5 mg/kg KA injection. The interval between treatments was three days. Neuronal degeneration, revealed by the silver impregnation method and analysis of cresyl violet staining, was markedly reduced in rats preconditioned with a sublethal ischemia or a 5 mg/kg KA treatment. TUNEL labeling and DNA laddering confirmed the component of DNA fragmentation in the death of ischemic and epileptic neurons and its reduction in all preconditioned animals.The present study supports the existence of bi-directional cross-tolerance between KA excitotoxicity and global ischemia and, suggests the involvement of adenosine A1 receptors and sulfonylurea-sensitive ATP-sensitive K+ channels in this protective phenomenon.

Neuroprotective effects of LY379268, a selective mGlu2/3 receptor agonist: investigations into possible mechanism of action in vivo.

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The possible mechanism of action underlying the neuroprotective effects of the Group II metabotropic glutamate receptor (mGluR) agonist, LY379268, has been investigated in the gerbil model of global ischaemia. Ischaemia, induced in gerbils by bilateral carotid artery occlusion (BCAO) for 5 min, produced a large increase in locomotor activity at 24 and 120 hr post-occlusion and a severe loss of CA1 cells in the hippocampus at 120 hr post-occlusion. LY379268 (10 mg/kg i.p.) 30 min or 60 min after BCAO attenuated the ischaemia-induced hyperactivity and provided protection in the CA1 cells. Further studies indicated that the compound was protective ($p < 0.001$) when histological analysis was performed 14 and 28 days after surgery.
To study the mechanism of action of LY379268 we used the Group II mGlu antagonist, LY341495, in combination with LY379268 and we also evaluated the bioavailability of LY379268. Results indicated that pre-treatment with LY379268, at 10 mg/kg, i.p., dosed 24 hr ($P < 0.001$) or 48hr ($P < 0.05$) prior to 5 min BCAO, reduced the damage to CA1 hippocampal neurones. However, when LY379268 (10 mg/kg, i.p., 24 h prior to occlusion) was dosed in combination with LY341495, dosed at 1 mg/kg, i.p., 1 h prior to occlusion, the neuroprotection afforded by LY379268 was prevented. In parallel studies treatment with LY379268 (10 mg/kg i.p.) failed to alter the expression of a number of neurotrophic factors (TGFβ, BDNF, NGF, bFGF) in the hippocampal regions of brains at 6, 24, 72 and 120 hours post-injection. Analysis of the brain concentration of LY379268 at 24h following dosing of 10mg/kg ip showed that a significant receptor-active concentration of the compound persisted at this time and is likely to account for the efficacy of the compound.

Opioid and somatostatin receptor systems in the hippocampus after a contusion trauma in rats.

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Traumatic brain injury can be divided into primary damage, which appears immediately after injury at the site of impact and secondary damage which occurs distant to the impact several hours or days after injury. We used a contusion trauma model in 7 weeks old Wistar-rats in order to investigate immunocytochemically secondary changes occuring in the hippocampus.
During nembutal narcosis a craniotomy of 4.5 mm diameter was performed over the right hemisphere1.5 mm posterior and 2.5 mm lateral to the Bregma. A 20 g weight was guided onto a footplate resting upon the surface of the dura. Animals were perfused with 4 % paraformaldehyde at different time points after lesion and 40 µm free-floating coronal sections were immunocytochemical stained. Several specific antibodies were used, recognizing the neuropeptide somatostatin and its receptor subtypes (sst2A, sst3 and sst4), the µ-opioid receptor and the endogeneous opioids dynorphin and enkephalin. Further we used Ox-42 a monoclonal antibody against the surface antigen CD 11b/c, which is a specific marker for activated microglia. One day after trauma, the number of somatostatin-positive neurons in the hilus of the damaged ipsilateral hippocampus was markedly reduced. The immunoreactivity for the sst4-receptor was drastically reduced in the pyramidal cells of the damaged hippocampus. Further, we find substantial cell loss of the sst3-positive neurons in the CA3 region and in the dentate gyrus. In contrast, the immunoreactivity after cortex contusion for the µ-opioid receptor remained nearly unchanged between ipsi- and contralateral hippocampus. Already 24 h after trauma we observed the infiltration of sst4 positive cells as well cells positive for a pan-opioid

antibody recognizing both dynorphins and enkephalins. Although, we found a dramatic activation of microglia, beginning at day 1 and lasting for at least 7 days, there is no colocalization of sst4 and CD 11b/c. Our findings indicate that opioid and somatostatin peptide systems play a differential role in modulating neuronal cell death and infiltration of cell populations in damaged brain regions. The precise nature of these cells has to be characterized further.

Mechanisms of FK506 neuroprotection following cerebral ischemia: effects on enzyme activity, microglia and hemodynamics

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The macrolid FK506, which is used in humans as immunosuppressant following (liver) transplantation, has been shown to be neuroprotective after nerve injury and cerebral ischemia. FK506 binds to FK506 binding proteins (FKBPs), which comprise a heterogenous family of meanwhile 15 members in mamalians. The FKBP -FK506 complex has different functions ranging from inhibition of protein phosphatase 2B and interference with peptidyl-prolyl cis/trans isomerase activity. After transient focal cerebral ischemia in rats, we analyzed the expression of FKBP12, 52 and 65, which did not change significantly in the peri-infarct area. However, changes of peptidyl-prolyl isomerase activity were detected in the necrotic infarct core 24 hours after ischemia and this enzyme activity could be blocked does dependently with FK506 (1mg/ kg and 5 mg/ kg). On cellular level, FK506 treatment decreased the c-Jun phosphorylation, a putative marker for stress kinase activity and attenuated the microglial activity. Neuroprotection by FK506 was observed in the peri-infarct area and in areas of secondary degeneration such as the substantia nigra, where FK506 diminished the neuronal loss of tyrosine hydroxylase expression in size matched infarcts. MRI tests to reveal the hemodynamic effect of FK506 in cerebral ischemia are under performance. FK506 (1 mg/kg) is neuroprotective following ischemia by reducing neuronal damage and glial activation. Reduction of peptidyl -prolyl cis/trans isomerase activity plays more likely an important role rather than alterations in the expression of FKBPs.

Contribution of ion channels to ischemia-induced damage in rat hippocampus in vitro is time-dependent

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The hippocampus is highly susceptible to neuronal injury by hypoxia and ischemia. Massive ion movements during such events, in particular the sustained influx of Ca^{2+} and Na^{+} and the efflux of K^{+} eventually lead to pronounced neuronal damage, especially in the CA1 region. A number of ion channels have been reported to be involved in these ion movements but their role in ischemia-induced neurodegeneration is still not completely clear. Using acutely isolated hippocampal slices from adult and organotypic hippocampal slice cultures (OSC) from 10-day-old male Wistar rats we investigated if the activation or inhibition of cation channels influences neuronal recovery after hypoxia/hypoglycemia. Recovery of the synaptically evoked population spike in the CA1 region in acutely isolated slices and propidium iodide staining after 24 h in OSC were taken as measures of neuronal viability. The AMPA-receptor antagonist NBQX (10 - 100 µM) and the Ca^{2+}-channel blocker nimodipine (10 µM) did not influence neuronal viability. In contrast, the Na^{2+}-channel blocker tetrodotoxin (3 -30 µM) significantly reduced damage when present during and the NMDA-receptor antagonist MK-801 (1 - 100 µM) as well as the novel K^{+}-channel opener Y-26763 (10 - 100 nM) also when present after an insult. In conclusion we suggest that the timing of activation of these cation channels is critical for neurodegeneration. *This study is supported by LSA grant 2507A-0086H.*

Apoptotic pathways that regulate glucose deprivation-induced cell death in cerebellar granule neurones

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Our recent work in CNS primary culture has shown that selected antagonists of P2 ATP receptor prevent neuronal cell death under glutamate-evoked excitotoxicity, hypoglycaemia, chemical hypoxia and selected mitochondria dysfunction. We have now examined the contribution to these stressful events of various biological pathways. We show that cerebellar granule neurones under hypoglycaemic insult undergo a combination of apoptosis and necrosis. This is demonstrated by morphological and biochemical features, such as TdT-mediated dUTP-biotin nick end-labelling, fluorescent staining of nuclear chromatin using Hoechst 33258, propidium iodide incorporation and direct count of intact viable nuclei. As measured by all these means, cell death results maximally prevented in the presence of the antagonist of P2 ATP receptor basilen blue. We furthermore find that

hypoglycaemia selectively stimulates the release of cytochrome c from mitochondria. It up-regulates heat-shock protein HSP70, but not HSP90, and glucose-regulated GRP75 and GRP78 proteins. The transcription and expression of caspase 2 are also modulated by hypoglycaemia. Supplying basilen blue differently regulates these effects. Our results show that basilen blue elicits its neuroprotective role by acting on both apoptotic and necrotic cell death and by discriminating between different apoptotic pathways.

A set of genes expressed in response to kainate in the adult hippocampus by using Gene Discovery Array

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The molecular mechanisms underlying the response to injury in the central nervous systems are incompletely understood. Here we used a model of central nervous system lesion in the mouse to induce recurrent limbic seizures and status epilepticus. Systemic treatment of NMRI-mice with kainate, an analogue of glutamate, was performed. Kainate-induced irreversible damage (develops 24 hours after kainate injection) results in reactive changes, which include modifications in gene expression and synaptic plasticity. One day after kainate treatment, the mRNA from mice hippocampus was prepared, while normal adult mouse hippocampus mRNA served as control. In order to identify and analyze the changed expression of molecules, cDNA was hybridized with Gene Discovery Arrays (Genomesystems) representing 18,000 genes or ESTs on nylon filter. Several differentially expressed genes can be assigned to the ones known to be associated with plasticity, whereas others can be assigned to pathways not previously associated with plasticity. Additionally, genes of unknown function were identified that may be involved in the plastic response. The utility of this approach is demonstrated for studying a set of complex biological processes in the hippocmpus underlying the response to excitation of neurons.

Contribution of muscarinic-Ach, alpha2-adrenergic and GABAB receptors to the depression of synaptic transmission induced by hypoxia

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Hypoxia markedly depresses synaptic transmission in hippocampal slices of the rat. This depression is attributed to presynaptic inhibition of glutamate release and is largely mediated by adenosine released during hypoxia acting through presynaptic adenosine A1 receptors. Paired pulse facilitation studies allowed us to confirm the presynaptic nature of the depression of synaptic transmission during hypoxia. We tested the hypothesis that activation of heterosynaptic inhibitory receptors localized in glutamatergic presynaptic terminals in the hippocampus, namely gama-aminobutyric acid subtype B (GABAB) receptors, alpha2-adrenergic receptors, and muscarinic M2 receptors might contribute to the hypoxia-induced depression of synaptic transmission. Field excitatory postsynaptic potentials were recorded in CA1 area of hippocampal slices from young adult (5-6 weeks) Wistar rats. Neither the selective antagonist for alpha2-adrenergic receptors, rauwolscine (10 microM), nor the antagonist for the GABAB receptors, CGP 55845 (10 microM), modified the response to hypoxia. The selective adenosine A1 receptor antagonist, DPCPX (50 nM), reduced the hypoxia-induced depression of synaptic transmission to 57.7±11.8%, and the muscarinic receptor antagonist, atropine (10 microM), in the presence of DPCPX (50 nM), further attenuated the depression of synaptic transmission to 48.5±9.6%. In the same experimental conditions, in the presence of DPCPX (50 nM), the muscarinic M2 receptor antagonist AF-DX116 (10 microM), but not the M1 receptor antagonist pirenzepine (1 microM), also attenuated the hypoxia-induced depression to 36.9±6.1%. Activation of adenosine A1 and muscarinic M2 may act in an additive way in order to maintain evoked neurotransmitter release under control during prolonged periods of hypoxia.

Effects of endogenous adenosine on short- and long-term phenomena of synaptic plasticity in young and old rats

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Adenosine acting through adenosine A1 receptors is able to limit long-term potentiation (LTP) (de Mendonça & Ribeiro, 1994), long-term depression (LTD) and depotentiation (DP) (de Mendonça et al, 1997) and also to increase paired-pulse facilitation (PPF) in the hippocampus of young rats. During normal aging, the hippocampal formation undergoes changes that might contribute to age-related differences in synaptic function and behaviour (Geinisman et al., 1995) and these alterations in processes that influence synaptic strength may underlie age-related memory deficits. Among these changes, modifications in adenosine neuromodulation may have particular importance. We compared the effects of the selective adenosine A1 receptor antagonist, 8-dipropyl-1,3-cyclopenthylxanthine (DPCPX), on LTP, LTD and DP elicited in hippocampal slices taken from young adult (6 weeks old) and old (2 years old) rats. Evoked field

excitatory post-synaptic potentials (fEPSP) were recorded extracellularly from CA1 stratum radiatum. DPCPX (50 nM) attenuated PPF both in young adult (1.64±0.05% vs 1.76±0.05 in control solution, n=6, P<0.05), and in old rats (1.33±0.05% vs 1.55±0.1% in control solution, n=6, P<0.05). LTD was only observed in the presence of the selective adenosine A1 receptor antagonist, DPCPX (50 nM), in both young (21.3±0.6%, n=4, P<0.05) and old rats (14.4±0.9%, n=4, P<0.05). A larger DP was observed in old rats in the presence of DPCPX (50 nM) (41.3±5.1%, n=6) than in its absence (16.1±2.7%, n=6, P<0.05). Similarly, in young rats a larger DP was observed in presence of DPCPX (50 nM) (27.6±4.4%, n=7) than in the control conditions (16.8±4.7%, n=7, P<0.05). LTP was induced by theta-burst pattern stimulation (3 bursts of 3 individual pulses at 100 Hz separated by 200 ms). In young rats, this pattern of stimulation induced LTP in the presence of DPCPX (50 nM) (53.9±4.9%, n=5), but not in control conditions (6.4±1.1%, n=5, P<0.05). In old rats this pattern of stimulation elicited LTP either in the presence (81.8±17.9%, n=7) or the absence of DPCPX (50 nM) (98.5±24.2%, n=7, P>0.05). Taken together the present results suggest that the modulatory role of endogenous adenosine on synaptic plasticity is maintained in aged rats. These findings reinforce the importance of testing adenosine antagonists as memory/cognitive enhancers.

Melatonin, riluzole, and 7-nitroindosole improve motor performance following experimental spinal injury

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Spinal injury induces early and latant neuronal damage resulting in irreversible motor disfunction. Majority of the proposed coping strategies target processes such as excitotoxicity, oxidative stress, oncotic and apoptotic neuronal cell loss, and inflammation. We aimed to test and compare the effects of melatonin (MEL), 7-nitroindosole (7-NI), and Riluzole (RIL) in a rat model of spinal cord injury. All of these agents are known to be more or less and directly or indirectly related with the ultimate control of Ca++ homeostasis in neurones. Ten groups of adult female Sprague Dawley rats were experimented in the following scheme: Naïve (n=6), Placebo (n=7), Lesion (n=6), Sham Operated (n=6), Melatonin (n=6), Melatonin + Lesion (n=7), 7-Nitroindosole (n=5), 7-Nitroindosole + Lesion (n=7), Riluzole (n=5), and Riluzole + Lesion (n=5). Experimental spinal injury was induced at level T7-T8 by five sec compression of total cord with an aneurism clip on anaesthetised and laminectomised animals which became and remained paraplegic following compression. All agents were obtained from Sigma and administered i.p.: 10mg/kg MEL, 10mg/kg 7-NI, 6mg/kg RIL Motor performance was assessed by Tarlov (inclined plane) and Open Field Walking Scales (OPWT) performed in the 40th hour and 7th day post-injury. Data was analysed by SPSS. Motor performance in both scales showed very significant (p=0.026) impairment in the lesioned group compared to all the other groups. Tarlov scores revealed significant benefits from MEL (p=0.005) and RIL (0.005) but none from 7-NI at the 40th hour and significant protection by MEL (p=0.002), RIL (0.004), and 7-NI (p=0.033) on the 7th day. OPWT results showed significant favourable effects of MEL (p1=0.005; p2=0.001) and RIL (p1=0.002; p=0.002) both in the 40th hour and 7th day of injury. 7-NI did not favour motor performance in the 40th hour whereas it became beneficial in the 7th day (p=0.003). Within a time course of one week, either significantly or not, the protective effects of all the agents became more potent in general in both Tarlov test (7-NI: p=0.0102; MEL: p=0.102; RIL: p=1) and OFWT (7-NI: p=0.008; MEL: p=0.041; RIL: p=1). In conclusion, the negative outcomes of spinal injury in the rat were remarkably attenuated by MEL and RIL in both short and long term, and by 7-NI particularly in the long term. (Supported by TUBITAK SBAG-U 15/4)

Melatonin and 7-nitroindosole improve motor performance following experimental focal ischeamia

