Oral communications

The molecular mechanisms underlying alpha-synuclein-evoked cell death; possible implications for the pathogenesis of Parkinson's disease

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alpha-Synuclein (ASN) play a key role in pathogenesis of Parkinson's disease (PD) and is implicated in the other neurodegenerative disorders. However, the underlying mechanism by which ASN affects neuronal function and death remains unknown. Our previous data indicated that ASN is secreted from synaptic endings into extracellular space and this pool of the protein is involved in dopaminergic cell death and may have a role in the propagation of synuclein pathology and progression of PD. A number of mechanisms have been proposed to explain the toxic effects of ASN. Our own data indicate that ASN leads to nitric oxide (NO) mediated mitochondria dysfunction and caspase-dependent programmed cell death. Previously, we have shown that ASN enhanced the release and toxicity of amyloid beta peptide and indicated an importance of ASN/amyloid beta interaction in neurodegeneration processes. Novel and most interesting data suggested the significance of ASN in Tau phosphorylation and microtubule instability, which could be involved in the mechanisms of dopaminergic cell death in PD brain. However, the exact relationship between ASN and Tau is unknown. Our preliminary data indicated that extracellular ASN affects glycogen synthase kinase-3beta (Gsk-3beta) and cyclin-dependent kinase 5 (Cdk5), two major protein kinases involved in abnormal phosphorylation of Tau. Herein we show that ASN activates phosphorylation of Cdk5 on tyrosine 15 (Tyr15) that stimulates Cdk5 activity. This phosphorylation has been described as a key point in controlling the activation of Cdk5. In addition, ASN affects Cdk5-dependent phosphorylation of Gsk-3beta. Our data suggest an important functional link between Cdk5 and Gsk-3beta. The specific and non-specific Cdk5 inhibitors (BML-259 and roscovitine) protect dopaminergic PC12 cells against ASN-evoked cells death. Summarizing, our results indicate that extracellular ASN-evoked NO dependent mitochondria dysfunction play important role in dopaminergic cells death. Moreover, our last data demonstrate that proapoptotic effects of ASN might be in part mediated via activation of Cdk5. Thus, extracellulary acting ASN may be an important factor in degeneration processes and it may provide therapeutic target for retarding the progression of the neurodegeneration.

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Eaten alive: neuronal death by microglial phagocytosis during inflammatory neurodegeneration

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Inflammatory neurodegeneration is neuronal degeneration due to inflammation, and is thought to contribute to neuronal loss in infectious, ischemic, traumatic and neurodegenerative brain pathologies. We have identified three mechanisms by which inflamed glia kill neurons: iNOS, PHOX and phagocytosis.

Inflamed microglia and astrocytes express inducible nitric oxide synthase (iNOS), producing high levels of NO. We find that this NO acutely and potently inhibits mitochondrial respiration at cytochrome oxidase in competition with oxygen, sensitising neurons to hypoxic death. In addition, NO-derivatives peroxynitrite and S-nitrosothiols inactivate mitochondrial complex I, stimulating oxidant production by mitochondria.

The phagocyte NADPH oxidase (PHOX) is constitutively expressed by microglia, and we find that H_2O_2 from PHOX activation is required for inflammatory activation of microglia. Alternatively, superoxide from PHOX can react with NO from iNOS to produce peroxynitrite, which can induce neuronal apoptosis.

However, the phosphatidylserine (PS) exposure, induced by inflamed glia or peroxynitrite, can be reversible in neurons. Phagocytosis of PS-exposed cells can be mediated by adaptor protein MFG-E8 binding to both PS on target cells and the vitronectin receptor (VR) on phagocytes. We found inflammatory activation of neuronal-glial co-cultures with LPS, LTA, TNF-α or β-amyloid results in progressive loss of neurons (without any apparent cell death), which is accompanied by microglial phagocytosis of neurons, and is prevented by blocking phagocytosis. LPS-induced neuronal loss is absent in cultures from MFG-E8 knockout mice, but is reconstituted by adding wild-type MFG-E8, but not mutants lacking VR binding. LPS-induced neuronal loss *in vivo* is reduced by co-injection of phagocytosis inhibitors or in MFG-E8 knockout mice. Nanomolar levels of β-amyloid induced neuronal and synaptic loss in culture that was prevented by phagocytosis inhibitors.

Cell death by phagocytosis may constitute a *'*new' form of cell death: *'*primary phagocytosis*'*.

The role of Cyclin Dependent Kinase 5 in mechanism of amyloid beta toxicity. Significance in Alzheimer's disease

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Cyclin Dependent Kinase 5 (Cdk5) belongs to the family of proline-directed serine/threonine kinases and plays a critical role in the development of the central nervous system. The activation of Cdk5 is triggered by the binding of the regulatory subunits, p35 (Cdk5r1) or p39 (Cdk5r2), and by phosphorylation at Tyr15 and Ser159. Deregulation of Cdk5 has been demonstrated in many pathological conditions, such as Alzheimer's disease (AD), Parkinson's disease, brain ischemia, alcohol-induced neurodegeneration. Many Cdk5 substrates, like MAP tau, APP, NMDAR, p53, Gsk-3 beta, STAT1/3, are related to pathomecha-

nism of AD. It was demonstrated previously that increased activity of Cdk5 may be responsible for the hyperphosphorylation of MAP tau, destabilization of the cytoskeleton and neuronal death. Our last data present the important role of Cdk5 in cell degeneration and death. We have observed an increase of Cdk5 activity by amyloid beta (AB), alpha-synuclein and it's neurotoxic fragment – NAC peptide (non-amyloid beta component of Alzheimer's disease).