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Focal cerebral ischeamia is an important cerebrovascular disorder which induces early and latant neuronal damage resulting in irreversible motor and / or cognitive disfunction varying according to severity, duration and locus of occlusion. Majority of the proposed coping strategies target processes such as excitotoxicity, oxidative stress, oncotic and apoptotic neuronal cell loss, swelling, and inflammation involved in the pathogenesis. We aimed to test and compare the effects of the antioxidant and free radical scavenging neurohormone, melatonin (MEL) and the neuronal NOS inhibitor, 7-nitroindosole (7-NI) on motor performance in a rat model of focal ischeamia. Both of the agents are known to be more or less and directly or indirectly related with the ultimate control of Ca++ homeostasis in neurones. Eight groups of adult male Sprague Dawley rats were experimented in the following scheme: Naïve (n=6), Placebo (n=6), Ischeamia (n=6), Sham Operated (n=7), Melatonin (n=6), Melatonin + Ischeamia (n=6), 7-Nitroindosole (n=5), and 7-

Nitroindosole + Ischemia (n=5). Using Tamura's technique, right cerebral artery of rats was occluded irreversibly following Tiopental sodium (40 mg/kg) anaesthesia. MEL and 7-NI were obtained from Sigma and administered i.p. in the following protocole: 10mg/kg MEL was injected in one fourth doses at 20 min before, during and at 1h and 2h post-occlusion. 10mg/kg 7-NI was injected in half doses at 20 min before and at 20 min post-occlusion. Motor performance was assessed by Tarlov (inclined plane) and Open Field Walking Scales (OPWT) performed in the 40th hour and 7th day post-ischeamia. One way ANOVA showed a significant (p=0.002) impairment of motor performance in the ischeamia group compared to all other groups. Student Newman Keuls Test and Mann Whitney U Test analysis of the Tarlov and and OPWT scores revealed significant benefits induced by MEL (p1=0.003 and p2=0.002) and by 7-NI (p1=0.005 and p2=0.004) both in the 40th hour and 7th day of ischeamia. Neither of the agents was significantly more beneficial than the other. Wilcoxon Signed Ranks Test did not reveal any significant difference between the measuremets done at different times. In conclusion, our results reflect remarkably favourable effects of MEL and 7-NI on motor performance following focal cerebral ischeamia in the rat. (Supported by TUBITAK SBAG-U 15/5)

Differential gene expression after mechanical optic nerve injury: I. genes putatively associated with axonal remodeling

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Mechanical damage of the optic nerve induced by a calibrated crush device leads to axon shear, stretch and membrane rupture. Despite this structural damage rat optic nerves exhibit extensive reorganisation processes at the ultrastructural level within 1 week post-injury. A combined subtractive hybridization / suppression polymerase chain reaction screen for genes differentially expressed in the inner retina after optic nerve crush was initiated and a method established for the rapid analysis of regulated transcripts in retinal ganglion cells after axon injury. From this screen in the retinal ganglion cell layer and the optic nerve we identified several candidate genes associated with cytoskeletal components, signal transduction and transcriptional regulation. Differential expression of genes possibly associated with axonal remodeling was analyzed in more detail. These experiments showed that α-actinin-2, kinesin light chain, MAP-1b and thymosin-ß4 seem to be differentially expressed. α-actinin-2 belongs to the actin-based membrane cytoskeleton which takes part in forming a meshwork beneath the cytoplasmic surface of the growth cone membrane and therefore is supposed to be involved in adhesion of growth cones and in neuronal plasticity. Kinesin is known to be involved in anterograde axonal transport. It is supposed to be a molecular motor that generates ATP-dependent movements along microtubules. Efficient transport is particularly important in neurons where vesicles and organelles move substantial distances from sites of synthesis in the cell body to sites of activity at the axonal termini. The up-regulation of members of the kinesin light chain family suggests that the anterograde axonal transport of vesicles, possibly providing material for neurite extension and axonal repair is increased. MAP1B is a major cytoskeletal protein in growing axons and is strongly regulated during brain development. Phosphorylated forms were shown to exist mainly in axons, whereas unphosphorylated forms were limited to cell bodies and dendrites. Phosphorylated MAP1B was quite abundant in developing axons, suggesting its essential role in axonal elongation. MAP1B is the only MAP to be found consistently in extending processes in both the developing and adult brain, making it a likely regulator of neurite outgrowth. Thymosinß4 (tß4), a gene encoding a major G-actin binding protein involved in neuronal migration and differentiation, is also dramatically upregulated in the inner retina after optic nerve crush. One of the major functions of tß4 is sequestration of G-actin and thus inhibition of actin polymerization. In line the peptide has been thought to participate in G-actin depolymerization needed for neurite extension and growth cone functions during development Furthermore, several kinases interacting with the cytoskeletal proteins (pp125FAK, PYK2) as well as members of the MAP-kinase and Jun-kinase pathway are regulated by crush injury.

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Activation of group-II metabotropic glutamate receptors increases transforming growth factor beta expression in vivo

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Activation of group-II mGlu receptors protects neurons against excitotoxic death or other forms of neurodegeneration. We have recently shown that neuroprotection mediated by group-II mGlu receptors involves a novel form of glial-neuronal interaction which is mediated in vitro by the activation of mGlu3 receptors. Cultured astrocytes respond to mGlu3 receptor activation by releasing one or more neuroprotective agents.

Transforming growth factor beta (TGF-beta) is one of these agents, because it is released in response to mGlu3 receptor agonists, and neutralizing antibodies against TGF-beta 1 or beta 2 prevent the neuroprotective action of the medium collected from astrocytes treated with mGlu3 receptor agonists. Both TGF-beta 1 and beta 2 are highly neuroprotective against excitotoxic death in culture as well as in vivo (Bruno et al, J Neurosci, 1998). Here we have examined the effect of group-II mGlu receptor agonists on TGF-beta 1 synthesis, by measuring TGF-beta 1 mRNA and protein levels. In cultured astrocytes, a transient exposure to the group-II mGlu receptor agonist, 4-carboxy-3-hydroxyphenylglycine (4C3HPG) increased TGF-beta 1 mRNA levels. A similar increase was observed in the rat corpus striatum 7 days after local infusion of 4C3HPG. The TGF-beta 1 protein levels were also increased in the corpus striatum 7 days after 4C3HPG local injection. We therefore conclude that the synthesis of TGF-beta 1 (a TGF-beta isoform that is expressed in very low amount in the CNS under normal conditions) is under the control of group-II mGlu receptors. Activation of group-II mGlu receptors may provide a mechanism for increasing the local production of TGF-beta in the brain, thereby protecting neurons against various toxic insults.

Bio-engineering of peripheral nerves

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Bio-engineering is considered to be the laboratory based alternative to human autografts and allografts. It ought to provide "custom made organs" cultured from patient's material. Venous grafts and acellular muscle grafts support axonal regeneration only to an certain extent due to the lack of viable Schwann cells in the graft. We created a biologic nerve graft in the rat sciatic nerve model by implanting cultured Schwann cells into veins and acellular gracilis muscles, respectively. Autologous nerve grafts, and veins and acellular muscle grafts without Schwann cells served as controls. After 6 and 12 weeks regeneration was assessed clinically, histologically and morphometrically. The PCR analysis showed that the implanted Schwann cells remain within all the grafts. A good regeneration was noted in the muscle-Schwann cell-group, while regeneration in both of the venous grafts and the muscle grafts without Schwann cells was impaired. The muscle-Schwann cell graft showed a systematic and organized regeneration including a proper orientation of regenerated fibers. The number of axons regenerating through the muscle-Schwann cell-grafts was significantly increased compared with the other grafts. The venous grafts with Schwann cells showed less fibrous tissue and disorganization than the veins without Schwann cells, but failed to show an excellent regeneration. This might be attributed to the lack of endoneural tube like components serving as scaffold for the sprouting axon. Although the conventional nerve graft remains the gold standard, the implantation of Schwann cells into an acellular muscle provides a biologic graft with basal lamina tubes as pathway for regenerating axons and the positive effects of Schwann cells producing neurotrophic and neurotropic factors, and thus, support axonal regeneration.

Gap junctional blockers reduce the extent of cell death after ischaemic or traumatic episodes

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A submersion model of ischemia in organotypic hippocampal slices was used to study the possible role of gap junctional communication (GJC) in spreading cell death during reperfusion. Cell death, measured with propidium iodide fluorescence, was significantly reduced in the slices pretreated with the gap junctional blocker carbenoxolone (120 M). In addition, two models of mechanical trauma injury were used to assess the role of gap junctional communication in spreading cell death after the impact damage. Primary traumatic injury was achieved by rolling a glass cylinder on the organotypic slice, and secondary traumatic injury by dropping onto the slice a weight of 0.137g from a height of 2 mm. The initial localised impact damage in the secondary injury model spread 24, 48 and 72 hours after the injury, cell death being 11% at 24 hours and 35% at 72. The gap junctional blockers carbenoxolone (120 M) and octanol (50 M) significantly reduced the spread of the cell death in both trauma models. Higher concentrations of octanol (200 M) were found to be toxic. To determine whether specific connexins (the proteins that form gap junctions) are involved in spreading the damage, we used organotypic brain slices prepared from mice with a mutation for connexin43 (a connexin present mostly in astrocytes). Cell death spread to a similar extent in slices from wild type, heterozygote (that express less than half of connexin43 expressed by wild-type) and knockout mice. These results indicate that GJC plays a critical role in the spread of ischaemia/trauma related injury in brain tissue and that these models of moderate mechanical trauma could be potentially exploited to investigate neuroprotective strategies.

Neuroprotective effects of a novel phenytoin derivative in an in vitro model of ischemia.

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Excessive calcium influx during anoxia leads to irreversible neuronal injury. Blocking non-inactivating sodium conductances by the anticonvulsant phenytoin may decrease calcium influx by preventing activation of calcium channels and attenuating reverse sodium/calcium exchange. The present abstract addressed the hypothesis that pretreatment of organotypic hippocampal slices with phenytoin and its novel derivative 5,5-diphenylthiohydantoin (5,5 DTH) will increase neuronal survival following in vitro anoxia. The neuroprotective efficacies of equipotent concentrations of phenytoin and 5,5 DTH were assessed using a submersion method of ischemia in organotypic hippocampal slices. The compounds were applied 1 hour before the insult and maintained during the experimental period. Both phenytoin and 5,5 DTH reduced neuronal death estimated by propidium iodide fluorescence (Table). Phenytoin (100 M) 5,5 DTH (125 M) Cell survival (% of control) 58±8.1% 60.6±4.5% 5,5 DTH ameliorated ischemic neuronal loss in a dose-dependent manner and prevented electrophysiologic functional deterioration as assessed by recovery of population spike in the CA1 region. Furthermore, lowering the ambient temperature to 33oC from 37oC significantly enhanced the neuroprotective effects of 5,5 DTH. However, no additive neuroprotection was determined when 5,5 DTH was used in combination with another potent neuroprotective agent, Cyclosporin A. These results suggest that preapplication of phenytoin and its novel derivative 5,5 DTH increased neuronal survival after anoxic insults. The advantage of 5,5 DTH is an anticipated low toxicity profile and a similar potency compared with its prototype.

Involvement of protein kinase B in neuronal cell death following transient forebrain ischemia

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Activation of protein kinase B (PKB, also known as Akt) is stimulated by treatment of cell with insulin or growth factors with concomitant phosphorylation at Thr-308 and Ser-473. Activation of PKB provides a survival signal that protects cells from apoptosis induced by various cytokines and stresses. We here found alteration of phosphorylation of PKB following transient forebrain ischemia and a neuroprotective action of stimulators of PKB. Adult Mongolian gerbils were anesthetized and a 5-min forebrain ischemia was carried out under normothermia. Immunoblotting analysis with the anti-phospho-Ser-473 antibody showed that phosphorylation of PKB was in the CA1 subfield decreased immediately after ischemia, following by increased 2 and 6 hrs after reperfusion. In contrast, the decreased phosphorylation of PKB was not evident in the CA3 subfield. The subsequent increases in the phosphorylation was still observed. We then tested effects of intraventricular injection of insulin-like growth factor 1(IGF-1) which is known to activate PKB. The treatment of IGF-1 30 min before transient ischemia ameliorated the delayed neuronal cell death in the CA1 subfield following ischemia. Interestingly, the injection of IGF-1 also prevents the decreased phosphorylation of PKB in the CA1 subfield observed immediately after ischemia. These results suggest that the decreased activity of PKB underlies the neuronal vulnerability in the CA1 subfield following forebrain ischemia and that activation of PKB by IGF-1 treatment rescues neurons from the delayed neuronal death in the hippocampus.

Estimation of astrocytes viability in cell culture exposed to hypoxia under influence of trimetazidine

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Hypoxia produces sequential morphological and biochemical changes in glia including increased number of mitochondria, increase of rough endoplasmic reticulum abundance, and their transformation into reactive astrocytes. It is known that in this condition in vitro astrocytes have a beneficial effect on neuronal survival and for themselves as well. The aim of the present study was to establish, whether it is capable to increase the viability of cultured astrocytes exposed to hypoxia. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinum bromide (MTT, Sigma Co., USA) conversion test and Live/Dead Viability/Cytotoxicity Assay Kit (Molecular Probes, USA). Astrocytes were prepared and cultured according to the method of Hertz et al. On the 21st day the cells placed in the medium deprived of glucose and serum were incubated for 8 hours in the following conditions: 92% N2, 5% CO2 and 3% O2 at 37 0C. For MTT assay trimetazidine was added to the medium at the concentrations of 0,1 μM, 1 μM, 10 μM and 100 μM 24 hrs before, during hypoxia and the consecutive 24 hrs period of reoxygenation. Besides, the drug in the above-mentioned

concentrations was added to cultured cells in the normoxic conditions. Live/Dead assay was performed on astrocytes treated with trimetazidine at concentration 10 μM in similar schema. Cells were examined by fluorescence imaging set MiraCal Pro III workstation (Life Science Resources Ltd., UK) comprising of inverted microscope Eclipse TE200 (Nicon, Japan) and high-resolution cooled CCD camera (Photonic Science Ltd., UK). It was observed a significant decrease of surviving astrocytes in hypoxia conditions after measurement by Live/Dead Kit. On the other hand trimetazidine displayed apparent protective action enhancing the percentage of surviving cells. The ratio of living to dead cells depended strongly on the sequence of drug adding and the onset of hypoxia/reoxygenation. The conversion of MTT reflecting the activity of mitochondrial electron transport chain reaction revealed to be in accordance with the data obtained by Live/Dead Kit. The drug by itself did not tend to activate the mitochondrial respiratory chain. In the hypoxic groups treated with trimetazidine where the augmented astrocytes kill was observed, the activation of mitochondrial function was noted.

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Considerations on Radioprotection of CNS tissue

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Background: Radiation oncologists consider brain tissue to be a late reacting tissue. Clinical observations indicate that neurophysiological changes can occur even later than three years after radiation. There must be processesresponsible for this that are currently unknown and different from those thzat cause normal cell death by ionizing radiation. Questions and goals: Is there a chance of a pharmacological protection of CNS tissue from late complications after radiotherapy? In order to develop protecting medication the underlying processes have to be evaluated thorroughly. Current hypothesis: The mechanisms of late changes in brain tissue after ionizing irradiation are nearly unknown whereas early side effects can be described by DNA damage and consecutive apoptosis or necrosis. The following hypothesis for late complications is based on histological findings: - Disturbance of the vessel intima (endothel) followed by a chronic edema. - Disturbance of the oligodendroglia in parallel with demyelinization of the white matter and liberation of glucosides - Immigration of lymphocytes and macrophages Due to destroyed blood-brain barrier resulting in a new microglia - Autoimmunological processes continuously driven by the microglia and the produced gangliosides – a process that is self-conserving and locally progressive. Investigational steps: Two different approaches are possible: Already in-vitro or in-vivo tested substances have to be evaluated in animal settings or clinical trials. Approved medications that were developed for a different purpose might be the first candidates. The basic knowledge of the radioinduced pathophysiology regarding signal transduction, stress response or apoptotic processes has to be improved. Regulating proteins seem to be of major interest. A specific experimental setup including a fast observation equipment after local irradiation of cells and whole organ preparations has to be developed.

In vitro ischemia causes induction of c-fos and c-jun in rat cell culture systems - comparison between primary neural cell cultures and PC 12 cells.

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BACKROUND: Cerebral ischemia causes many physiological, biochemical and morphological changes on cellular level in the brain. Using techniques of molecular biology, recent experimental studies have shown that cerebral ischemia also induces a variety of changes in gene expression. Although during the early postischemic stage the protein synthesis in the brain is generally suppressed specific genes as the immediate early genes (IEG) and their corresponding proteins are synthezised. However, there are only limited experimental data on c-fos and c-jun expression in primary and secondary cell cultures after in vitro ischemia. MATERIAL AND METHODS:Therefore, we developed a model using rat primary cultured cells (neuron rich and astrocyte rich cell cultures) and a cell line (PC 12 cells) to study the effect of one hour in vitro ischemia followed by 1 hour of reoxygenation on the c-fos and c-jun mRNA levels. RNA was analysed by RT-PCR using GAPDH as an internal standard in a competetive PCR assay. RESULTS: One hour of in vitro ischemia causes a significant increase in c-fos and c-jun mRNA in all cell culture systems, which was most pronounced in neuron rich cultures with a 10 fold increase in c-fos mRNA level (17,93 ± 6,66 compared to 1,73 ± 0,32; $p< 0,05$) and a 7 fold increase in c-jun mRNA level (35,58 ± 4,16 compared to 4,99 ± 1,0; $p<0,05$), respectively. In astrocyte rich cultures there was an about 4 fold increase for c-fos mRNA (21,65 ± compared to 5,58 ± ; $p<0,05$) and a weaker increase of about 2,5 fold for c-jun mRNA (13,24 ± 5,43 compared to

4,80 ± 1,40; p<0,05) respectively. For PC 12 cell cultures we found similary results with an about 5,5 fold increase for c-fos mRNA (8,27 ± 1,94 compared to 1,49 ± 0,31; p<0,05) and for c-jun mRNA an 2 fold increase (13,86 ± 5,63 compared to 6,34 ± 1,95; p<0,05) respectively. The addition of the non-competitive NMDA receptor antagonist MK 801 to the cell culture medium during and after ischemia significantly reduced the induction of c-fos and c-jun in neuronal cultures, while no effect on c-fos and c-jun mRNA levels was detected in PC 12 cells. CONCLUSIONS: This in vitro model offers new possibilities to study ischemia induced gene-expression in various cell culture systems and to investigate the effects of neuroprotective compounds with respect to their influence on specific gene expression and necrotic and apoptotic pathways in ischemic cell culture systems.

PAR ligands and S1P involvement in Ca^{2+} regulation in human epithelial (HBE) cells.

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There is growing evidence that endogenous serine proteases like thrombin and trypsin may have cytoprotective properties in neural and non-neural cells. These effects are transduced via activation of the G protein-coupled protease-activated receptors (PAR) 1 and 2 , respectively. On the other hand sphingomyelin metabolites can induce (ceramide) or prevent (sphingosine-1-phosphate (s1p)) apoptosis in various tissues. The protective effect of PAR-2 activation is well documented in airway epithelial cells. Therfore, we initially wanted to elucidate the Ca^{2+} signalling induced by trypsin and s1p alone or in combination in human bronchial epithelial (HBE) cells, before extending our gained knowledge to cells of neural origin, e.g. astrocytes.

In HBE cells typsin evoked an intracellular Ca^{2+} response, whereby the sensitivity of the cells as well as the amplitude of the response strongly depend on the agonist concentration with a maximum change at 50 nM trypsin. From the dose-response curve we calculated an IC_{50} value of 10 nM. Furthermore we could show that the amplitude of the trypsin-elicited response depends on extracellular Ca^{2+}, showed a concentration- and time-dependent desensitization, and could be mimicked by the PAR-2 activating peptide SLIGRL. In contrast to trypsin the ability of s1p to induce Ca^{2+} responses, revealed a great variabilty, which seems to depend on the culture period. Comparison of the two agonists showed that Ca^{2+} was released from different stores, since prior addition of the SERCA-inhibitor cyclopiazonic acid (CPA) abolished the Ca^{2+} response to a subsequent addition of trypsin but not of s1p. Taken together, our results show that trypsin and s1p differentially affect the cellular Ca^{2+} homeostasis in epithelial cells and now should be compared with the Ca^{2+} responses induced by these agonists in glial cells.

Electrophysiology in brain slices

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Properties, communication and pathophysiology of nerve cells and their networks as well as glia are studied in brain slices since about 30 years. Here we want to discuss the validity of the methods for neuroprotection and –repair. After submitting an animal to acute or chronic damaging influences slices from its brain may be studied at various time intervals and in comparison with animals exposed to some protective treatment. The standard paradigms of interest are excitability and conductivity of neurons, synaptic transmission (exciting, inhibiting, modulating) and synaptic plasticity. Experiments with extracellular stimulating and recording electrodes, preferably at dendritic and somatic sites can provide reliable and salient information, offering themselves for many screening purposes. Intracellular recording with sharp or patch electrodes, pharmacological or mechanical isolation of cells and fast application systems allow more sophisticated and mechanistic investigations. Morphological identification of living cells, uncaging of compounds at restricted spots and visualisation of signaling molecules (Ca, cAMP) are also available. Typical examples for strength and weakness of the brain slice methods are discussed.

Immortalized rat nigral progenitor cells of the cell line CSM14.1 as a model to study the influences of trophic factors on neural and glial development in vitro

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Immortalized stem cells transfected for the secretion of trophic factors are potential candidates that can be used as cell transplants in the treatment of Parkinson's disease. In this study we wanted to establish an in vitro differentiation procedure for the nestin expressing rat nigral cell line CSM14.1 to study the influences of various trophic factors (for example GDNF) on the differentiation and protein expression patterns of these cells.

CSM14.1 cells were cultured and expanded in DMEM/10% FCS and incubated at 33°C. Cells were passaged on L-lysine coated culture slides and allowed to sit down for six

hours at 33°C in DMEM/10% FCS. Then the medium was replaced by neurobasal medium supplemented with B27 (serum free), DMEM/1% FCS (serum reduced) or DMEM/10% FCS and incubation continued at 39°C (nonpermissive temperature). After 3, 7, 10 and 14 days the cells were fixed with 4% PFA and prepared for immunocytochemistry.
The cell numbers in the serum free and serum reduced media decreased, whereas increased in the medium containing 10% FCS. Cell proliferation occured in spite of the nonpermissive temperature in all three experimental approaches. Tyrosine-hydroxylase (TH), NF68, NeuN and MAP2 were not expressed in detectable amounts, whereas the expression of MAP5, NF160, NF200, NSE, GFAP and oligodendrocytic epitopes increased during incubation time. The serum reduced medium (1% FCS) showed the highest rate of differentiated cells. In previous in vitro studies of the CSM14.1 cells TH and NF68 were only detectable using northern blot techniques.
Using the established differentiation protocol, our laboratory has now a tool to investigate in further studies the influences of extracellular GDNF (for example cocultures with GDNF-transfected striatal ST14A cells) on the differentiation and protein expression (especially TH) of the immortalized rat nigral cell line CSM14.1 in vitro. We thank D.E. Bredesen (Los Angeles) for providing us with the CSM14.1 cells.

Slices cultures: a valid tool to study neuronal differentiation and the formation of connections.

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Slice cultures of various brain regions are widely used to examine the differentiation of neurons and the establishment of functional connections under experimental conditions. We used the entorhino-hippocampal system to study axonal growth and the formation of specific synaptic contacts.
Slices of hippocampi and entorhinal cortices from neonate (P0-P3) rats and mice were cultivated for up to 20 days. Anterograde tracing, intracellular biocytin-filling, Golgi-impregnation and immunocytochemistry were carried out to identify neurons and axonal projections that have developed in vitro. Cultured hippocampal neurons differentiate their typical dendritic arbors. Dendrites of hippocampal and dentate principal neurons are covered with spines. Like in vivo, the dentato-hippocampal mossy fiber projection develops its layer-specific distribution pattern and the giant presynaptic boutons. Labeled entorhinal axons terminate in their appropriate target layers in the co-cultured hippocampus. Axons that had been transected due to the dissection procedure were able to reenter the hippocampus and distribute in their correct termination zone.
Our data demonstrate that neurons develop both, their structural characteristics and their specific interneuronal connections in slice co-cultures of entorhinal cortex and hippocampus. Thus, this in vitro model provides an useful tool to analyze the factors that influence axonal growth during development and regeneration.
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Still confusing: Role of metabotropic glutamate receptors in ischaemia

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To date it is commonly accepted that glutamate is crucially involved in many forms of brain damage. Unfortunately, however, drugs targeting glutamate receptor-gated ion-channels (NMDA, AMPA) have not led to a breakthrough in clinical therapy. Since metabotropic glutamate receptors (mGluRs) were assumed rather to have modulatory functions on basal synaptic transmission, they were considered to be suitable targets for novel pharmacological strategies leading to neuroprotective drugs with less side effects. mGluRs can be subdivided into three groups which are distinct by signal transduction pathways, amino-acid sequence and pharmacology: group I includes mGluR 1 and 5, group II includes mGluR 2 and 3, and group III includes mGluR 4, 6, 7 and 8. We used in vivo models of global and focal ischaemia to investigate the involvement of mGluRs in postischaemic neuronal degeneration.
In our hands, group I antagonists like MCPG cause a weak improvement of neuronal survival after global ischaemia in gerbils. (S)-4C3HPG (mGluR-I-antagonist/-II-agonist) strongly protected hippocampal neurones after transiently clamping both common carotid arteries, however, the effect was - at least partially - due to induction of hypothermia. Interestingly, (S)-4C3HPG reduced the infarct size after focal ischaemia in rats *without* a reduction of body-temperature. The influence of group-I-agonists are even more opposing. (1S,3R)-ACPD (mGluR-I and -II-agonist) leads to a significant enhancement of ischaemic damage, whereas DHPG (mGluR-I-agonist) leads to a slight neuroprotection (but at the same time induces seizures). Interestingly, the neuroprotective effect of DHPG can be enhanced by co-application of mGluR-I-antagonists. With DCG-IV (mGluR-II-agonist) we could not find neuroprotection, however, this might be due to the fact that we could only apply very low dosages as this compound shows NMDA agonistic effects in higher concentrations.

Regarding mGluR-II, we can also not rule out that the neuroprotective effect of (S)-4C3HPG is partially mediated via mGluR-II. Up to now we could not find pronounced neuroprotective effects with mGluR-III-agonists in our model of transient global ischaemia, however, data from models of excitotoxicity indicate that this compound might be beneficial in case of a challenge which is dominated by excitotoxicity.
In summary, we could reduce ischaemic damage by application of several mGluR-ligands. However, the interpretations of these neuroprotective effects is not yet clear. To investigate the mechanisms by which (S)-4C3HPG reduces infarct size in focal ischaemia and the mechanisms by which DHPG rescues neurones in global ischaemia awaits further investigations.

Hydroxyeicosatetraenoic acids and isoprostanes as markers of lipid peroxidation in brain mitochondria, homogenates and cultured glial cells

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Arachidonic and docosahexaenoic acid are the most important polyunsaturated fatty acids in brain lipids. In a number of neurodegenerative diseases associated with oxidative stress these fatty acids are strongly diminished. Specific oxidation products, as monohydroxylated derivatives and isoprostanes are formed, which are known as important in vivo biomarkers of lipid peroxidation with potent biological activities. It was the aim of our work to introduce two procedures for the analysis of monohydroxy fatty acids and isoprostanes. Monohydroxy fatty acids and isoprostanes were quantified with high sensitivity using GC-MS in the negative ion chemical ionization mode, allowing detection in the femtomol range. To establish and to apply these methods different models of oxidative stress were used. The monohydroxy fatty acids detected in isolated functionally intact rat brain mitochondria were 2-, 3-, 5-, 8+9-, 11+12- and 15-hydroxyeicosatetraenoic acid (HETE). After induction of an oxidative stress with iron/ascorbate especially 5-, 8-12- and 15-HETE were highly increased.
F2-isoprostanes were identified as stable products in mitochondrial samples in the femtomol range (8-epi-PGF2a and 9a,11ß-PGF2a) and exhibit remarkable increases following iron/ascorbate induced oxidative stress, too. Furthermore we introduced the analysis of F4-isoprostanes, derived from docosahexaenoic acid, an essential constituent of nervous tissue. We oxidized normal human brain cortex homogenates by 2,2′-azobis-2-amidinopropane and could demonstrate the presence and increase of two F4-isoprostane isomers, which correlated to F2-isoprostanes and HETEs, respectively.
HETE content in cultured glial cells was found to be very small. Paraquat induces an increased 5- and 15-HETE-formation in these cells. Additional stimulation of NO-synthase expression in glial cells with
LPS/g-interferon (with and without inhibition of NO-formation) strongly suppressed the HETE-formation.
A reason for the inhibition of lipid peroxidation may be the simultaneous expression of antioxidative enzymes as demonstrated for MnSOD.

Electrophysiological changes and cell death in juvenile organotypic hippocampal slice cultures after hypoxia followed by different reperfusion protocols

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Organotypic hippocampal slice cultures are becoming increasingly important in substitution of animal experiments for studies of hypoxia, especially for studying the prominent resistance of immature tissue to hypoxia.
We used entorhinal hippocampal slice cultures (div 7-9) of juvenile Wistar rats (postnatal day 7; prepared after Stoppini) to investigate electrophysiological changes during 30 min of hypoxia (95 % N_2/ 5 % CO_2) followed by two different reperfusion protocols (high oxygen group: 95 % O_2/ 5 % CO_2, low oxygen group: 19 % O_2/ 5 % CO_2/ 76 % N_2) in an interface chamber (n=22). During hypoxia field potential responses in area CA3 to hilar stimulation were dimished by about 75 %. The time up to disappearance varied to a much higher degree than in acute slice experiments. Moreover, only 32 % of slices showed the characteristic anoxic depolarisation (AD).
Cell death measured as Propidium Iodide (PI) fluorescence intensity after hypoxia and reperfusion showed a large and highly variable number of death cells with no differences according to the appearance of AD. However in the high oxygen reperfusion group cell death was significantly higher than in the low oxygen group. This result was confirmed by supplement experiments of different posthypoxic reoxygenation protocols in an oxygen adjustable incubator. Our results could be of significance for newborn resuscitation protocols after asphyxia.

Functional establishment of the perforant pathway in an organotypic co-culture monitored over two weeks with a microelectrode array

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Injury to the adult mammalian central nervous system (CNS) caused by trauma, ischemia or neurodegenerative diseases results in serious and permanent functional deficits. Evaluation of treatments that may lead to neural regeneration require a model system that correlates long-term recordings of physiological activity with morphological changes. By combining organotypic co-cultures with extracellular multielectrode recording technology the development of new connections and their regenerative ability can be repeatedly monitored on a functional level in the same culture for days to weeks. Slices of entorhinal cortex (EC) and dentate gyrus (DG) taken from 6 to 7-days old Wistar rats or BalbC mice were cultured on a microelectrode array (MEA) of 60 substrate-integrated electrodes. Electrodes had a spacing of 200µm and a diameter of 30µm. Beginning from div7, the overall spontaneous activity of the co-cultures as well as the response to extracellular electrical stimulation of layer II of the entorhinal cortex and of the stratum granulosum of the dentate gyrus were monitored every three days. After seven to ten days in vitro, 12 of 14 co-cultures that were spontaneously active in both parts showed correlation in this spontaneous activity, suggesting a functional connection between the two explants. Stimulation of the EC elicted responses in the EC and also in the DG, whereas stimulation of the DG produced responses only in the DG itself. Additionally, DiI staining of the DG retrogradely labled cells in layer II of the EC. We therefore conclude that the axons from the entorhinal cortex form correct and functional connections with the dentate gyrus resembling the perforant pathway known in vivo, regardless of the loss of the intermediate subicular tissue. The observed connections are unlikely to arise from unspecific axonal sprouting, because the DG axons also show extensive outgrowth from the explant but do not establish functional connections with the cortex. Our future goal is to lesion the newly established connection after development in vitro proceeded to an adult-like stage. This provides a test for the regenerative potential of the differentiated neurons.

Neuronal Toxicity of the Sodium Channel Blocker, Lidocaine

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Background: The use of lidocaine and other sodium channel blockers as neuroprotective agents during ischemia has been proposed on the basis of their ability to decrease neuronal metabolic requirements. However, recent studies have shown that higher concentrations of lidocaine can have a direct neurotoxic effect during spinal anesthesia, independent of sodium channel block, and possibly related to elevation of cytoplasmic calcium by an incompletely characterized mechanism. Therefore, we have tested the direct neurotoxicity of lidocaine using the ND7 cell line, derived from rat dorsal root ganglion. Methods: Cultured cells were exposed to lidocaine for 2, 4, or 24 hr in growth medium at pH 7.4, then loaded with indo-1 AM, stained with propidium iodide, and analyzed by flow cytometry. Dead cells were counted as those whose DNA was stained by propidium iodide (emission >630 nm). Cytoplasmic calcium was determined from the ratio of indo-1 emission at 390 and 500 nm, gating only on live cells. Lidocaine concentrations of 2.3-9.3 mM were tested, to cover the range that gives maximal sodium channel blockade. Results: Two hr exposure to lidocaine was essentially nontoxic. At 4 hr, 2.3 mM lidocaine was nontoxic, 4.6 mM showed mild toxicity (<25% increase in Ca2+ and cell death), and 9.3 mM showed significant toxicity (250% increase in Ca2+ among live cells, with >60% cell death). After 24 hr exposure, all lidocaine concentrations tested were toxic (50% to 300% increase in Ca2+, with 55%-95% cell death). Conclusion: Lidocaine, at high concentrations which completely block sodium channels, is neurotoxic after 4 hr exposure, but not at 2 hr exposure. Lidocaine neurotoxicity is associated with an increase in cytoplasmic calcium, although this data do not establish a cause-effect relationship.