The aim of the present study was to evaluate the expression, phosphorylation and activity of Cdk5 in cells overexpressing Amyloid Precursor Protein. In our studies we used PC12 cells stably transfected with human APP gene, wild-type (APPwt) or bearing Swedish mutation (APPsw). Real-time PCR and Western blotting were used for analysis of expression and phosphorylation of Cdk5, Cdk5r1, Cdk5r2, Gsk-3 beta (Gsk-3B) and MAP tau.

Our data demonstrated the increased level of mRNA for Cdk5 gene in APPsw cells. However, we observed significantly decreased phosphorylation of Cdk5 on Tyr15 in APPwt and APPsw cells, what suggests the lowering of Cdk5 activity. In agreement with this observation, Cdk5-dependent phosphorylation of Gsk-3B on Ser9 was decreased, what leads to the increase of Gsk-3B activity. Concomitantly, the increase of Gsk-3B-dependent MAP tau protein phosphorylation on Ser396 was detected.

These results indicated the sequence of molecular events where interaction between Cdk5 and Gsk-3B plays the important role in mechanism of amyloid beta toxicity. The overexpression of APP and enhancement of Aβ production may be responsible for the deregulation of Cdk5, leading in consequence to overactivation of Gsk-3B and to hyperphosphorylation of MAP tau, impairment of cytoskeleton function, cell degeneration and death. These all processes may play a key role in pathomechanism of AD.

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Mitochondrial pathways of neuronal cell death as therapeutic targets in neurological diseases

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Mitochondria are highly dynamic organelles playing an important role in life and death of neurons because of their functions in energy metabolism, calcium buffering and signaling pathways of programmed cell death, respectively. In particular, fragmentation of mitochondria, loss of mitochondrial membrane integrity, Ca^{2+}-deregulation, formation of reactive oxygen species and, finally, the release of mitochondrial proteins into the cytosol and the nucleus are major features causing neuronal death in various neurodegenerative diseases and after acute brain damage. Therefore, better understanding of the mechanisms upstream of mitochondrial damage is essential to identify promising targets for neuroprotective strategies. The presentation highlights our recent insights into key mechanisms of pathological mitochondrial fragmentation triggered by glutamate toxicity in neurons, which involve the concomitant activity of Drp1 and pro-apoptotic proteins of the bcl-2 family mediating mitochondrial membrane permeabilization and AIF-dependent cell death. Based on these insights into key mechanisms of mitochondrial death pathways, siRNA approaches and small molecule inhibitors targeting Bid, Drp1 or potassium channels were validated as potent neuroprotectants in neuronal cells and in models of ischemic brain damage in vivo. Overall, our data clearly demonstrate that inhibiting mechanisms of pathological mitochondrial fragmentation is a promising strategy for therapeutic approaches in neurodegenerative diseases.

Cerebral glucose metabolism in Alzheimer neurodegeneration: cause, risk factor or consequence?

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Brain makes only 2% of body mass but it uses 20% of oxygen and 50% glucose taken up by the whole human body at rest. Uninterrupted glucose supply is critical for brain metabolism and function. In healthy adult human brain 85% of glucose is used directly for the energy metabolism and the rest serves other purposes, eg. synthesis of acetylcholine. Alzheimer's disease (AD) is associated with neuropathological hallmarks: amyloid plaques and neurofibrillary tangles, and dementia. In AD brain glucose metabolic uptake is progressively depressed, perhaps even more than oxygen metabolic uptake, and debate continues whether this phenomenon is primary – i.e., the cause of the disease, important – i.e., the risk factor of the disease, or secondary and of no independent importance – i.e., the epiphenomenon merely reflecting neurodegeneration which already happened. Depression of glucose metabolism in some brain regions occurs early in AD and preceeds neurological symptoms. Group studies identified significant regional metabolic impairments in asymptomatic individuals at increased risk for dementia. Impairment of fluorodeoxyglucose uptake in temporoparietal association cortices is considered a reliable predictor of rapid progression to dementia in mild cognitive impairment patients which could serve as a biomarker for the diagnosis of prodromal AD. The most popular pathogenetic concept of AD is known as the "Amyloid Cascade Hypothesis". This concept is based on the assumption that the primary cause of AD is the development of neurotoxic beta-amyloid fibrils wheras degeneration of neurons and disturbances of brain glucose metabolism are secondary phenomena. However, several recent experimental data support the idea that sporadic AD (SAD) may develop as a consequence of desensitization of cerebral insulin receptors and SAD may be "brain-specific (type 3) diabetes". A rodent model of intracerebroventricular injection of streptozotocin, toxin selectively destroying insulin-producing cells and insulin receptors, is supposed to produce brain insulin receptors destruction and desensitization. The result is deep brain hypometabolism, memory disturbances and in longer observation formation of cerebral amyloid deposits. Brain glucose hypometabolism may also activate kinases responsible for tau protein hyperphosphorylation (DYRK1A, GSK3-beta). Further studies with this model may be useful for preclinical assessment of novel therapeutic concepts.