Requirement of glycolytic and mitochondrial energy supply for loading of Ca^{2+} stores and $InsP_3$-mediated Ca^{2+} signaling in rat hippocampus astrocytes

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A major consequence of hypoxia and hypoglycemia is impaired ATP supply of brain tissue which induces the detrimental effects of stroke. However, it is not yet clear to which degree the signaling of glia cells is affected by reduced cellular ATP production. Here we studied in hippocampal astrocytes the influence of loss of cellular energy supply on Ca^{2+} load of intracelluar stores and on Ca^{2+} -mediated signal transduction. Inhibition of glycolysis resulted in an average drop of intracellular ATP levels by 35% if substrates for mitochondrial respiration were provided. Specific inhibition of mitochondria reduced intracellular ATP on average by 16%. With inhibition of

both glycolysis and mitochondrial ATP production, intracellular ATP level was drastically reduced (84%). Remarkably, the moderate reduction of ATP levels found with inhibitors of glycolysis caused a severe loss of Ca^{2+} from cyclopiazonic acid (CPA)-sensitive Ca^{2+} stores. In Ca^{2+}-free buffer a dramatic increase of cytosolic $[Ca^{2+}]_i$ was found with glycolytic inhibitors, even in the presence of mitochondrial substrates, whereas only a minor increase of $[Ca^{2+}]_i$ was observed with mitochondrial inhibitors. Inhibition of glycolysis reduced P2Y receptor- or thrombin receptor-evoked Ca^{2+} responses on average by 95% whereas an average reduction of only 26% was found with mitochondrial inhibitors. In conclusion, loss of energy supply by suppressed glycolysis can be compensated for maintaining the bulk ATP content in hippocampal astrocytes. Glycolysis, however, is the major source of ATP for the maintenance of Ca^{2+} load in stores which are required for transmitter-induced signaling. These results are consistent with the concept that a local ATP source in the vicinity of endoplasmic reticulum Ca^{2+} pumps is required.

Inhibition of NF-kB potentiates Ab mediated neuronal apoptosis

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NF-kB is an inducible transcription factor important in several neurological diseases (1). One mechanism leading to neurodegeneration during Alzheimer's disease (AD) is Amyloid b peptide (Ab) neurotoxicity. Previously we reported that NF-kB is activated by low doses of Ab only (2). Here we show, that NF-kB activation leads to neuroprotection. In primary neurons we found that a pretreatment with 0.1 µM Ab (1-40) protects against neuronal death induced with 10 µM Ab (1-40). As a known neuroprotective agent we analyzed the effect of TNF a. Maximal activation of NF-kB was found with 2 ng/ml TNF. Pretreatment with TNF protected cerebellar granule cells form cell death induced by 10 µM Ab (1-40). Trans-dominant negative IkB-a blocks NF-kB activation and potentiates Ab-mediated neuronal apoptosis. Similarly we find that nuclear NF-kB immunoreactivity around various plaque stages of AD patients is reduced in comparison to age-matched controls. These data suggest that pharmacological NF-kB activation may be a useful approach in the treatment of AD and related neurodegenerative disorders (3).

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Recovery of brain stem functions after severe brain injury in humans as indicated by coma duration

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Objectives: Recent studies on early magnetic resonance tomography (MRI) after severe brain injury indicate a 50-70% rate of traumatic brain stem lesions. The dominant predictor of brain stem affection is a prolonged coma due to disturbances of the formatio reticularis (FR). Nevertheless, structural damage of the FR has never been investigated in humans after BI aside from autopsies. Methods: This prospective study on 100 consecutive patients after brain injury compared duration of coma with data from early magnetic resonance imaging (MRI). MRI was performed within 7 days postinjury. All patients were comatose after admittance for 24 hours or longer despite maximal reduction of sedation. Due to international classifications patients were defined as "comatose" when not opening their eyes consistently and not obeying commands. Results: 1. Coma duration was significantly increased (mean: 5.4 days) in patients with a lesion on the mesencephalon, pons or upper medulla oblongata. It was significantly decreased (mean: 2.2 days) in patients with lesions above the mesencepahlon or of the caudal parts of medulla oblongata. 2. Patients with a bilateral lesion of mesencepahlon showed a maximal prolongation of coma (13.6 days). Patients with a bilateral lesion of the pons always died without emerging from coma prior to death. Patients with an unilateral brainstem lesion might die by extracerebral complications, but always emerged from coma prior to death. 4. Coma duration of 5-10 days in 85%, > 10 days in 100% was associated with a lesion of FR. Conclusions: The study proves structural damage of formatio reticularis as the basis of prolonged posttraumatic coma in humans. Analysis of coma duration in correlation to MRI findings leads to a precise prediction of the potency of recovery after traumatic brain stem damage.

Neuroprotection by mild postischemic hypothermia: Impact on functional recovery in neocortex slices of immature rat brain

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Introduction: Mild hypothermia of 3-4°C has been shown to be neuroprotective after a hypoxic/ischemic insult

in numerous animal studies and neuronal death was found to be diminished or even prevented in vitro after oxygen and glucose deprivation when temperature was lowered before or during the simulated ischemia. Various mechanisms of action seem to be responsible for the neuroprotective potential of hypothermia, among them abolishion of primary and secondary energy failure, postischemic hyperperfusion and brain edema and inhibition of excitotoxicity and free radical synthesis. Necrosis as well as apoptosis of neurons could be reduced by hypothermia. A hypothermic intervention after ischemia has been studied far less often. We investigated a mild hypothermia as therapeutic option after a simulated ischemia in the immature rat brain slice. Methods: Functional integrity was evaluated in neocortex slices of neonatal Wistar rats (age 6-8d) before, during and after simultaneous oxygen and glucose deprivation (duration 16min) by recording of orthodromically stimulated population spike in layer II-III of the somatosensory neocortex. Recordings were performed in an interface-chamber by calcium-sensitive microelectrodes. The recovery of the population spike amplitude after complete intraischemic suppression and anoxic depolarisation served as measure for neuroprotection. Temperature in the slice was 35°C in normothermia and was lowered to 31°C for 60min during hypothermic intervention. Results: An oxygen and glucose deprivation of 10min resulted in a complete suppression of the population spike amplitude from am mean of 3.4 ± 1.04mV and an anoxic depolarisation with an average amplitude of 5.43 ± 2.42mV after a latency of 10.72 ± 2.05min (mean ± SD). Extracellular calcium concentration fell to one fifth of the initial value. Recovery of 40, 60 and 80% of the initial population spike amplitude was on average 25-30% faster and thus significantly differed in the hypothermia-treated group in comparison to the control group. Conclusion: Functional recovery was faster in neocortex slices of neonatal rats that were postischemically treated by mild hypothermia of 4°C. This may imply a therapeutic option concerning perinatal asphyxia.

Blockade of glutamate-, NMDA-, or AMPA-induced Ca2+ signaling is not sufficient to cause neuroprotection in primary neuron cultures

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We studied five different types of ionotropic glutamate receptor antagonists. We determined their binding affinity for the glycine-, glutamate-, polyamine- and channel-pore site of the NMDA receptor and their binding affinity for the AMPA receptor in rat forebrain tissue. We measured the potencies of the compounds to inhibit agonist (glutamate, NMDA, AMPA)-induced Ca2+ signaling and their potencies to protect against neurotoxicity caused by these agonists in primary neuron cultures of embryonic rat cortex. For the five classes of compounds, different relationships between potencies in the various tests were found. Glycine site antagonists inhibited Ca2+ signaling with potencies that best reflected their affinity for the L689,560 site; their potency for neuroprotection against glutamate or NMDA was significantly lower. Glutamate site antagonists, channel-pore blockers, and polyamine-site antagonists in general bound to the NMDA receptor with a potency equal to that for inhibition of Ca2+ signaling and neuroprotection against NMDA. None of the pure NMDA receptor antagonists was able to block AMPA-induced Ca2+ signaling or toxicity in these cultures. The affinity of AMPA receptor antagonists for the AMPA receptor corresponded to their potency to inhibit AMPA-induced Ca2+ signaling but they were much weaker in protecting against AMPA-induced neurotoxicity. Several AMPA receptor antagonists blocked glutamate-induced Ca2+ signaling, but were inactive against glutamate toxicity in these cultures. We conclude that protection against the rise of intracellular Ca2+ does not necessarily corresponds to neuroprotection and that different types of glutamate antagonists show different profiles in the functional tests.

A 13C-NMR Study on the Neuroprotective Action of Dichloroacetate (DCA) in Murine Cerebral Malaria

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Cerebral malaria (CM) is the major life-threatening complication of Plasmodium falciparum infection. The pathogenesis of CM remains controversial. A prominent clinical feature of human CM infection is increased concentrations of lactate in both brain and cerebrospinal fluid, with sustained lactic acidosis being an important prognostic indicator. We considered that DCA, an activator of the pyruvate dehydrogenase complex, may be neuroprotective in CM. To test this hypothesis, Plasmodium berghei ANKA (PbA) infection was studied in CBA mice infused with DCA (60 mg/kg) on days 5 and 6 post-inoculation (p.i.). Following this, on day 7 p.i., animals were injected with [1-13C]-glucose and sacrificed after 15 and 30 min. Cerebral metabolites were analysed using both 1H and 13C-NMR spectroscopy.

Brain metabolite pool sizes in PbA/CBA mice showed definite metabolic perturbations, including significant increases in lactate and alanine, the hypoxic indicators.

In short, the use of DCA improved neurological outcome in CBA mice infected with PbA, providing a beneficial

decrease in acidosis and facilitating recovery of aerobic metabolism.

Ischemia/reperfusion-induced changes of intracellular calcium in organotypic hippocampal slice cultures

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An increase of the intracellular Ca2+ concentration has been proposed to play a major role in ischemia-induced neurodegeneration. We are going to perform a detailed investigation of intra- and intercellular Ca2+ signals during experimental ischemia and reperfusion. For this reason, transient exposure of organotypic hippocampal slice cultures to oxygen and glucose deprivation (OGD) is used to mimic brain ischemia in vitro. This study has been focused on CA1 pyramidal neurons, because of their special vulnerability by ischemia. Organotypic slices cultures resemble better the in vivo situation than isolated cells used frequently, due to their ability to conserve interneuronal circuits until several days in culture. Two different fluorescent Ca2+ dyes, fura-2 and mag-fura-2, with Kd-values in the nanomolar and micromolar range, respectively, have been used. We found that the vulnerability of hippocampal CA1 neurons is directly related to their intracellular Ca2+ response during OGD and reperfusion. In particular, the extent of the Ca2+ elevation corresponded to the duration of OGD. A short-lasting and therefore, subletal OGD (5 min) caused an early increase of Ca2+ within the nanomolar concentration range that was detected exclusively by fura-2. In contrast, only a longer-lasting, severe OGD (e.g. 20 min) led to a delayed Ca2+ elevation into the micromolar range that was detectable by mag-fura-2 at the end of OGD and during the first minutes of reperfusion. We conclude that distinct intracellular Ca2+ signals define neuronal survival or death following ischemia.

A comparison of the neurodegenerative changes in the striatum of Huntington diseased patients and in animal model

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The main histopathological characteristic of Huntington's disease (HD) is loss of neurones, occuring mainly in the neostriatum, pallidum and cerebral cortex, followed by rarefaction of the neuropil. This results in shrinkage of the entire striatum and compensatory enlargement of the lateral ventricles. The neurotoxic lesion of the rat brain, induced by stereotaxic infusion of kainic (KA) or ibotenic (IA) acids, is commonly used model of HD in animal studies. In this study, we demonstrate that the experimentally induced neurodegenerative changes in the striatum closely resemble the pathology seen in HD patients. Bilateral injection of the KA or IA cause partial necrosis of the striatum. However, the selective destruction of striatal neurones, accompanied by rarefaction of the neuropil, is not followed by the reduction of the blood vessels. The bundles of myelinated nerve fibres passing through the striatum are usually only slightly damaged. The number of subsequently proliferating glial cells, represented mostly by GFAP-positive astrocytes, is relatively small. The shrinkage of the caudate nuclei and compensatory dilatation of lateral ventricles are present in most of animals, but the level of dilatation differs depending on the severity of the striatal damage. The only morphological observation that doesn't correlate to the findings from the HD patients is from the needle-track area, as it is transformed into a conspicuous glial or fibro-glial scar. Concerning the use of IA and KA in the animal lesion model, there are no substantial differences between these two neurotoxic compounds, except that the fine structure of the neuropil is better preserved when using IA. This animal model possesses all the main characteristics seen in HD patients and is suitable for our experimental studies on the use of neural tissue grafts to ameliorate the HD pathology.
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Effect of Cyclosporin A against toxicity caused by hypoglycemia in striatal neurones.

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Cyclosporin A (CsA) is a potent immunosuppressant but has been shown to attenuate cell death in models of ischaemia and traumatic brain injury. Under circumstances of oxidative stress and high calcium content, the mitochondrial permeability transition pore (MTP) opens, disrupting the mitochondrial membrane potential. CsA is thought to inhibit the opening of the MTP, thereby maintaining the mitochondrial membrane potential. In this

study, we tested the effects of CsA on hypoglycemic toxicity in primary cultured striatal cells. Cultures were prepared from e-17 embryos and maintained for 16-18 days. Culture medium containing glucose was removed and replaced with glucose-free medium with the addition of CsA for 24 hours. Cells were fixed and then stained with anti-MAP-2 antibodies for the assessment of neuronal viability. Our results indicate that hypoglycemia causes 40% cell death. CsA, by itself, showed toxicity to striatal cells at higher doses. Furthermore CsA did not produce any neuroprotective effects at concentrations 1 to 5 uM after 24 hours. These findings suggest that CsA may not be neuroprotective against hypoglycemic damage in the striatum.

Poly(adp-ribose) polymerase inhibitors and neuroprotection against post-ischemic neuronal death

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Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that plays a key role in the restoration of integrity of damaged DNA. PARP catalyzes the covalent attachment of ADP ribose units from NAD to nuclear proteins, including PARP itself. An excessive activation of PARP, with the subsequent depletion of cellular NAD and ATP stores, and a neuroprotective activity of PARP inhibitors have been shown in cerebellar granule cells exposed to excitotoxins and in focal ischemia in vivo. In this study, we examined the neuroprotective effects of three PARP inhibitors [benzamide (BA), 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), and 6(5H)-phenanthridinone (PND)] in primary cultures of murine cortical cells and in rat organotypic hippocampal slices exposed to oxygen and glucose deprivation (OGD) in vitro. We also investigated the effects of systemic administration of these inhibitors against the selective loss of CA1 hippocampal pyramidal cells after 5 min of forebrain ischemia in gerbils. In previous experiments, we demonstrated that antagonists of NMDA, AMPA or group I metabotropic glutamate receptors display significant neuroprotective activity in all three models (Pellegrini-Giampietro et al., Eur. J. Neurosci. 11: 3637-3647, 1999). When added to the incubation medium during the OGD insult (60 min) and the subsequent 24 h recovery period, BA (1-5 mM), DPQ (1-100 μM), and PND (10-100 μM) significantly reduced the extent of neuronal death in cortical cells by 90 ± 4%, 85 ± 6%, and 59 ± 5%, respectively, but no neuroprotection was observed in organotypic hippocampal slice cultures exposed to 30 min OGD. Systemic administration of appropriate doses of PARP inhibitors (BA, 160 mg/kg 2 X after reperfusion; DPQ, 30 mg/kg 4 X after reperfusion) was not neuroprotective against the CA1 pyramidal cell loss induced by transient forebrain ischemia in gerbils. In view of the fact that OGD induces necrotic cell death in cortical cultures, while most of the dying neurons in the CA1 region of OGD-exposed hippocampal slices have apoptotic features (see Peruginelli et al., this meeting), our findings suggest that PARP inhibitors may attenuate ischemia-induced neuronal death of the necrotic but not of the apoptotic type.

Ischaemia-induced glutamate efflux in vitro: inhibition by nociceptin

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Nociceptin (NC) is the endogenous ligand for the G-protein coupled nociceptin receptor (NCR) whose activation inhibits K^+ evoked glutamate release from adult rat cerebrocortical slices (Okawa *et al.*, 1999). Here we have examined the effects of NC and the competitive NCR antagonist [$Nphe^1$]nociceptin(1-13)NH_2 ($Nphe^1$) on hypoxia/aglycaemia (H/A) induced glutamate efflux in the same model. Slices (350μm^2) were prepared and incubated in mesh baskets for 30 min in either control or H/A HBS as described previously (Nelson *et al.*, 1999). The H/A medium contained NC (1nM-30μM), peptidase inhibitors (capotril, amastatin, bestatin and phosphoramidon, 30μM each) and $Nphe^1$ (30μM) in various combinations. Glutamate was measured fluorimetrically using the reduction of $NADP^+$ by glutamate dehydrogenase (Okawa *et al.*, 1999). There was a marked increase in glutamate efflux under H/A conditions which was inhibited in a concentration dependent manner by NC with an EC_{50} of 0.26μM (C.I.=0.056-1.2μM) and an E_{max} of 58.1% (s.e.mean ± 4.7%) respectively (n=4). The inhibition by NC (1μM) was abolished by the antagonist $Nphe^1$ (n=4) which was per se inactive. These data suggest a neuroprotective action of NC and may implicate a role for NCR agonists in the treatment of stroke.

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Neuroprotective effect of s-benzoylthiamine-o-monophosphate observed in rats after chronic ethanol administration

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Chronic ethanol consumption leads to degenerative changes in the brain, especially in the cerebellum, including spine elongation of Purkinje cell dendrites. Spine elongation could be regarded as compensative growth of spines in search of new synaptic contacts due to ethanol cell loss. The contribution of thiamine deficiency, which is often associated to chronic ethanol consumption, to the pathology of ethanol related brain damage is discussed. The effect of ethanol on Purkinje cell dendritic spines and brain thiamine-status of rats fed with conventional (water-soluble) thiamine-hydrochloride (thiamine-HCL) or lipid-soluble S-benzoylthiamine-O-monophosphate (BTMP), was the aim of the present study. 24 male wistarrats were randomly divided into 3 groups (8 animals each) for 3 months: (1) ethanol-free control; (2) thiamine-ethanol (11.9 mg thiamine-HCL/kg diet and 20% ethanol-solution); (3) BTMP-ethanol (16.45 mg BTMP/kg diet, equimolar to thiamine-HCL in 2 and 20% ethanol-solution). Spines of Purkinje cell dendrites in rat cerebellum showed a significant elongation (34%) after ethanol exposure only in group 2 (thiamine-ethanol), whereas this neuromorphological alteration could not be detected in group 3 (BTMP-ethanol) and controls (1). Furthermore, a significantly decrease (6%) of thiamine-diphosphate (TDP / the biological active coenzyme of thiamine) in brain tissue could be observed in group 2. In contrast, TDP and thiamine in the BTMP-ethanol-group were significantly increased to the equimolar-dosed thiamine-ethanol-group (18% / 9%) and the ethanol-free control (12% / 7%). This demonstration of increased TDP- and thiamine-concentration, together with a normalised spine length of Purkinje cell dendrites in rat brains after chronic ethanol administration as a result of BTMP therapy provides the first direct evidence of the efficacy of this pro-drug in neuroregenerative respectively neuroprotective processes.

Role of adenosine A1 receptors during hypoxia on rat cortical pyramidal cells

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Hypoxia results in a rapid increase of the extracellular lebel of adenosine. It acts as an extracellular signal molecul by occupying specific receptors. In the present experiments, the involvement of adenosine A1-receptors on hypoxic changes of electrophysiological parameters were examined in rat cortical pyramidal cells by means of intracellular recordings. The only response observed was a hypoxic depolarization. The adenosine A1-receptor agonist N^6-cyclopentyl-adenosine (CPA) decreased the hypoxic depolarization. The effect of CPA was antagonized by the adenosine A1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Both, CPA and DPCPX had no apparent effects on resting membrane potential or input resistance. In a further series of experiments monophasic synaptic potentials (PSPs) were evoked by electrical stimulation. CPA in the range from 0.01 to 10 μM inhibited the PSPs. The maximum inhibition at a concentration of 10 μM was 55.5 %. The selective A1 receptor antagonist DPCPX (0.1 μM) abolished the inhibitory effect of CPA.
We conclude that the reduction of synaptic transmission by activating of presynaptic adenosine A1 receptors provides a primary mechanism resulting in hypoxic neuroprotection on cortical neurons.

The study of the products of lipid peroxidation in the membrane components of the blood of rats after acute gamma irradiation.

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Tba-test (thiobarbituric acid) was used to determine the content of malondialdehyde (mda) in the membrane components of blood of normal and irradiated rats by acute dose of 0.5gy. The consequences of radiation were observed on day 3, 10,20 and 30th.The obtained results showed that the content of mda reached a maximum level on the tenth day. The gradual decrease of mda was odserved during the next following days not reaching the level of the intact rats even at the 30th. day. These results were discussedin relation to the effect of acute low radiation induced peroxidations recovery in rats.

Influence of ionizing radiation on the homeostasis of intracellular cytoplasmic calcium in thymocytes of rats at different periods of observation.

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The methods of fluorescence probes were used to study the content of intracellular cytoplasmic calcium in the thymocytes of irradiated rats in doses of 0.25, 0.5 and 1.0 Gy. The comparative analysis of the effect of small doses of gamma-radiation exposure has shown, that the chronic exposure of low-power radiation doses expressed more violations of the content of intracellular cytoplasmic calcium stores in thymocytes, as in contrast to acute

exposured rats. The effect is more maximized on the 10 day after the irradiation. The same regularity was detected on the research of effect of radiation, on the state of lipid component of cytoplasmic membranes of thymocytes irradiated rats. Thus obtained data testify, that effect of an operation of small doses of gamma-radiation on the homeostasis of intracellular [Ca2+]i depends first of all, on power of sectional radiation, time after radiation effect and exposure dose.

The novel neuronal calcium channel blocker, LY393615, is neuroprotective following global and focal cerebral ischaemia.

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Several studies have reported that ω-conotoxin MVIIA (SNX-111) is highly effective in reducing the neocortical infarct volume in rat models of focal cerebral ischaemia. We have examined the effects of a novel, non-peptide calcium channel blocker, LY393615 ((N-Butyl-[5,5-bis-(4- fluorophenyl)terahydrofuran-2-yl]methylamine hydrochloride, NCC1048) in the gerbil model of global and in two rat models of focal cerebral ischaemia.

Global ischaemia was induced in gerbils by clamping both carotid arteries for 5 min. LY393615 was administered 15 mg/kg i.p. 30 min or 60 min after occlusion followed by two doses of 5 mg/kg i.p. Five days later the CA1 hippocampal cells were histologically analysed. The compound was then evaluated in the endothelin-1 model of focal ischaemia in rats [1] where LY393615 was administered 15 mg/kg i.p. 30 min or 60 min after infusion followed by two doses of 5 mg/kg i.p. Finally the compound was evaluated in the intraluminal model (2hr ischaemia) of focal ischaemia. In both focal models the brains were taken and histological analysis of infarct carried out using image analysis.

Results indicated that LY393615 provided significant protection in the CA1 hippocampal cells in global ischaemia and in the infarct volume following Et-1 middle cerebral artery occlusion. Some reduction in infarct volume was also observed in the intraluminal monofilament model.

We have reported that, LY393615, protects in models of global and focal cerebral ischaemia. The compound is neuroprotective when administered post-occlusion a may therefore be a useful anti-ischaemic agent.

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Apoptotic cell death in the chick embryo following spinal cord injury

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Chick embryos are capable of functional spinal cord regeneration prior to embryonic day 13 (E13). Developmental changes occurring thereafter result in a failure of regeneration following injury. The molecular events characterising this transition from regeneration competence to incompetence, however, have not been fully characterised. This study aimed to compare apoptotic cell death at regenerative competent and incompetent stages using TdT-mediated dUTP nick end labelling (TUNEL). Following injury at E11, where the spinal cord is largely mature, yet capable of functional regeneration, apoptosis was localised to the immediate injury site and was undetectable after 3 days. In embryos injured at E15 however, cell death was noted to extend cranially and caudally and persisted 4 days post injury. In addition extensive cavitation was observed in all spinal cords injured at E15, but was absent in those injured at E11. Morphological analysis of dying cells was indicative of apoptosis rather than necrosis. These results suggest a causal relationship between the extent of injury induced cell death and regenerative capability. Basic fibroblast growth factor (FGF2) has been shown to be neuroprotective following spinal cord injury. Interestingly, we found that the level of FGF2 protein detected by immunocytochemistry in the spinal cord gradually declined during development. Therefore, it is conceivable that changes in FGF2 expression through development may play some role in the transition from regeneration permissive to regeneration restrictive state and that altering FGF2 levels may be of future therapeutic use in reducing levels of apoptosis following human spinal cord injury. We are currently attempting to manipulate the expression of FGF2 in the chick spinal cord at E11 and E15 to test these hypotheses.

Involvement of inter-neuronal signaling in induction of collateral sprouting of sensory axons

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Collateral sprouting of cutaneous sensory axons depends on the nerve growth factor. Due to its increased availability in partially denervated skin, peripheral mechanism of sprouting induction has been proposed. However, cell bodies of the neurons with injured axons usually lie in the same dorsal root ganglion (DRG) as the cell bodies of non-injured sensory neurons whose axons sprout into the skin. We hypothesized that, signaling

between the injured and non-injured neurons within a DRG could be involved in induction of collateral sprouting of sensory neurons. To test this hypothesis, collateral sprouting into the end-to-side nerve anastomosis in rats was examined. In Group A, only degenerated neural pathways were available, but no neurons in the relevant DRGs were injured to activate signaling. A 20 mm long segment of the peroneal nerve was excised from the left hind limb and coapted to the intact sural nerve in the right hind limb in an end-to-side fashion. In Group B, in addition, dorsal cutaneous nerves (DCNs) from the L4-L6 spinal segments on the right side were cut and their regeneration prevented, so that injured neurons, pertaining to the DCNs, were present in the DRGs from which the sural nerve arise. Eight weeks later, the collateral sprouting of nociceptive axons into the anastomosed peroneal nerve segment was examined by the nerve pinch test and counting of myelinated axons. In group B, the number of animals (eight out of ten) with positive nerve pinch response was significantly greater than that in the group A (one out of ten) ($p < 0.001$). Also the number of myelinated axon sprouts was significantly higher than in the group A ($p < 0.002$). Therefore, proliferating SCs in degenerating neural pathways of the anastomosed nerve segment (secreting growth factors) are not sufficient to induce collateral sprouting of sensory axons. It seems that direct or indirect signaling between injured and non-injured neurons in the DRG is required in this respect.

Is in vitro post-ischemic neuronal death necrotic or apoptotic?

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Post-ischemic neuronal cell death has been considered for years a clear case for necrosis and the discovery of the implication of excitotoxicity as an important component of ischemic neuronal death further strengthened this hypothesis. More recently, reports have appeared suggesting that apoptosis can significantly contribute to post-ischemic neuronal damage both in vitro and in vivo. To date, the prevalence and the relevance of either type of cell death, apoptosis or necrosis, in these pathologic conditions is still unclear. In this study, we investigated the contribution of apoptosis to post-ischemic neuronal death in vitro using primary cultures of murine cortical cells and rat organotypic hippocampal slices exposed to oxygen and glucose deprivation (OGD). In order to distinguish between apoptotic and necrotic cell death we used the nuclear fluorescent dye Hoechst 33258, which revealed fragmentation of DNA in CA1 hippocampal pyramidal cells exposed to 30 min OGD but not in cortical cell cultures exposed to a similar (60 min) OGD insult. An enzymatic assay evaluating caspase 3 activity, an important component of the apoptotic process, demonstrated that this enzyme was strongly activated in hippocampal slices 3h after the OGD insult, while OGD-exposed cortical cells had only a modest and transient increase of enzyme activity at 6h after the insult. Morphological data obtained with electron microscopy showed that cortical cell cultures following OGD underwent the typical features of necrosis (breaks in plasma membranes, swelling of mitochondria and other organelles and modest, irregular clumping of chromatin) while cell death in organotypic hippocampal slices was mostly apoptotic (integrity of plasma membrane, condensation of cytoplasm, intense clumping of chromatin and formation of apoptotic bodies). These results demonstrate that OGD induces a mostly necrotic neuronal damage in cortical cell cultures and a significant apoptotic damage in the CA1 region of organotypic hippocampal cultures. Our results show that apoptotic neuronal death can be detected in selected in vitro models of cerebral ischemia and may be helpful to explain conflicting results obtained with a number of pharmacological agents, including poly(ADP-ribose) polymerase inhibitors (see Meli et al., this meeting) and caspase 3 inhibitors in different ischemic models.

Modified neocortical synaptic plasticity in a model of focal cortical dysplasia in the rat

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Freeze lesion induced cortical malformations mimick human cortical dysplasia and are used as a model for focal epilepsy. The epileptiform activity and hyperexitability in the vicinity of a freeze lesion is partly due to an imbalance between excitatory and inhibitory receptors, especially AMPA and $GABA_A$ (1). Neocortical long-term potentiation (LTP) depends on AMPA- and $GABA_A$-mediated synaptic transmission. This study was aimed to investigate wether the enhanced irritability in the freeze lesion model is associated with an alteration in neocortical LTP-induction.
Cortical slices (400 µm) from male Wistar rats (250-330 g), which received a cortical freeze lesion at P1 ($n = 8$), and controls ($n = 8$), were used. In freeze-lesion animals a stimulation electrode was placed in layer VI, 2-3 mm lateral from the lesion (Par I), in control animals at a corresponding location. Field potentials with approximately one half of the maximal response were evoked. ACSF-filled micropipettes were placed in layer II/III. After a stable

baseline for at least 15 min was achieved, LTP-induction was initiated by application of a theta-burst sequence.
Amplitude and slope of the field potential measured in layer II/III were used to investigate differences between slices from control and freeze-lesion animals. In slices from control animals slope and amplitude remained almost stable after LTP-induction on a slightly elevated level between 110 and 120 % of baseline. In freeze lesion animals field potentials started to increase a few minutes after LTP-induction. After 30 min the amplitude reached a level 165 ± 5 % of baseline and slope was increased up to 217 ± 19 %. Both parameters peaked approximately 50 min after theta burst (amplitude: 185 ± 10 %; slope: 273 ± 23 %).
While LTP-induction in layer III neurons of healthy animals is restricted to stimulation of layer IV (2), we could evoke pronounced LTP in deeper layers. We conclude that enhanced LTP in the freeze lesion model is attributed to a long lasting modification of receptors and synaptic transmission leading to a modified neocortical plasticity.

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Increased oxygen tension leads to cell damage in organotypic hippocampal slice cultures

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Considering the appearance of high oxygen tensions due to increased cerebral blood flow during a number of pathological conditions like seizures, migraine attacks, postanoxic periods as well as during spreading depressions we investigated the influence of different oxygen tensions on cell integrity of rat hippocampal organotypic slice cultures using extracellular recordings and Propidium Iodide (PI) staining. Slices were prepared following the Stoppini technique (postnatal days 6-8) and incubated in culturing medium for 9-10 days. Experiments were performed during perfusion with artificial cerebrospinal fluid for a 150 min period. Electrophysiological responses in area CA1 to hilar stimulation were recorded every 15 min after an initial equilibration period of 60 min. Slices maintained in 95% oxygen showed a 53% (SEM=17%; n=10) run-down in amplitudes of the evoked responses over the observation time course. In contrast slices maintained in 19% oxygen showed no run-down in amplitudes (SEM=9%; n=18). PI staining of the slices carried out immediately after the electrophysiological measurements indicated a dramatic cell death in the high oxygen group compared to those maintained in 19% oxygen. Interestingly, paired pulse behavior of evoked responses suggested a loss of GABAergic function in the 95% oxygen-group. Our findings indicate that in contrast to acute slices of juvenile and adult hippocampus, in slice cultures of juvenile hippocampus high oxygen deteriorates tissue function and increases cell death.

The Role of Growth-promoting and Growth-inhibiting Factors in Neurorepair after Stroke.Effect of Age.

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Although stroke in humans usually afflicts the elderly, most experimental studies on the nature of cerebral ischemia have used young animals. This is a particularly important issue when studying restorative processes that are age-dependent. To explore the potential of older animals to initiate regenerative processes following cerebral ischemia, we studied the expression of the growth-promoting cytoskeletal protein, microtubule associated protein 1B (MAP1B), dendrites-specific protein, microtubule-associated protein 2 (MAP2), and growth-inhibiting protein, ß-amyloid (Aß) fragment, in male Sprague-Dawley rats at 3 months and 20 months of age. Focal cerebral ischemia, produced by reversible occlusion of the right middle cerebral artery, resulted in a large decrease in the expression of both MAP1B and MAP2 in the infarct core at the mRNA and protein levels. However, at one week following the stroke there was vigorous expression of MAP1B and its mRNA, as well as MAP2 protein, in the border zone adjacent to the infarct of 3 month- and 20 month-old male Sprague Dawley rats. The upregulation of these key cytological elements was generally diminished in aged rats compared to young. Previous studies have shown that the ß-amyloid precursor protein (ßAPP) is upregulated following cerebral ischemia, and that the ß-amyloid (Aß) fragment may be toxic to brain cells. Aß immunoreactivity was evident in GFAP-positive astrocytic somata and processes, and also in clusters of small, spherical structures in the penumbra. These Ab immunoreactive minispheres were more numerous in aged rats than in young rats. These results suggest that (i) the regenerative potential of the aged rat brain appears to be competent, although somewhat attenuated, at least with respect to MAP1B and MAP2 expression up to 20 months of age, (ii) cerebral ischemia promotes conditions that are favorable to the focal

accumulation of the growth-inhibiting Aß, especially in the aged brain.