Zn as a trigger of acetyl-CoA-dependent cholinergic neurodegeneration

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Excessive accumulation of Zn in postsynaptic cholinergic neurons is an early neurotoxic-excitoxic signal in different neurodegenerative processes. We investigated whether excess of Zn is capable inducing

impairment of energy and transmitter metabolism in cholinergic neurons. Short term exposition of SN56 cells to Zn increased their mortality in a concentration-dependent manner. Zn caused direct reversible inhibition of pyruvate dehydrogenase (PDH) and ketoglutarate dehydrogenase (KDH) and irreversible one aconitase, isocitrate dehydrogenase (IDH) activities, with Ki values equal to 0.058, 0.004, 0.010, 0.0015 mM, respectively. Activities of succinate, malate dehydrogenases and fumarase were not affected. Also ChAT activity was not suppressed by Zn. On the other hand, the potency of Zn inhibitory effects on activities of same enzymes *in situ* in intacted cells appeared to be about 10 times weaker than the direct ones. Despite of that cytoplasmic levels of acetyl-CoA, ACh as well as ACh release were decreased in these conditions. Acute inhibition of ACh synthesis and release correlated with decrease of cytoplasmic acetyl-CoA concentration. Moreover, long time Zn-induced acetyl-CoA deficits caused adaptative suppression of ChAT activity. These effects were modified by pH of extracellular medium. At pH 7.4 0.10 mmol/l Zn caused no neurotoxic effects. Lactic acidosis of pH 6.5, as a single factor simulating conditions in hypoxic brain, caused no alterations in the SN56 counts and in activitie of their key enzymes of energy and acetylcholine metabolism. Also acetyl-CoA levels were not depressed by acidosis. However, the pH 6.5 0.1 mmol/l Zn caused 60% increase of nonviable cell fraction and 50% decrease of cell count irrespective on the degree of their differentiation. The 50% inhibitions of PDH, IDH as well as aconitase activities were observed, along with decrease in whole cell acetyl-CoA level. These data indicate that Zn exerts its neurotoxic effects through inhibition of key enzymes of acetyl-CoA metabolism, which are aggrevated by acidotic conditions due to decreased metal affinity to its intracellular binding sites.

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Acetyl-CoA – key target in thiamine deficits evoked cholinergic encephalopathies

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Thiamine deficiency (TD), causes inhibition of key enzymes of energy metabolism: pyruvate and ketoglutarate dehydrogenase complexes (PDHC, KDHC), yielding different syndromes of peripheral neuropathies and central cholinergic encephalopathy. However, more frequently subclinical TD overlays several other cholinergic encephalopathies including Alzheimer's (AD) and Parkinson's (PD), apparently attenuating their onset and worsening outcome. Therefore, the aim of this work was to investigate alterations in concentration and distribution of acetyl-CoA and ACh metabolism in cellular and whole brain models of TD. Pyrithiamine-induced TD, caused decrease of PDHC and KDHC activities in brain nerve terminals along with decrease of acetyl-CoA levels in their cytoplasmic and mitochondrial compartments. These alterations correlated with inhibition of citrate lyase pathway of acetyl-CoA transport from mitochondria to cytoplasm and suppression acetylcholine synthesis and its quantal release. In cultured clonal septal cholinergic SN56 cells, amprolium-induced TD caused inhibition of metabolic flux of pyruvate through PDHC step and decreases acetyl-CoA in cytoplasmic compartment. However, neurons with low expression of the cholinergic phenotype appeared to be more resistant to TD than those with high expression of cholinergic parameters. Mortality of differentiated cholinergic cells was about two times greater that nondifferentiated ones at the same levels of thiamine diphosphate deficits. Zn-glutamate induced ex-

citotoxicity is a preliminary nonspecific step of several encephalopaties. Amprolium-TD aggravated detrimental effects of Zn on cholinergic cell viability, their acetyl-CoA content, energy metabolism and transmitter functions. Significant correlations have been found between alterations of acetyl-CoA in differentiated TD cells and their viability and transmitter functions. No such correlations existed in nondifferentiated cells. These data indicate that TD deficiency may be both sole and complementary factor contributing to development of various cholinergic encephalopathies through the acetyl-CoA depletion-dependent mechanisms.

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Why do patients with hyponatremia develop brain symptoms? A view from the perspective of experimental studies

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According to the epidemiologic studies, hyponatremia (decrease of plasma Na^+ concentration below 135 mM) occurs in more than 15% of hospitalized patients, in approximately 18% of elderly population and in 13% of marathon runners. The symptoms of hyponatremia are primarily neurologic and are related to osmotic brain cells swelling. These symptoms correlate with the severity and duration of sodium ions imbalance. Despite the importance of blood vessels function for the adequate brain supply of nutrients, surprisingly little is known about the vascular effects of hyponatremia.