Ac-YVAD.cmk, the caspase-1 inhibitor, blocks ischemia-induced neurodegeneration by inhibition of caspase-induced cell death and pro-inflammatory cytokines release

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The interplay between apoptosis and inflammation is a key event in the development of ischemic brain damage. Some early step in the progression of the two processes is common. For example IL-1beta, a neurotoxic mediator, originates from pro-IL1beta through caspase-1 activity. On the other hand caspase-1 is also an up-stream activator in the caspase cascade leading to cell death. These data suggest that caspase-1 inhibition is a potentially useful approach to reduce neural damage after an ischemic insult. We tested this hypothesis by treating ischemic rats with Ac-YVAD.cmk, an irreversible, caspase-1 inhibitor. Treatment significantly reduced infarct volumes of about the 30% ($p<0.05$) both at 24 h and at 6 days after middle cerebral artery occlusion. Consistently, at 24 hours, caspase-1 activity was almost completly inhibited (caspase-1 activity expressed as difference between ipsilateral and contralateral hemispherein in pmol/min.mg of protein was: vehicle; 199±40; Ac-YVAD.cmk; 7±20; $p< 0.01$) and IL-1beta cortical levels were significantly reduced (IL-1beta difference between ipsilateral and contralateral hemisphere, measured in pg/ml.mg of protein was: vehicle; 82±20; Ac-YVAD.cmk; 11±15; $p< 0.05$). In order to evaluate the effects of caspase-1 inhibition on apoptosis, we measured the levels of histone-associated DNA fragments in cortex homogenates with a quantitative biochemical approach. Drug treatment significantly decreased free nucleosome formation at 24 hours (Delta Absorbance at 405 nm/mg of protein; vehicle: 1.7±0.2; Ac-YVAD.cmk: 0.8 ± 0.1; $p< 0.01$, n=17-19]. We also investigated the effects of Ac-YVAD.cmk on cortical levels of TNF-a, another mediator involved in ischemic damage progression. TNF-a level was elevated 24 hours after ischemia and treatment with Ac-YVAD.cmk significantly reduced it, but the effect disappeared at 6 days. The level of other inflammatory mediator, such as IL-10, MCP-1, MIP-2 and NO, were not affected by treatment. The present study, showed that caspase-1 inhibition leads to a long-lasting neuroprotective effect by the inhibition of both caspase-mediated cell death and release of proinflammatory mediators.

Cross-Talk between calpain and caspase-3 in response to cerebral ischemia

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A central challenge in apoptosis research is understanding the mechanisms by which apoptotic cascades are initiated and affected. While the involvement of the caspases in apoptosis has been demonstrated in a variety of systems, there are no facts concerning the implication of the cystein protease µ-calpain (µ-CP). We tested a potential role for µ-CP and eventual interactions with caspases in ischemia.
Rats were subjected to transient global ischemia. The proteolytic activity of µ-CP and caspase were measured in the hippocampal CA1-subfield at different times post-ischemia by the immunolocalisation and immunoblotting of µ-CP, calpastatin, spectrin-breakdown products and caspase-3. The time course of apoptotic DNA fragmentation was assessed by TUNEL-staining. For pharmacotherapy calpain inhibitor III and z-VAD-fmk were used. We found that µ-CP is (1) activated and translocated to the nucleus before DNA-laddering. (2) that calpastatin, the endogenous inhibitor to calpain, is downregulated (3) pre-treatment with CPI-III and z.VAD attenuate synergistically the cleavage of calpastatin and cell death.
In conclusion, these results contribute additional evidence that proteases may play a functional role in apoptosis. Moreover, the neuroprotective synergistic effect of caspase- and calpain-inhibitors suggests, that there is a cross-talk between caspase and calpain during apoptosis.

The generation of free hydroxyl radicals is involved in hypoxia preconditioning

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Hypoxia preconditioning states that a sublethal hypoxia periode will afford neuroprotection against a second harmful event. In our experiments, we carried out a procedure for the development of hypoxia preconditioning in adult male Wistar rats using a hypoxic exposure (9% O_2; 91% N_2) for one hour. The neuroprotection was analysed by performing an acute pentylenetetrazol (PTZ) seizure model. For this, rats were tested with PTZ (48 mg/kg i.p.)

on 1-14 days after hypoxia exposure. The hypoxia exposure prevented significantly the development of acute PTZ convulsion. The present study was designed to determine the effect of N-t-butyl-a-phenylnitrone (PBN), an electron-trapping agent and free radical scavenger, on hypoxia preconditioning against PTZ seizures 7 days after hypoxia exposure. PBN abolished the neuroprotective action of hypoxia exposure. In a second experiment, the generation of free hydroxyl radicals in brains of animals exposed to hypoxia was determined. For this purpose, the rats were i.p. pretreated with 30 mg/kg PBN and NaCl, respectively, 20 min before the start of hypoxia exposure. 45 min later the rats were i.p. injected with 300 mg/kg sodium salicylate and once again exposed to hypoxia for 15 min. Immediately after that the animals were decapitated and the free hydroxyl radicals and the salicylate content were estimated in the whole brain without cerebellum. Hypoxia preconditioned animals which were pretreated with NaCl showed a significantly higher extent of free hydroxyl radicals in the brain compared with PBN injected preconditioned animals and with naive and sham exposed controls. The results pointed out that the generation of free reative oxygen species under hypoxic conditions in the brain is involved in the development of the hypoxic preconditioning phenomenon.

Dopaminergic neurotoxicity produced by repeated administration of psychostimulant drugs: a possible role of extracellular dopamine and hydroxyl radical

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Microdialysis technique and HPLC/ED were used to test neurotoxic effects of Sydnocarb (SYDN), an original psychostimulant, and D-amphetamine (D-AMPH) on dopamine (DA), DOPAC, HVA and hydroxyl radical (detected by 2,3-dihydroxybenzoic acid, 2,3-DHBA) extracellular levels in rat dorsal striatum. The male Wistar rats were treated with four equal doses of drugs each given i.p. two hours apart. D-AMPH (5 mg/kg) caused the sharp increase in DA level up to 950%. Subsequent D-AMPH injections were followed by moderate increase in DA level (about 300%). DOPAC and HVA levels decreased within 1 hour after the first dose of D-AMPH. D-AMPH produced marked increase in 2,3-DHBA level most pronounced 80 min after the second injection (up to 700%). SYDN at the dose 23,8 mg/kg (equimolar to 5 mg/kg D-AMPH) gradually increased DA level up to 200%. DOPAC extracellular concentration was increased after the first two doses of SYDN. HVA level failed to be changed. 2,3-DHBA concentration appeared to be less increased (up to 200%). It is concluded that Sydnocarb might have lower neurotoxic potential in comparison to D-amphetamine.

Thrombin and its receptors in pathophysiology of global cerebral ischemia

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The serine protease thrombin serves as an extracellular signaling molecule in the nervous system. Its action is at least partly mediated by distinct protease-activated receptors (PAR-1 and PAR-3). In this study, we investigated the involvement of endogenous thrombin in neurodegenerative and/or neuroprotective processes *in vivo* following global ischemia. A second goal was to examine ischemia-induced changes in the gene expression of PAR-1, PAR-3, protease nexin-1 (PN-1, a potent thrombin inhibitor) and prothrombin. Ischemia was induced by transient occlusion of both common carotid arteries of Mongolian gerbils for 6 min. This treatment consistently caused a delayed neuronal death in the hippocampal CA1 region. The number of surviving hippocampal CA1 neurons was quantified by toluidine blue/fuchsin acid staining of brain slices 7 days after the ischemic insult. If hirudin, a specific thrombin inhibitor, was injected intracerebroventricularly (i.c.v.) 20 min before clamping both vessels, the neuronal cell death was significantly reduced. Furthermore, two relatively short-lasting (2 min) occlusions at 1 day intervals 2 days before a longer-lasting (6 min) occlusion exhibited a striking ischemic tolerance of hippocampal CA1 neurons. However, the pre-conditioning effect was attenuated if hirudin was applied 20 min before each mild insult but not before the severe occlusion. This finding strongly suggests that endogenous thrombin, besides its participation in ischemia-induced degeneration, is also involved in the neuroprotective mechanisms leading to ischemic tolerance. In order to perform a relative RT-PCR analysis, transient global ischemia was induced by a two-vessel occlusion combined with systemic hypotension lasting for 15 min in rats. We found a marked increase of prothrombin and PAR-1 mRNA and a smaller but significant increase of PN-1 mRNA level 1 day after the ischemic insult, whereas PAR-3 was not affected. Taken together, our results suggest that thrombin and its receptors are components of neuroprotective as well as degenerative mechanisms in the context of cerebral ischemia.

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Neuroprotective effect of 17B-estradiol on the glutamate-induced damage in the rat cerebral cortex.

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Estrogen plays an important role in neuronal function and in protecting neurons in the cerebral cortex against pathological conditions. Estrogen has been shown in vitro to protect primary neurons from toxic glutamate exposure. An in vivo model of glutamate neurotoxicity in which glutamate is applied to the cortex of rats through a microdialysis probe has been used to investigate the neurochemical processes initiated by glutamate. Using this model, we investigated the neuroprotective effects of 17b-estradiol against glutamate–induced damage. Rats were pretreated with 17b-estradiol before application of glutamate through a microdialysis probe. Dialysate was collected every 30 min for 4h and extracellular glucose and lactate concentrations determined using an enzymatic assay. A lactate peak was seen immediately after glutamate application, which was significantly higher and more prolonged after the 17b-estradiol pretreatment than with glutamate alone. Glucose markedly decreased concomittantly to the lactate increase. At the end of the experiment the brains were removed and sectioned and the volume of the lesion determined by immunohistochemistry. We observed a significative reduction of the size of the glutamate-induced lesion after 17b-estradiol pretreatment. These results suggest a new important role of 17b-estradiol in neuronal and glial survival which might be mediated by high lactate production.

Identification of a neuritogenic ligand of the first Ig module of NCAM by means of a combinatorial library of synthetic peptides

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The neural cell adhesion molecule, NCAM, plays a key role in neural development, regeneration and learning. In order to identify functional ligands of NCAM, a combinatorial library of synthetic peptides was screened with a recombinant form of the first immunoglobulin homology module (Ig1) of NCAM. A peptide, termed C3, was identified and shown to bind the NCAM-Ig1 module by plasmon surface resonance analysis. By nuclear magnetic resonance (NMR) spectroscopy, the C3 binding site in the NCAM Ig1 module was mapped and shown to be different from the binding site of the NCAM Ig2 module, the putative endogenous ligand of NCAM-Ig1. The C3-peptide was shown to modulate NCAM-mediated cell adhesion and signal transduction with a high potency. In cultures of dissociated neurons, the C3-peptide stimulated neurite outgrowth, with a maximal effect at a concentration of 5*10-7 M by activating a signalling pathway presumably identical to that activated by homophilic NCAM-binding. The NCAM-Ig2 module was shown to promote neurite outgrowth in a similar manner. Thus, we have identified a synthetic ligand of NCAM that upon binding to a novel binding site in the NCAM Ig1 module mimics homophilic NCAM-binding inducing neurite outgrowth. The C3-peptide may be of interest in relation to functional regeneration of neuronal connections in neurodegenerative disorders.

Effect of increased extracellular lactate on glutamate-induced lesion : an in vivo animal model.

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INTRODUCTION : Glutamate has been implicated in brain tissue injury after ischemia, hypoglycaemia and experimental traumatic brain injury. Using a microdialysis technique, the clinical observations have shown the correlation between the increased glutamate concentrations and the severity of the clinical conditions. Firthermore the increase of glutamate is accompagnied by a increase of lactate and decrease of glucose. To investigate the role of glutamate in neuronal damage we used our in vivo animal model in which we perfused glutamate through a microdialysis probe implanted into the left cortex of the rat . To produce a lesion we needed to use a glutamate concentration 10-fod greater than those found in pathological cases. This suggests that other factors max influence the neurotoxicity of glutamate. We have chosen to investigate the effect of one of this factors : Lactate , since a fall in pH has been shown to be injurious to neurones. However, there is now much evidence that lactate

has an important role as a fuel under normoxic conditions and is released in response to glutamate uptake.
MATERIALS AND METHODS: Animals were treated using a microdialysis probe implanted into the left frontoparietal cortex (n=24) as follows: 1)- Control-Mock-CSF.(n=8).during whole experiment 2)- Lactate (6 mM) for 30 minutes. (n=3) followed by mock-csf 3)- Glutamate (100 mM) for 30 minutes.(n=5) followed by mock-csf 4)- Glutamate and lactate (100 mM , 6 mM, respectively) for 30 minutes (n=8) followed by mock-csf
At the end of the experiment, the brains were removed, frozen in isopentane at –40°C and keeped at –80 °C until use. Cryosections through the lesion were stained with a marker for damage (Map2 antibody).
RESULTS Glutamate produces a lesion significantly bigger than Mock-CSF and lactate (6.02 ± 1.42, 1.9±1.03, 0.77±0.20mm3 respectively). The lesion produced by the co-infusion of glutamate and lactate (4.17 ± 1.20 mm3) is significantly bigger than Mock-CSF and smaller than glutamate alone.
CONCLUSION We have shown the neuroprotective effect of lactate on glutamate-induced lesions.

Adaptive versus lethal calcium activity rise in retinal ganglion cells after optic nerve crush

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Predicting which neurons will survive after injury, an essential step in any attempts to repair the central nervous system, requieres that individual neurons can be followed over time in vivo to determine their ultimate fate. This is possible with the in vivo confocal neuroimaging (ICON) method which allows repeated visualization of retinal ganglion cells (RGCs). To determine the relationship of calcium influx and cell survival in the living rat, we follow calcium activity over time in RGCs after optic nerve crush (ONC). RGCs are retrogradely labeled with a calcium marker (oregon green BAPTA) and/or a cell marker (TransFluoSpheres 488/645). A mild or moderate ONC on the left or both sides is carried out. Thereafter RGCs are imaged with ICON at different time points until post surgery day 15 (P15) to visualize calcium activity. Depending on their calcium-related fluorescence intensity change (FI) after ONC, 3 different RGC types are found: (1) RGCs with no change in the FI or soma size are observed after both ONCs, comprising 17% of the total RGC population. (2) After mild ONC, an additional 23% of the RGCs survive also, but they show a FI decrease at P4 (26% below baseline) and a delayed, rather rapid FI increase until P15 (169% above baseline). These cells also show no significant soma size change. (3) Finally, in both ONCs, 60-65% of RGCs die within the first 6 days after having undergone a fast and massive FI (287% above baseline) and a cell size increase (138% above baseline). Thus, retrograde cell death is inevitable when calcium activity increases immediately after ONC but, when it appears with a time-lag at P6, an intracellular calcium rise may participate in an adaptive response of surviving RGCs.

TNF-a induced changes in sphingomyelin cycle and cholesterol level in rat brain.

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TNF-a has been shown to play an important role in the pathogenesis of number of CNS diseases such as MS, AIDS dementia and Alzheimer's disease. Receptors for TNF-a are situated on the surface of both nerve and glial cells. TNF-a activates receptors linked to multiple effector systems, including a shingomyelin products pathway. It has been shown that the depletion of cell membrane shingomyelin may leads to the mobilisation of cell membrane cholesterol and that neutral shingomyelinase regulates synthesis of cholesteryl esters. We have determined the changes of N-shingomyelinase activity (SPMase), shingomyelin (SPM) and ceramide contents, cholesterol level and LP products within 5,15,30 min, 1, 2 and 5hs after intraperitoneal TNF-a injection in the cerebral cortex, hippocampus and cerebellum of rats. SPMase level was found to be significantly higher in hippocampus as compared to cerebellum and cerebral corterx in normal rats. Maximal changes in SPMase activity, SPM, ceramide and cholesterol contents after TNF-a administration were found in the hippocampus, and were less expressed in the cerebral cortex and cerebellum. Cholesterol was increased in different brain regions, its content correlates with sphingomyelin level during the 1st h after TNF-a injection. Production of the LP products were registered in cerebellum and hippocampus during 1hr after TNF-a injection. Our findings suggest that TNF-a may participate in the regulation of cholesterol and sphingomyelin metabolism in brain.

Desferrioxamine mediates tolerance against oxygen/glucose deprivation via induction of lactate dehydrogenase in neurons.

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In the CNS, tolerance against ischemia can be elicited by a number of stimuli, including ischemia/hypoxia itself, respiratory chain inhibition, etc. In this study we elucidated

a possible role in induction of tolerance by the iron chelator desferrioxamine (DFO) in a rat neuronal-cell enriched culture system. DFO was given 48 hours in different concentrations (15, 50, 100, 150, 300, and 600 μM) prior to oxygen-glucose deprivation (OGD). Only 150 μM DFO caused a statistically significant reduction of neuronal damage (morphologically) and reduced lactate dehydrogenase (LDH) release compared to sham treated sister cultures. When comparing 48, 24, 12, 6, and 1 h of pretreatment intervals (150μM DFO) only the 48 h interval was effective. DFO was reported as a strong inductor of LDH gene expression via mimicry of intracellular hypoxia and activation of the transcription factor HIF-1. We found in parallel to tolerance induction an increased intracellular neuronal LDH activity, which might contribute to the higher viability against OGD. This prompt us to test the effect of protein synthesis inhibition by cycloheximide (CHX). CHX given immediately before treatment with DFO suppressed the induction of intracellular LDH and also blocked the protection mediated by DFO. We conclude that upregulation of LDH is involved in DFO induced tolerance against OGD in neuronal enriched cultures.
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The group III mGluR agonist (R,S)-PPG protects CA1 neurons in hippocampal slices against hypoxic/hypoglycemic injury

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Background and Purpose The role of group III metabotropic glutamate receptors (mGluR) in ischemic neurodegeneration is still unsettled. In order to examine a possible modulatory effect of presynaptic mGluR on glutamate excitotoxicity the novel selective group III agonist (R,S)-4-phosphonophenylglycine [(R,S)-PPG] was tested in an in vitro ischemia model based on rat hippocampal slices.

Methods Hypoxia/hypoglycemia was induced by changing the carbogen atmosphere in an interface chamber to a gas mixture containing 95% N_2/5% CO_2 in the presence of a Ringer solution in which glucose was replaced by mannitol. (R,S)-PPG was bath applied 1.) during the hypoxia/hypoglycemia and in the recovery period or 2.) from the time of re-establishment of oxygen and glucose supply until the end of the experiment. The recovery of population spike amplitudes in the CA1-region was used as parameter for neuronal viability.

Results 1.) (R,S)-PPG significantly improved the recovery of synaptic transmission in the CA1-region when applied during the hypoxia/hypoglycemia and in the recovery period. 2.) Interestingly (R,S)-PPG also significantly improved the recovery of synaptic transmission when applied solely during the recovery period, i.e. in a different and clinically more relevant time window after the insult.

Conclusions The results imply that presynaptic glutamate release after an insult contributes to neurodegeneration. Since agonists of group III mGluR reduce neurotransmitter release - probably via presynaptic autoreceptors - we interpret the results obtained in our in vitro model of hypoxia/hypoglycemia as support of the hypothesis that group III mGluR agonists might be beneficial drugs against diseases where excitotoxicity is one of the dominant pathological mechanism.

MAPKs inhibition increases kainate-induced toxicity in neuronal retina cells

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In this work we studied the toxicity induced by glutamate receptor agonists in neuronal retina cells. The toxicity was assayed by the MTT test, and the type of cell death (apoptosis versus necrosis) was determined by analysis of the nuclear morphology, using fluorescence microscopy and the DNA stains propidium iodide and SYTO-13. The cultured chick neuronal retina cells died by apoptosis upon glutamate receptor stimulation and were particularly sensitive to the non-NMDA glutamate receptors agonists. In our studies, one hour stimulation with 100 □M kainate or 100 □M glutamate induced a reduction of cell viability, assayed 24 hours after stimulation, by about 45% and 26% respectively. The toxicity induced by kainate was completely inhibited by LY303070, a specific antagonist of the AMPA receptors, whereas the toxicity induced by glutamate was completely inhibited by MK801. We studied post-receptor signalling events triggered by glutamate receptor stimulation and found that the DNA binding activity of the transcription factor AP-1 was increased in cells challenged with glutamate. In cells treated with kainate we observed a transient, smaller, increase in the DNA binding activity of AP-1. However, we found that MEK inhibitors, 250 nM U0126 or 20 □M PD098059, increased the toxicity induced by kainate but not by glutamate. These results suggest that in primary cultures of retina neurons glutamate and kainate cause toxicity through activation of different intracellular mechanisms, and that the MAPK pathway could be a survival pathway in cultured retina cells stimulated with kainate.
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Growth hormone and hypoxic ischemic brain injury

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Although the Growth Hormone Receptor / Binding Protein (GHR/BP) is found throughout the brain its specific functions are largely unknown. There is evidence however that GH deficiency impairs both cerebral growth and myelination. Previously, we have shown the neural GHR/BP is specifically upregulated following a unilateral hypoxic ischemic (HI) brain injury in the juvenile rat. Now we show that that GH-like immunoreactivity is also increased after HI brain injury and specifically observed on stressed and dying neurones, glia and axons in regions of cell loss. We hypothesized that this may be an endogenous neuroprotective response by the injured brain, similar to the induction of other growth factors after brain injury. We subsequently treated HI brain injured juvenile rats (n=12/group) with 20μg rat GH (rGH) versus vehicle only by intracerebroventricular infusion starting two hours after a moderate (15 minute) HI injury and quantified the neuronal survival after three days.
Results: rGH treatment conveyed significant neuroprotection exclusively in regions previously shown to contain GHR/BP by others, namely in the hippocampus ($P=0.05$), the cortex ($P<0.001$) and thalamus ($P=0.006$) while no neuroprotective effect was seen in the striatum where little or no GHR/BP is found.
Conclusion: These results suggest that a) following HI brain injury, GH-like immunoreactivity is strongly increased within and closely surrounding, regions of cell loss, b) that central treatment with rGH can provide neuroprotection to brain regions expressing the GHR/BP and c) that the spatial distribution of the neuroprotection offered by rGH is distinct from that offered by IGF-1 in this model, suggesting an at least partially IGF-1 independent effect.

Extracellular Changes of Taurine and Glutamate during various cerebral insults: A comparison

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In brain physiology, glutamate plays a dual role: It is involved in many metabolic pathways and at the same time it is the most wide spread excitatory and sometimes excito-toxic neurotransmitter in mammalian brain. On the contrary, taurine represents a metabolic endpoint and may act as an inhibitory transmitter and/or as an osmoregulator. In several studies, we observed that taurine in the extracellular space changes in a close relation to glutamate. To get a closer insight into this relationship, this paper aimes at summarising and comparing some of these observations in search for a common denominator. By means of microdialysis, glutamate and taurine were monitored in the rat cerebral cortex during high K+ or NMDA -application, during irreversible ischemia or after induction of a thrombotic infarct. A microelectrode inserted nearby served to follow electrical activity. K+-application triggered a cortical spreading depression (CSD) reproducibly. Both, taurine and glutamate, increased more or less concomitantly. The time of recovery to baseline, however, was different with glutamate returning to basal level quickly whereas taurine was about 4-fold slower. After NMDA application, CSD also was reproducibly induced, but only taurine increased. Ischemia elicited a faster increase of taurine than that of glutamate which even started several minutes after onset of ischemia. During the initial ischemic period, glutamate exhibited a very small, but transient increase concomitantly with the anoxic, negative shift of the DC signal in about 50 % of the experiments. Within the peri-infarct zone, taurine also started to increase first and rapidly whereas glutamate again exhibited a slow and delayed increase. These data illustrate a close but timely distinct relationship between the extracellular changes of glutamate and taurine which will provide important clinical information when monitored together: A rise of glutamate after a period of elevated taurine seems to indicate the point of no return of cell damage after excitotoxic or ischemic periods.

Permeability transition pore indenpendent release of the proapoptotic factor cytochrome c from rat brain mitochondria

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The release of cytochrome c from mitochondria into the cytosol is accepted to be a key step linking the triggering phase of apoptosis to the executioning phase by activation of caspase 9. Two mechanisms for cytochrome c release have been proposed. One involves the opening of the unspecific permeability transition pore (PTP) followed by osmotic disequilibrium and the expansion of the matrix space leading to the disrupture of the outer membrane. This mechanism is commonly favored. The other recently became a matter of interest and is based on the opening of channels in the mitochondrial outer membrane without

subsequent matrix swelling. Here we demonstrate that Ca2+, even at low micromolar concentrations, is able to induce the release of cytochrome c from isolated rat brain mitochondria. This process was cyclosporin A insensitive and is therefore not mediated by opening of the PTP. Rather, mitochondria did not swell and remained morphologically intact. The intactness of the outer membrane could be illustrated by electron microscopy analysis as well as by ATP induced polarisation of the mitochondrial membrane in presence of respiratory chain inhibitors. Furthermore, the Ca2+-induced cytochrome c release from isolated rat brain mitochondria was not mediated by NO since we were not able to show any sensitivity to inhibitors of the nitric oxide synthase. In contrast, 25% (w/v) dextran which is known to inhibit the transport of low-Mr solutes through the mitochondrial outer membrane completely prevented the cytochrome c release. This possibly points to the involvement of the voltage dependent anion channel (VDAC). Taken together, an increase of cytosolic Ca2+ into the lower micromolar range is suggested to be sufficient to induce the release of cytochrome c whereas the PTP is not required for the induction of apoptosis in neurons. Our findings might be important for the understanding of several neurodegenerative disorders, e. g. brain ischemia and neurotrauma.

The NMDA receptor antagonist flupirtine directly protects oxidative phosphorylation against oxidative stress

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Problem: Extensive membrane lipid peroxidation is associated with neurodegenerative processes (cerebral injury, Parkinson´s disease, Alzheimer´s disease). Recently, it has been reported that the analgetic drug flupirtine (Katadolon(R))- an NMDA receptor antagonist of the widely used analgetic triaminopyridine type - acts as an antioxidative compound at oxidative stress [1]. To examine the potential antioxidative property of flupirtine in more detail, we exposed isolated rat liver mitochondria to oxidative stress induced by Fe2+/ascorbate. We applied the technique of paramagnetic resonance spectroscopy (EPR) to study changes in the protein integrity during peroxidation. Peroxidative damage of membrane lipids was followed by determination of thiobarbituric acid reactive substances (TBARS). Changes in mitochondrial functions were examined by measuring their respiration properties. Results: (1) Flupirtine effectively protects lipids against peroxidation at clinically relevant concentrations. (2) Proteins are also protected, but to a lesser extent than lipids. (3) With flupirtine the peroxidation- induced decline in the respiratory control ratio of RLM is delayed. (4) The protective activity of flupirtine is in line with its OH-radical trapping property. (5) Contrary to flupirtine, the highly water-soluble antioxidant DMPO (5,5-dimethyl-1-pyrrolidone-1-oxide) is unable to protect lipid peroxidation, indicating that flupirtine acts near the membrane environment. (6) Flupirtine increased the W/S ratio - a relevant EPR parameter indicating the physical state of membrane proteins. Conclusion: Flupirtine incorporates in the phospholipid-protein bilayer of the inner membrane of mitochondria, thereby protecting membrane lipids from peroxidative damage.

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Arachidonic acid modulates Ca^{2+} signalling evoked by thrombin and ATP: Implications for a neuroprotective role

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Concentrations of polyunsaturated fatty acids are increaased rapidly during inflammatory processes in brain tissue. Arachidonic acid (AA) is the most important because it is metabolized to prostaglandins, leucotrienes, and other bioactive peptides. Several reports indicate a role of AA and its metabolites in the regulation of cellular Ca^{2+} homeostasis. Therefore, we investigated the influence of AA on agonist-evoked Ca^{2+} signalling in astrocytes. After 30 min preincubation with AA the amplitude of the Ca^{2+} response elicited by thrombin or ATP was significantly reduced compared to untreated astrocytes. In the same way, simultaneous addition of AA together with thrombin or ATP attenuated the amplitude of the elicited Ca^{2+} transient. Furthermore, the ability of ATP to induce $[Ca^{2+}]_i$ oscillations was totally suppressed by AA. The effect of AA on the agonist-induced Ca^{2+} responses was concentration-dependent and could be mimicked by the non-hydrolyzable AA analogue ETYA, indicating that the effect is mediated by AA itself. To assess what step in the Ca^{2+} signalling cascade is influenced by AA, we investigated the effect of AA on the amount of Ca^{2+} released by SERCA inhibitor CPA. Preincubation of astrocytes for 30 min with AA resulted in a strong reduction of the CPA-evoked Ca^{2+} transient compared to untreated cells. However, simultaneous addition of AA displayed no influence on the amount of Ca^{2+} released by cyclopiazonic acid (CPA), indicating that long-and short-term effects of AA on agonist-evoked Ca^{2+} signals are mediated by different mechanisms. Taken together these

results show that AA actively participates in agonist-induced Ca^{2+} responses, thus regulating the reactivity of astrocytes during states of increased AA levels, e.g. inflammation.

Development of a mechanical in vitro trauma model using thalamocortical organotypic slice cultures

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Slices of dorsal thalamus and occipital cortex of postnatal rats (P0) maintained under organotypic conditions according to Gähwiler are the basis for the presented mechanical *in vitro* injury-model. During cultivation period reciprocal axonal connections between cortex and thalamus are generated in a time-span between 7 to 10 days in vitro (7-10DIV). This axonal network reveals single fibers or axons organized in fascicle structures. Thalamocortical co-cultures are cultivated for 14-20DIV before the mechanical insult. The mechanical injury is made by a PVC-stylus under electromagnetic control. The stylus (for a few milliseconds) hit inflicts a mixed mode of axonal damage consisting of compression and transection.
Spatio-temporal gradients of neurodegenerative events occurs within 24 hours after axonal injury. A highly increased cell death-rate detected by application of vital stains (PI; Syto 21) becomes obvious in superficial and infragranular layers of cortical tissue. The intermediate cortical layers reveal no recognizable cell death. Adjacent to mechanical insult severe injury of axonal compartments within thalamic areas (55% of total axons are fragmented or reveal swellings) can be observed. This damage must concern the corticothalamic or thalamocortical excitatory projection neurons because inhibitory calretinin-ir neurons do not reveal axonal or cellular injury neither in the thalamus nor in the cortex. Moreover we investigated MAP-2 expression patterns by semi-quantitative western blot analysis. Dendritic MAP-2a,b isoform expression declines after injury while MAP-2c localized in axons is elevated in its expression level within investigated 14DIV cultures. More matured tissue does not reveal any MAP-2c expression elevation. This effect corresponds to the regenerative capacity of thalamic neurites after mechanical insult within 14DIV co-cultures which can be not observed within 20DIV thalamocortical cultures.

ATP-sensitive ion channel in the inner mitochondrial membrane

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Mitochondrial ion channels are often involved in apoptosis. The permeability transition pore is the best studied example. Our aim is to study whether mitochondrial channels contribute to neuronal apoptosis, as well. As neuronal mitochondria are difficult to patch clamp we started with T-lymphocytes, a cell type known for a role of mitochondrial ion channels in apoptosis.
Mitoplasts, i.e. small vesicles of inner membrane, were prepared from mitochondria of Jurkat T-lymphocytes as described by Siemen et al. (1999, Biochem. Biophys. Res. Commun. 257: 549). The preparation procedure includes a hypotonic treatment that causes the inner membrane to swell and thereby rupturing the outer membrane. Thereafter isotonicity was restored and mitoplasts were studied by patch-clamp methods using isotonic potassium solutions at both sides. Approaching the mitoplast with the patch pipette yielded seals of about 1.5 GOhm. Two types of channels were observed, a small one with a conductance of 7 pS or multiples of 7 pS. This channel was active in the positive and in the negative voltage range. In addition, we saw a voltage-dependent channel with a single channel conductance of about 50 pS. It appeared at a density of 0-3 channels per patch. The channel showed rectification into the direction of prefered opening. Activity was reduced to a short flickering by addition of 2 mM ATP but it was not inhibited by a reduction of free calcium or by margatoxin blocking other mitochondrial channels. Ockaili et al. (1999, Am. J. Physiol. 277:2425) showed that in other cells ATP-sensitive mitochondrial channels are involved in suppression of apoptosis.
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Ionotropic glutamate receptors-mediated toxicity in cultured rat hippocampal neurons

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We investigated the role of ionotropic glutamate receptors in the neuronal toxicity. Cultured rat hippocampal neurons were exposed to agonists and/or antagonists for 30 min, and the cell viability was evaluated using the MTT assay 24 h later. Kainate (KA, 100 µM) or/and AMPA (100 µM) or NMDA (100 µM) plus glycine (10 µM) were ineffective in inducing cell death by themselves. However, in the presence of cyclothiazide

(CTZ, 30 μM), which prevents AMPA-receptor desensitization, the two non-NMDA receptor agonists had a neurotoxic effect. The neurotoxicity induced by kainate or/and AMPA plus cyclothiazide was completely prevented in the presence of LY303070 (15 μM), a non-competitive AMPA receptor antagonist. Moreover, the NMDA receptor antagonist, MK-801 (10 μM), protected neurons from KA plus CTZ toxicity, since the MTT reduction was 86.8±2.5% of the control.

It has been reported that concanavalin A (Con A) blocks agonist-induced desensitization of kainate receptors. Thus, we used this lectin to unmask the possible role of kainate receptors in neuronal toxicity. Concanavalin A, by itself, induced neuronal toxicity in a dose-dependent manner. At 250 μg/ml, the MTT reduction decrease to 68.6±2.1% of the control. Moreover, we observed that MGA (10 μM), a selective kainate receptor agonist, was toxic in the presence of Con A (250 μg/ml), since the MTT reduction was decreased to 52.9±2.3% of the control. In single cell studies, the increase in the $[Ca^{2+}]_i$ mediated by stimulation with kainate (10 μM) was potentiated in the presence of Con A (250 μg/ml).

Taken together, these results indicate that kainate induced excitotoxicity in rat hippocampal cultures was mainly mediated by AMPA and NMDA receptors. However, in the presence of MGA plus Con A, the activation of kainate-preferring receptors can also induce neuronal toxicity in the hippocampal cell cultures.