Present study was undertaken to investigate the effect of hyponatremia on cerebral vascular functions. Isolated rat middle cerebral arteries (MCA) were subjected to acute (AH) or prolonged (PH) hyponatremia. To assess their function, the responses to changes in perfusion pressure, to acidosis and to the increase in extravascular concentration of potassium ions were studied in MCAs mounted in the small organ chamber filled with 3-(N- morpholino) propanesulfonic acid – buffered physiological saline solution. To imitate hyponatremic conditions, Na^+ concentration in the chamber was lowered to 120 mM. Hyponatremia, irrespective of its duration, did not affect the response of MCA to the changes in perfusion pressure. It has, however, abolished the response to acidosis both in AH and PH groups. The response to hyperkalemia was abolished in AH but preserved in PH group. Thus, AH impairs regulation of the MCA more than PH. In order to elucidate the mechanism behind MCA dysfunction in AH, the response of MCA in AH to endothelium-dependent vasodilator acetylcholine, nitric oxide inhibitor (L-NAME), nitric oxide donor (SNAP) and analogue of cyclic guanosine monophosphate (8-Br-cGMP) was studied. The results of this part of the study point to the disordered function of K^+ channels in vascular smooth muscles in acute hyponatremia.

Our results demonstrate that hyponatremia selectively disturbs the regulatory mechanisms of cerebral blood vessels.

They suggest that impairment of the regulation of cerebral blood vessels may, in addition to cellular swelling, be a reason why patients with hyponatremia develop neurologic symptoms.

The role of the blood-brain barrier in the formation of brain metastases

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Brain metastases constitute the most significant part of intracranial tumors. The majority of brain metastases originate from lung cancer, breast cancer and melanoma. Among these malignant tumors melanoma is the one which metastasizes to the brain with one of the highest frequencies: brain metastases are diagnosed in 40–50% of the cases. Since the central nervous system lacks a lymphatic system, the only possibility for melanoma cells to reach the brain parenchyma is *via* the blood stream and the blood-brain barrier. Despite the great clinical importance, the mechanisms of transmigration of melanoma cells through the blood-brain barrier are incompletely understood.

For our investigations we have used an *in vitro* blood-brain barrier model system based on the culture of cerebral endothelial cells (CECs) and A2058 or B16/F10 melanoma cells, respectively. Immunofluorescence studies have shown that the TJ proteins occludin, ZO-1 and claudin-5 disappeared from the membrane of endothelial cells which were in contact with melanoma cells suggesting that melanoma cells can transmigrate through the blood-brain barrier using the paracellular route. Transmigration was accompanied by an increased proteolytic activity, and we have shown that the serine protease seprase plays a role in this process.

Furthermore, we have demonstrated that transmigration is significantly enhanced by the Rho-kinase inhibitor Y27632. This supports the hypothesis that melanoma cells preferentially use the mesenchymal-type of movement during their transmigration through cerebral endothelial cell layers. Atomic force microscopic measurements indicate that individual binding force between melanoma and endothelial cells is in the range of 100–1000 pN and is significantly increased in the presence of the Rho-kinase inhibitor. Further investigations are necessary to clarify the molecular details of the transmigration of melanoma cells through the blood-brain barrier.

The influence of a low dose LPS preconditioning on the paraquat-induced toxicity

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Prolonged inflammation, oxidative stress and protein aggregation are the main factors contributing to the Parkinson's disease (PD) pathology. Pesticide paraquat (PQ) is known as the environmental substance increasing the incidence of PD and has been used to model this disease. We investigated if a combination of these factors in subthreshold doses would increase the modelled neuropathology.

We examined how acute or prolonged inflammation would influence PQ toxicity in the nigrostriatal dopaminergic pathway or change the expression of major Lewy bodies proteins as well as microglial activation.

A small dose of LPS (10 μg/kg, *ip*) was administered either once, 3 hours before 4 weekly doses of PQ (10 μg/kg, *ip*) or 4 times, before each of PQ doses. Animals were killed 7 days after the last dose of PQ. The body temperature was measured in different times. The density of dopaminergic (TH+) and non-dopaminergic neurons as well as microglial cells (Iba1+) was calculated stereologically in the substantia nigra (SN). The expression of alpha-synuclein and synphilin-1 and activated microglia marker CD11b were measured densitometrically in SN.

Single LPS pre-treatment aggravated PQ toxicity in SN. Also neurodegeneration of dopaminergic neurons after prolonged inflammation itself was observed. Interestingly, repeated LPS administration combined with PQ did not enhance this effect. PQ induced long-term deactivation of microglia and prevented the glia activation and an increase in the body temperature while combined with LPS treatment. The expression of alpha-synuclein decreased after repeated LPS injections while only combined treatment lowered the levels of synphilin-1. We observed different results in younger and older animals treated repeatedly with LPS and PQ.

Our results indicate that both PQ exposure and inflammation can induce toxicity but they act through different, not additive mechanisms, implying the microglia activation. Moreover, those degenerative mechanisms are different in dopaminergic *vs.* non-dopaminergic neurons. The alpha-synuclein level is influenced by inflammatory processes while that of synphilin-1 expression is changed only when both inflammation and oxidative stress processes are involved, although expression of both proteins increases with aging. Our results corroborate that even small but repeated inflammation itself is sufficient to evoke neurodegeneration.

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Treatment of spasticity on the basis of the data on pathogenesis of spinal cord injury

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The intrathecal baclofen therapy has a good clinical benefit for spinal spasticity when oral medication is found to be ineffective or presents serious side effects. In a previous study, we have demonstrated that L5 and L6 dorsal rhizotomy caused degeneration of nNOS-IR Ia fibers in the dorsal funiculus of L5 and L6 segments, and that nitrergic proprioceptive afferents originating from quadriceps femoris muscle are related to monosynaptic α-motoneuron stretch reflex circuit. Here we hypothetized that nNOS may play a key role in setting the excitability of the α-motoneurons after spinal cord transection at the low thoracic level receiving inputs from skeletal muscle afferents. We performed RT-PCR, western blotting and immunohistochemical analyses to examine the changes in the level of neuronal nitric oxide synthase (nNOS) protein, nNOS mRNA and the expression of nNOS immunoreactivity (nNOS-IR) in the lumbosacral segments (L2-S1) after thoracic spinal cord transection and baclofen and/or NNLA treatment. Baclofen (3 μg/2 × per day/*i.t.*) was applied 3-times from the 7th day after spinal cord transection. In addition, we monitored tail-flick response in order to investigate a pain-related behavior. For the treatment of trauma-induced pathology we used nNOS blocker, NNLA in three different ways: (1) NNLA applied during first 3 days following spinal cord transection in dose 20 mg/kg/day caused the loss of nNOS-IR in neurons of dorsal horn and around central canal, but nNOS expression remained unchanged in motoneurons. This result sup-

ports the finding that strong blocking of NO production early after spinal injury may have unfavorable effect. (2) The NNLA/baclofen therapy effectively modulated trauma-evoked nNOS expression in a-motoneurons. Baclofen whether applied itself or in combination with the NNLA has beneficial effect but repeated application of NNLA is harmful for the tissue and does not influences the reflex response. (3) The intensity of nNOS staining in α-motoneurons was decreased after single dose of NNLA (60 mg/kg *im*), i.e. when NO blocator was applied 1 hour before the end of experiment. We can conclude that the intrathecal treatment with baclofen provides a significant suppression of spasticity and that post-traumatic NNLA administration attenuates the trauma-induced increase of nNOS-IR in motoneurons.

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Pro-inflammatory micro RNA (miRNA) signaling in Alzheimer's disease (AD)

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Human brain cells require a specific and selective subset of micro RNAs (miRNAs) to maintain intrinsic gene expression patterns, and miRNA effects on gene expression are mediated through their regulation of messenger RNA (mRNA) complexity. In recent studies (a) in short *post-mortem* interval Alzheimer' disease (AD) brain tissues versus healthy age-matched controls, (b) in cytokine- (IL-1β, TNFα) and Aβ42 peptide-stressed human neuronal-glial (HNG), astroglial (HAG) and micro-glial (HMG) cells in primary culture, and (c) in several murine transgenic AD (Tg-AD) models including Tg2576 and 5xFAD, we have identified several brain-abundant miRNA species that are consistently up-regulated, including miRNA-146a. This inducible, NF-κB-regulated, 22 nucleotide non-coding RNA targets the mRNA of the key, innate-immune- and inflammation-related regulatory protein, complement factor-H (CFH), resulting in significant decreases in CFH expression ($p < 0.01$, ANOVA). Our results further indicate that HNG, HAG and HMG cells each respond differently to cytokine- and Aβ42-peptide-induced stress, in part by variation in their intrinsic miRNA-mediated responses. The complex interactive signaling of NF-κB and miRNA-146a further underscore the interplay between inducible transcription factors and pro-inflammatory miRNAs that regulate CFH expression in degenerating brain tissue. The combinatorial use of NF-κB inhibitors with antisense miRNAs (antagomirs) may have potential as a novel therapeutic strategy directed against CFH-driven pathogenic signaling in progressive neurodegenerative disease, and may have further potential as a diagnostic marker for inflammatory neurodegeneration.

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Mitochondrial DNA variation in Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia. Among its early symptoms are cerebral hypometabolism, mitochondrial dysfunction and oxidative stress. Since mitochondria and brain bioenergetics have been increasingly recognized as important contributors to AD pathology, we investigated the potential involvement of mitochondrial DNA (mtDNA) variants and mtDNA haplogroups in AD risk.

We found that mtDNA haplogroups and subhaplogroups with a putative role in partial uncoupling of oxidative phosphorylation (OXPHOS) are significantly associated with a decreased AD risk (OR < 1). Conversely, mtDNA haplogroup associated with OXPHOS coupling, H, and HV cluster are significant AD risk factors (OR = 1.22, OR = 1.25, respectively). Haplogroup K was demonstrated to exert a neutralizing effect on the high risk associated with carrying at least one APOE4 allele (p = 0.014). Altogether, our data positively verified Wallace et al. (2003) hypothesis on coupling-uncoupling haplogroups role in neurodegenerative disorders.