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Expression of erythropoietin and erythropoietin receptor in human brain after ischemia / hypoxia

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Erythropoietin (EPO) is a hypoxia-inducible trophic factor with well-documented neuroprotective properties. Using immunohistochemistry, we investigated whether EPO and its receptor (EPOR) are present in human brain after ischemia and/or hypoxia. Autopsy brains of neuropathologically normal subjects were compared to those with ischemic infarcts, hypoxic brain damage, or acute perinatal hypoxic-ischemic pontosubicular necrosis (PSN). In normal adult brain, EPO/EPOR-immunoreactivity (ir) was restricted to neuronal somas. Acutely after stroke (<5 days), EPO-ir was seen in vascular tissue and inflammatory cells, EPOR in blood vessels and neuronal fibers within the infarcted tissue, while staining of neuronal somas was faint. In older ischemic infarcts (>18 days) EPO/EPOR-ir was strongest in reactive glia within ischemic lesions. Hypoxic brain damage was associated with strong EPO/EPOR expression in blood vessels. In PSN lesions, neuronal EPO-ir was weak while EPOR was seen in glia surrounding apoptotic neurons. The data support a role for EPO in human hypoxic-ischemic brain injury and suggest a therapeutic potential of EPO/EPOR stimulation in stroke.

Riluzole reduces brain swelling and contusion volume in rats following controlled cortical impact injury

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Modulation of the glutamatergic and excitotoxic pathway may attenuate secondary damage following traumatic brain injury by blocking sodium channels in their inactivated state and reducing glutamate release. The aim of the present study was to investigate the neuroprotective potential of riluzole in traumatic brain-injured rats. A cortical contusion was induced in 70 male Sprague-Dawley rats (Controlled Cortical Impact Injury). Riluzole (8 mg/ kg body weight) was given 30 minutes, 6, 24, and 30 hours after trauma while control rats received NaCl. Experiments were performed at two different degrees of trauma severity as defined by penetration depth of the impactor rod (1 vs. 1.5 mm) with the aim of investigating impact of tissue damage on the neuroprotective potential of riluzole. At 48 hours after trauma, brains were removed to determine hemispheric swelling and water content and to assess contusion volume. Before brain removal cisternal CSF was collected in all rats to determine the effects of riluzole on substances associated with edema formation. For this, the excitatory transmitter glutamate, the volume- regulatory amino acid taurine, and the ATP-degradation product hypoxanthine were analyzed by HPLC. Differences in results (mean ± SEM) were rated significant at $p<0.05$. Overall, degree of tissue damage influenced the neuroprotective potential of riluzole substantially. In rats with a less severe trauma, hemispheric swelling, cerebral water content of the traumatized hemisphere and contusion volume were significantly reduced under riluzole compared to controls ($p< 0.005$). Following a more severe trauma, riluzole, however, failed to be neuroprotective. After trauma, CSF glutamate, taurine, and hypoxanthine were significantly increased compared to non-traumatized rats ($p< 0.001$). However, these neurochemical parameters failed to reflect trauma-dependent increases in severity of tissue damage and did not reveal riluzole-mediated neuroprotection. Under the present study design, riluzole

significantly reduced brain edema formation and contusion volume in rats subjected to a mild focal cortical contusion.

The correlation between neuronal apoptosis and expression of P53-inducible genes following focal cerebral ischemia in rat

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Expression of P53-inducible protein Gadd45, the growth arrest DNA damage-inducible protein, has recently been reported to be induced by a wide range of stimuli (e.g. cerebral ischemia), especially that produce a high level of base pair damage. Purpose: to investigate the molecular mechanisms of DNA damage and repair and the correlation between neuronal apoptosis and p53-inducible genes gadd45, bax following transient cerebral ischemia. Methods: Focal cerebral ischemia was induced by the transient right MCA occlusion with a poly-l-lysin coated filament in Wistar rats and the brains were collected after 2, 6, 18, 24, and 48h after reperfusion. In situ hybridization and semiquantitative RT-PCR analysis were carried out for gadd45 mRNA. Both Gadd45 and Bax protein were examined with immunohistochemistry and apoptosis was assessed with TUNEL in the sham-operated group and 2,6,18,24,48h reperfusion after 2h cerebral ischemia. Results: Gadd45 mRNA, examined by semiquantitative RT-PCR, was elevated beginning from 2h ($p<0.05$) after and continuing to 48 h , with highest level at 18-24 h. In situ hybridization histochemistry indicated that these increases in gadd45 mRNA occured in neurons mainly located on the edge of the infarcted cortex. Gadd45 immunostaining was sequentially increased in the injured neurons from 6h to 24 h with maximal protein staining detected at 18 h after reperfusion. DNA fragmentation, shown by in situ TdT-biotin nick end labeling (TUNEL) was sequentially increased from 18 h to 48 h after reperfuaion. Bax immunoreactivity is consistent with DNA fragmentation. Conclusion: These findings suggest that p53-inducible gene Bax and Gadd45 contribute to DNA damage and repair following transient middle cerebral artery occlusion. Furtherfore, repairing processes seem to be more active in the penumbra and therefore gadd45 could have also a protective role in cerebral ischemia.

Activation of Caspase-3 in Neuronal Apoptosis After Focal Cerebral Ischemia in Rat

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Background Apoptosis is a physiologic mechanism by which a cell death through activation of an intrinsic cell death or suicide program. Activation of this cell death program leads to a cascade of intracellular events that include a commitment point, followed by the appearance of characteristic ultrastructural changes in the nucleus. Recent studies have showed that caspase-3 activation participates in programmed cell death after traumatic brain injury. However it is no clear whether caspase-3 activation is related to ischemia brain injury. We examined the changes of caspase-3 mRNA and the protein activity in ischemic brain region after MCAo in rats.

Objective To observe the role of caspase-3 activation in neuronal apoptosis after middle cereberal artery occlusion in rats.

Method Using the model of focal cerebral ischemia in Wistar rats, DNA fragmentation, caspase-3 mRNA and enzymatic activity were measured in the sham-operated group and 2,6,18,24,48h after 2h cerebral ischemia and reperfusion.

Result DNA fragmentation was observed in samples from injuried area. beginning from 6h after and continuing for at least 2d by FCM (Flow Cytometer). Caspase-3 mRNA levels, estimated by semiquantitative RT-PCR, were elevated threefold by 6hr and fivefold by 24hr after reperfusion; Increased caspase-3 enzymatic activity was found in cytosolic extracts from ischemic area, but to a lesser extent than caspase-3 mRNA. The changes of caspase-3 enzymatic activity is consistent with DNA fragmentation.

Conclusion Our result suggest that activation of caspase-3 contributes to neuronal apoptosis after middle cerebral artery occlusion and that the development of brain-penetrable inhibitors of caspase-3 may be a useful therapeutic approach to preventing the delayed neurodegeneration that occurs after global or focal ischemic brain injury.

Synthesis and neurochemical properties of substituted tribenzylamines (open analogs of MK-801) as potent NMDA-receptor linked ion channel blockers.

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Specific blockade of excitotoxic action of glutamate related to hyperactivation of NMDA-receptor and calcium ions massive influx is considered as a promising approach for treating and prevention of number of neurodegenerative disorders such as ishemia, Alzheimer's disease, etc. Compound MK-801 - well-known noncompetitive NMDA-receptor antagonist shows strong neuroprotective properties in cell culture experiments, but it therapeutic potential are totally diminished by remarkable side psychotomimetic effects, connected to its high-affinity interaction with PCP-binding site in NMDA-receptor linked ion calcium. In the present study we discuss structure-activity relationships of different groups of MK-801 open analogs ("mono-benzyl", "di-benzyl" and "tri-benzyl" series) which side effect could be significantly minimized due to much higher mobility of benzene fragments. Opening analogs of MK-801 (especially tribenzylamines) have shown the tendency to inhibit glutamate-induced Ca+2 uptake and manifested anti-MNDA activity. The effect of tribenzylamines depends appreciably on their substituents in benzene rings. The introduction of bulky orto-group into the each benzene rings leads to the increase of inhibition effect. The bioisosteric substitution of benzene ring on heteroaryl moiety (furyl, pyridyl, etc.) leads mainly to the appreciable stimulation of uptake. The lead compound (LS-3101) demonstrated strong calcium-blocking property (IC50 = 6.0 mM) and anti-NMDA activity (ED50 = 30 mg/kg). The original synthetic method was proposed for preparation of symmetrical tribenzylamines.

Induction of activin A is essential for the neuroprotective action of bFGF in vivo.

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Consistent with its well-established neuroprotective effect, basic fibroblast growth factor (bFGF) was found to prevent the neuronal damage in hippocampal area CA3 typically associated, with kainic acid (KA) lesion. We found that coinjection of bFGF with KA was accompanied by a substantially stronger expression of activin beta-A mRNA compared to injection of KA alone. We hence speculated that activin A, a member of the TGF-beta superfamily, might be involved in the neuroprotective effects ascribed to bFGF. This hypothesis was corroborated by the following observations: Firstly, recombinant activin, either coinjected with KA or continuously applied, mimicked the beneficial action of bFGF. Secondly, icv. application of the activin-binding protein follistatin abolished the benificial effect of bFGF. These data suggest that activin A is not only a neuroprotective agent in vivo, but might actually mediate the therapeutic effects of bFGF. As it is becoming evident that activin A is essential for the neuroprotective effects of bFGF, development of substances that influence activin expression or binding to its receptors should offer new venues to fight neuronal loss in brain injury.

Effect of valproate on the preoptico-pituitary GABA-LH feedback system

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The pharmacodynamic mode of action of valproate (VPA), an effective drug used in the therapy of generalized seizures and, more recently, also of affective disorders, has been explained by three different hypotheses: 1. the increased-gamma-aminobutyric acid (GABA)-level-hypothesis(1), 2. the postsynaptic-GABA-potentiation-hypothesis(2), and 3. the direct-membrane-effect-hypothesis(3). Recently, we demonstrated a suppressive effect of locally applied 40-100 mg VPA/ml CSF on presynaptic GABA release. For further investigation we utilized the neuroendocrine GABA-luteinizing hormone (LH) feedback system of the hypothalamo-pituitary axis. Methods: The in vivo-effects of VPA on preoptic GABA release and simultaneous pituitary LH secretion were studied perfusing the preoptic area of unanaesthetized, freely moving ovarectomized rats through push-pull cannulae at a flow rate of 20 µl/min with a fraction period of 15 minutes. Results: Local treatment with 40, 100, 400, and 1600 µg VPA/ml CSF differently affected preoptic GABA release and pituitary LH secretion. GABA levels were suppressed by treatment with 40 and 100 µg VPA/ml CSF, respectively, whereas no significant change could be observed at the highest concentration used (1600 µg VPA/ml CSF). Pituitary LH secretion was reduced by perfusion with 100 µg VPA/ml CSF. Using higher VPA concentration this effect became more pronounced. Conclusion: the present data put forward our view that the mechanism of action of VPA generates an enhancement of GABAergic transmission different from involving elevated extracellular GABA. References: 1. Godin et al., 1969; 2. Macdonald and Bergey, 1979; 3. Slater and Johnston, 1978.

Differential effects of Nitric oxide synthase inhibitors administrated intrastriatally on glutamate and GABA uptake in the striatum and cortex after intrastriatal kainate lesioning

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Kainate (a glutamate-receptor agonist, 4nmol in 1¦İl) was directly injected unilaterally into striatum. Two days after kainate injection, a significant neuronal damage was found as determined by Nissl staining in the injected striatum and also in the ipsilateral neighboring cortex. Meanwhile, glutamate uptake and GABA uptake was also decreased significantly both in the striatum (26% and 67% respectively) and cortex (29% and 33% respectively). When kainate was co-injected with L-NAME (non-specific NOS inhibitor), 7-NI (nNOS specific inhibitor), L-NIO (eNOS specific inhibitor), AMT or L-NIL (iNOS specific inhibitors), it was found that L-NAME, 7-NI and L-NIO would significantly attenuate the lesion in the striatum as indicated by a smaller decrease in glutamate and GABA uptakes (10%~16% and 51%~53% respectively), but worsen the lesion in the cortex as indicated by a greater decrease in glutamate and GABA uptakes (43%~48% and 42% respectively). These findings suggest that NO produced by nNOS and eNOS in the striatum promotes local damage but reduces remote damage in the cortex after intrastriatal kainate injection. In contrast, the iNOS inhibitors did not alter the kainate effects except that they can attenuated the decrease in striatal glutamate uptake.

Kainate injected into the striatum increases the expression of APP in neurons through AMPA/kainate- and NMDA-receptors directly and indirectly

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Glutamate-mediated excitotoxicity is an important mechanism responsible for neuronal injury and death. Alterations in the expression of beta-amyloid precursor protein (APP) is involved in neurodegenerative conditions, such as Alzheimer¡¯s disease and in the neuronal response to injury. It has been shown that kainate (an analogue of glutamate) could cause increased expression of APP in neurons. However, the relevant pathways still remain unclear. Using immunohistochemistry, we have investigated the changes in the expression of APP, and the effects of MK-801 and CNQX on the changes of APP in striatal neurons after unilateral microinjection of kainite. Kainate dose-dependently increased the expression of APP in the neurons surrounding the centre of the injection site where significant neuronal death occurred. MK-801 (a selective NMDA receptor channel blocker) and injected 15min before the injection of kainate (2nmol in 1microl) could dose-dependently but partially block the effect of kainate. CNQX (a selective non-NMDA receptor blocker), however, at 24nmol in 2microl could completely block the effect of kainate. Our results show that intrastriatal kainate increases the expression of APP through two pathways: a direct pathway by activating the AMPA/kainate-receptors of the postsynaptic membrane and an indirect pathway by first activating the presynaptic kainate receptors causing the release of glutamate, which then acts on the postsynaptic NMDA- receptors.

Prenatal hypoxia as a neuropathological model for investigation of the plasticity and repair of nervous tissue

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As we have shown previously, prenatal hypoxia resulted in changes in the functional properties of the mediator and signal transduction systems of the sensorimotor cortex (SMCx) and striatum of adult rats and influenced their lateralized motor reactions, especially under conditions of emotional stress. In the present study we analysed effects of prenatal hypoxia on the development of the SMCx and motor reactions of rat pups in early postnatal period. For this, pregnant female Wistar rats (13th day of gestation) were subjected to hypoxic hypoxia (O_2=7%, 3 hours) and the newborn pups were taken for morphological analysis and behavioural experiments. Morphological analysis of the SMCx was performed using Nissl's method. In the SMCx of the three days old pups small necrotic areas (NAs) were observed. The necrosis became more pronounced on the 5th day after birth and was accompanied by chromatolysis of many cells. The density of neurons in the NAs was 3 times lower than in surrounding tissue of the same layers and 3.5 times lower than in the same brain layers of control animals. The NAs were still observed on the 12th day of postnatal development and disappeared on the 16th day but the total density of neurons in the SMCx remained significantly reduced compared to the SMCx of control animals. The pups subjected to prenatal hypoxia have also demonstrated a reduced body weight and decreased spontaneous motor activity in the period from 7th to 11th days after birth. From 5th to 14th days the ability of these pups to maintain position on the rotating grid was also lower. The data obtained demonstrate that prenatal hypoxia leads to considerable impairment of the brain SMCx and motor deficit of newborn animals but these disturbances are partially compensated during maturation of the brain. The present animal model of prenatal hypoxia is suggested as a beneficial tool for further elucidation of neuronal mechanisms underlying cognitive and motor deficit in children suffering prenatal hypoxia and for searching for therapeutic strategies for early correction of brain dysfunction. Supported by RFBR (99-04-49751).

The rat brain $P2Y_1$ receptor expressed in HEK 293 cells: Pharmacological characterization of 2- and 8-substituted adenine nucleotides by studying Ca^{2+} signaling.

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Extracellular adenine nucleotides exert significant biological actions on various peripheral tissues as well as in the central nervous system. They mediate both short term events such as neurotransmission and regulation of immune cell function and long term events such as cell growth, differentiation and proliferation in development and regeneration. Effects are mediated via plasma membrane receptors for ATP (P2 receptors). Whereas P2X receptors represent ligand-gated ion channels P2Y receptors are G protein-coupled and induce the formation of various second messengers like release of Ca^{2+} from internal stores. Different types of P2Y receptors were described, such as $P2Y_1$ receptors which were characterized on many different cell types. However, due to the lack of specific $P2Y_1$ receptor agonists the significance of this receptor subtype remains to be elucidated. Therefore the aim of this study was to characterize the efficacy of some newly synthesized ATP- and AMP-analogues in a recombinant expression system with high receptor expression level. In HEK 293 cells stably expressing the rat $P2Y_1$ wild type receptor we investigated the rise in cytosolic free calcium after stimulation with ATP- and AMP-analogues substituted in 2- or 8-position of the adenine ring. We found an up to 100 fold decreased EC_{50} value with 2-substituted ATP-analogues in comparison to ATP (2-BuS-ATP<2-BuNH-ATP<2-BuO-ATP<ATP) whereas 8-substituted analogues showed considerably increased EC_{50} values. AMP induced Ca^{2+} release with a low potency; likewise 2- and 8-substitutions caused no significant affinity shift except for 2-BuS-AMP (EC_{50} = 100 nM). Moreover we found a great sensitivity for 2-(6-biotinylamido)-hexylthio-ATP (EC_{50} = 2.7 nM) offering a new approach for further receptor studies. To our knowledge the system investigated here represents the most sensitive tool to study $P2Y_1$ receptor agonists and provides insight into new strategies for the development of $P2Y_1$ receptor agonists of high potency and selectivity.