Impaired complex I activity enhances Aβ production: The link between brain aging and specific Alzheimer's pathology

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Sporadic Alzheimer's disease (AD) is the most common age-related neurodegenerative disease characterized by initial memory impairment progressing towards total loss of mental and physical abilities. The key histopathological features are amyloid-beta (Aβ) containing plaques and microtubule-associated tau-containing neurofibrillary tangles. The main risk factor for sporadic AD is aging. Aging itself is associated with a long time exposure of our brain to oxidative stress leading to accumulation of oxidized proteins and nucleic acids. The mitochondrion, as major center of energy production, is the main source of reactive oxygen species (ROS). The majority of ROS derive from complex I and III of the respiratory chain in form of superoxide anion radicals. Importantly, complex I activity declines substantially during normal brain aging whereas complex III activity is nearly unchanged probably making complex I the major player of the brain aging scenario. Not only in familial AD but also in sporadic AD, a gradual increase of Aβ over years or even decades is considered to play an important role in the pathogenesis. Enhanced amyloidogenic processing of APP by β-site APP cleaving enzyme (BACE) and the γ-secretase complex leads to increased intracellular soluble oligomeric Aβ levels resulting in pronounced synaptic failure and finally in memory decline. The mechanism why APP processing is altered in sporadic AD patients is still not yet known and probably represents the most important missing link in the understanding of this devastating disease.

Several recent findings show that mitochondrial dysfunction is one of the earliest pathogenic alterations in AD. In *post-mortem* brain tissue of sporadic AD patients, a deficiency of cytochrome c oxidase

(complex IV) activity is consistently reported. In several AD animal models, mitochondrial dysfunction compromising decreased mitochondrial membrane potential (MMP), reduced ATP levels, declined complex IV activity, and enhanced oxidative stress was detected. Furthermore, mitochondrial dynamics and morphology are discussed to be altered in AD. These pathological changes are mostly observed when oligomeric Aβ could be detected and fibrillar plaques were not yet present stressing the early contribution of mitochondrial dysfunction to the progression of the disease. We report that mitochondrial dysfunction especially complex I dysfunction associated with aging might be the initial trigger for altered APP processing and enhanced Aβ production might result in a vicious cycle which further impairs mitochondrial dysfunction leading to apoptosis, synaptic dysfunction and memory decline.

Blood-brain barrier transporters – checkpoints for energetic and neuroprotective compounds

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Delivery of important energetic substrates and a control of brain homeostasis depend on functioning of many specialized transporters, often localized asymmetrically in endothelial cells forming the blood-brain barrier. Accessibility of nutrients depends on transporters from SLC superfamily, as amino acids, hexoses, monocarboxylates, amines, carnitine and glutathione transporters, while efflux is controlled by ABC pumps and multiple organic anion transporters. Both the members of SLC and ABC families have been studied as potential targets for drug delivery to the brain, as well as proteins controlling drug removal. The role of these transporters in several pathological states leading to brain dysfunction will be presented. A special emphasis will be put on neuroprotective role of carnitine, the function of this compound in brain cells at molecular level, as well as the role of its transporters from SLC6 and SLC22 families under physiological conditions and in pathology.

Sphingolipid signaling and neuronal function

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Neuronal activity greatly influences the formation and stabilization of synapses. Although receptors for sphingosine-1-phosphate (S1P), a lipid mediator regulating diverse cellular processes, are abundant in central nervous system, neuron-specific functions of S1P remain largely undefined. Here I present novel actions of S1P using primary hippocampal neurons as a model system, i.e., S1P triggers neurotransmitter release in a dosen-dependent manner. Sphingosine kinase 1 (SK1), a key enzyme for S1P production, was enriched in hippocampal neurons. Silencing SK1 expression by siRNA resulted in a strong inhibition of depolarization-evoked glutamate release. FRET analysis demonstrated that S1P1 receptor at the pre-

synaptic membranes was activated during depolarization and that depolarization-induced S1P1 receptor activation was inhibited in SK1-knockeddown cells. Importantly, exogenously added S1P at nanomolar concentrations by itself elicited glutamate release from hippocampal neurons even when Na^+-channel was blocked by tetrodotoxin, suggesting that S1P acts on presynaptic membranes. These findings indicate that S1P, through its autocrine action, facilitates spontaneous glutamate release from hippocampal neurons. I will also present data using electrophysiological approaches and behavioral analysis using SK1-knockout mice showing a vital role of SK/S1P signaling in memory formation and learning in the hippocampus.

New insight into pathomechanisms of Parkinson's disease

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Degeneration of dopaminergic (DA) nigrostriatal neurons is generally accepted to result in appearance of primary motor symptoms (bradykinesia, muscle rigidity, tremor at rest) of Parkinson's disease (PD). However, PD is a progressive multisystem disorder where neuropathological alterations – α-synuclein aggregates (Lewy bodies and neurites) appear at different levels of the central nervous system and may contribute to its symptomatology. Although the cause of PD is not known, several processes e.g., oxidative stress, mitochondrial dysfunctions, protein misfolding and aggregation, impairments of ubiquitin-proteasome system and autophagy, disturbed calcium homeostasis, neuroinflammation and others, have been suggested to underlie degeneration of DA neurons. Aging and environmental toxins (e.g., pesticides) are risk factors of PD. Among the above processes mitochondrial dysfunctions seem to play the critical role in PD and occur early in its pathogenesis. A deficit of the complex I of respiratory chain have been consistently found in the substantia nigra pigmented neurons, in the frontal cortex, as well as in non-neural tissues: platelets, muscle fibers, or fibroblasts in PD. In patients suffering from the sporadic PD the level of clonally expanded mtDNA deletions encoding subunits of respiratory complexes is higher than in their age-matched controls, which may result in increased vulnerability of DA neurons to exogenous toxic insults. On the other hand, mutations in several genes have been linked to genetic forms of PD (alpha-synuclein, parkin, DJ-1, PINK1, LRRK2 and others). At least some of these monogenic mutations lead to mitochondrial dysfunctions. Recent studies have shown that PINK-1 and parkin play crucial roles in the regulation of mitochondrial dynamics and function. PINK1/parkin promote destruction of dysfunctional mitochondria by authophagosomes and protects the mitochondrial network against accumulation of somatic mtDNA mutations. DJ-1 and parkin deficiencies render DA neurons more susceptible to oxidative stress and mitochondrial toxins. A small proportion of alpha-synuclein is imported into mitochondria where it accumulates in the brain of PD patients and may impair respiratory complex I activity.

The lecture will summarize recent discoveries in the contribution of mitochondrial abnormalities to PD etiology and pathogenesis.

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Alterations of blood-brain barrier (BBB) permeability in hyperammonemia: an overview

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Ammonia is a neurotoxin involved in the pathogenesis of hyperammonemic encephalopathies, including hepatic encephalopathy (HE), a highly complex neurological condition associated with acute (ALF) or chronic liver failure. Ammonia affects both the transcellular and paracellular passage of low-to medium-size molecules across the blood brain barrier (BBB). Aberrations of the transcellular passage mainly involve interrelated dysfunctions of the amino acid carriers located at the BBB mediating the transport of large neutral amino acids (LNAA) and aromatic amino acids (AAA), leading to impaired synthesis of the catecholamines dopamine and serotonin and neurotransmitter imbalance. Recent evidence indicates that subtle increases of the transcellular passage of molecules of different size (BBB "leakage"), may be responsible for the vasogenic component of cerebral edema associated with ALF. Since ammonia evokes oxidative/nitrosative stress (ONS) in the brain we tested the hypothesis that the ammonia-induced transcellular passage occurs by a mechanism secondary to ONS in the BBB-forming cerebral capillary endothelial cells. Treatment of a rat brain endothelial cell line (RBE-4) with ammonia (5 mM, 24 h) caused accumulation of ONS markers: reactive oxygen species (ROS), nitric oxide (NO) and peroxidation products of phospholipid-bound arachidonic acid, F2-isoprostanes (F2-IsoPs). Concurrently, ammonia increased the activity of extracellular matrix metalloproteinases (MMP-2/MMP-9), and increased cell permeability to fluorescein isothiocyanate (FITC)-dextran (40 kDa). The increase of cell permeability was ameliorated upon co-treatment with a MMP inhibitor, SB-3CT and with an antioxidant, glutathione diethyl ester (GEE), which also reduced F2-IsoPs. Ammonia-induced ONS was attenuated by cytoprotective agents L-ornithine (Orn), phenylbutyrate (PB), and their conjugate L-ornithine phenylbutyrate (OP), an ammonia-trapping drug used to treat hyperammonemia. The results support the concept that ONS and ONS-related activation of MMPs in cerebral capillary endothelial cells contribute to the increased transcellular permeability of the BBB which may contribute to the vasogenic component of cerebral edema, which is the major cause of death of ALF patients.

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Endothelium – therapeutic target in stroke and neurodegenerative disorders

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Endothelium – an internal cell layer in the vessel wall plays a vital role in control of several physiological mechanisms such as regulation of vessel diameter, thrombosis and inflammation. These cells have therefore major impact on blood perfusion in every organ including the brain. Besides these physiological func-

tions, endothelium controls flux between vessel lumen and organ cells that include metabolic substrates, hormones, small and macromolecular toxins and pathogens. This function is particularly important and unique in the brain, as endothelium is a major contributor to blood-brain barrier. There are many unique features of the cerebrovascular endothelium such as high number of mitochondria and therefore increased potential for reactive oxygen species production. In many pathological conditions of the nervous system destruction of endothelium is known to contribute e.g., Alzheimer or Parkinson diseases, amyotrophic lateral sclerosis, multiple sclerosis, human immunodeficiency virus associated neurocognitive disorder and traumatic brain injury. Therapy directed to protect and regenerate endothelial layer could be therefore useful addition to optimal treatment in these conditions. While several common treatments are known to protect endothelial function (e.g., statins), drugs to specifically treat endothelium are not currently available. There is extensive research to develop therapy that would specifically target endothelium. Nicotinamide derivatives – in particular N-methylnicotinamide was proven to protect endothelium and increase antithrombotic and anti-inflammatory prostacyclin production. It was shown to reverse endothelial injury in diabetic animals or in lipid metabolism disorders. Recruitment of endothelial progenitor cells represents another promising strategy to regenerate impaired vessels in diabetes or in atherosclerosis. Endothelium protection strategy has been little tested in experimental models of neurodegenerative disorders or ischemic brain injury. There is considerable evidence that such strategy could provide substantial benefit, but more research is needed to verify this concept.

Lipid rafts regulate HIV-1 and amyloid beta interactions at the blood-brain barrier

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Due to the success of combination antiretroviral therapy (cART), which changed the clinical picture of HIV-1 infection from acute to chronic disorder, there is a sharp increase in infected patients 50 years old and older. This increase in age of the HIV-1 infected population constitutes a new challenge in the HIV epidemic in the affluent countries. Indeed, older HIV-1 infected patients are more susceptible to neurocognitive impairments associated with the disease. HIV-1 infected brains are characterized by increased deposition of amyloid beta (Aβ) in perivascular space, indicating the importance of brain microvessels and the BBB in amyloid accumulation. We hypothesize that lipid rafts and functional caveolae are critical structures involved in HIV-1-induced Aβ accumulation at the blood-brain barrier and in human brain microvascular endothelial cells (HBMEC). Indeed, both silencing of caveolin-1 (cav-1) and disruption of lipid rafts by pretreatment with beta-methyl-cyclodextrin (MCD) protected against Aβ accumulation in HBMEC. Exposure to HIV-1 and Aβ activated caveolae-associated Ras and p38. While inhibition of Ras by farnesylthiosalicylic acid (FTS) effectively protected against HIV-1-induced accumulation of Aβ, blocking of p38 with SB203580 did not have this effect. We also evaluated the role of caveolae in HIV-1-induced upregulation of the receptor for advanced glycation end products (RAGE), which regulates Aβ transfer from the blood stream into the central nervous system. In control cultures, RAGE immunoreactivity showed a distinct cytoplasmic staining pattern that appeared to imitate an intricate intracellular linear network. A 24 h exposure to HIV-1 resulted in a markedly stronger RAGE immunoreactivity with more detailed ramification of this staining pattern. Exposure of HBMEC to HIV-1-infected or control monocytes also resulted in increased RAGE mRNA levels. Importantly, HIV-1 induced increase in RAGE expression

was prevented by infecting HBMEC with cav-1 specific shRNA lentiviral particles or by pretreatment of cells with FTS. Overall, the present results indicate that Aβ accumulation in HBMEC is lipid raft/caveolae dependent and involves the caveolae-associated Ras signaling pathway.

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Neuroprotective properties of adenosine A2A receptor antagonists in animal models of Parkinson's disease

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Parkinson's disease (PD) is a chronic neurodegenerative disorder with cardinal clinical features of rest tremor, rigidity, and bradykinesia attributed to an underlying neurodegeneration of dopaminergic neurons of the substantia nigra (SN) and a drop in dopamine (DA) level in the striatum (CP). The current pharmacotherapy (L-DOPA) constitute "the DA replacement" strategy, however, it does not halt progression of the degeneration process. Recently A2A receptors have been shown to be involved in several neurological disturbances in humans and their animal equivalents, including PD and essential tremor. Antagonists of A2A receptors not only improve all motor symptoms of PD but also counteract the loss of dopaminergic neurons in the SN in animal models of this disease. These receptors form heterooligomeric complexes with dopamine D2 receptors and are present both on neuronal and glial cells mainly in DA innervated areas such as the striatum.

Caffeine, a non-selective adenosine antagonist provides neuroprotection against degeneration induced by 6-OHDA, MPTP, and paraquate in animals. This action was mimicked by selective adenosine A2A (KW6002, CSC, SCH58261, ST1535) but not A1 receptor antagonists. Similar neuroprotection including attenuation of striatal DA depletion and inhibition of DA cells neurodegeneration in the SN has been observed in genetic models of global and forebrain deletion of A2A receptors in mice. These compounds prevented also MPTP-induced astroglial (GFAP-ir) and microglial (CD11b-ir) cell activation in the SN. Moreover, pharmacological blockade of A2A receptors with KW6002 and MSX-3 partially attenuated the depletion of DA and its metabolites in the CP as well as a decrease in the number and density of TH-ir neurons in the SN, induced by chronic, 28-days infusion of MPP^+ (0.284 mg/kg/day) into the cerebral ventricle using an ALZET osmotic minipumps.

The available experimental data provide evidences in support of neuroprotective potential of caffeine and selective A2A antagonists in animal models of PD and suggest that these effects might be related to both neuronal and glial mechanisms. Therefore, it seems that blockade of adenosine A2A receptors may offer a new strategy to slow or halt degeneration of dopaminergic neurons.

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Regulation of poststroke brain inflammation by sensori-motor activation

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After stroke, inflammation hampers beneficial mechanisms important for tissue reorganization in the ischemic hemisphere. We found that EE profoundly attenuated the level of the pro-inflammatory cytokines interferon-γ, TNFalpha, IL-1β, IL-4, and IL-5. Importantly, cytokine levels in the cerebro-spinal fluid and serum were not altered in respective animals. Along with changes of pro-inflammtory cytokines we found a significant reduction of the otherwise upregulated chemokine receptor CXCR4 and its natural ligand stromal-derived-factor 1 (SDF-1) in rats housed in EE after pMCAO. Treatment with a CXCR4 antagonist for 3 consecutive days starting 2 days after tMCAO improved recovery compared with saline treated rats, and was accompanied by a reduction of infiltrating immune cells. We conclude that attenuation of poststroke inflammation obtained by housing rats in an enriched environment or specific inhibition of the SDF-1/CXCR4 pathway significantly improves functional recovery and prevents detrimental secondary systemic effects in rats subjected to experimental stroke.