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Early survival signaling responses to neurodegenerative diseases: significance for new therapies and diagnosis

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The significance of the selective enrichment in omega-3 essential fatty acids (docosahexaenoyl [DHA] chains of membrane phospholipids, 22C and 6 double bonds) in the nervous system (e.g. synaptic membranes and dendrites) has remained, until recently, incompletely understood. While studying mechanisms of cell survival in experimental stroke and neurodegeneration, we found PLA2 activation followed by DHA release. In turn, docosanoid is synthesized by 15-lipoxygenase-1, which we dubbed neuroprotectin D1 (NPD1,10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15E,19Z hexaenoic acid). This mediator is a docosanoid because it is derived from a 22C precursor (DHA), unlike eicosanoids, which are derived from the 20 C arachidonic acid family of essential fatty acids not enriched in the nervous system. We found that NPD1 is promptly made in response to oxidative stress, seizures and brain ischemia-reperfusion, and in the presence of neurotrophins. NPD1 is neuroprotective in experimental stroke, oxidative-stressed retinal pigment epithelial (RPE) cells, and in human brain cells exposed to amyloid-β peptide in a Parkinson's disease in a dish using MPTP or MiPP+. Thus we envision NPD1 as a protective sentinel, one of the very first defenses activated when cell homeostasis is threatened by neurodegeneration. We provide here recent experimental examples that highlight the specificity and potency of NPD1, spanning beneficial bioactivity during events critical during the initiation and early progression of neurodegeneration:

1) We used epileptogenesis as a model to explore key mechanisms that sustain neuronal network integrity under adverse conditions. Using LC-MS/MS-based mediator lipidomic analysis we found that NPD1 increases during seizures in the hippocampus, and when we administered this docosanoid during pharmacologically induced epileptogenesis it elicited a remarkable attenuation of pathological brain oscillations. This effect reflects attenuation of aberrant neuronal network activities that lead to spontaneous recurrent seizure. We used multi-microelectrode arrays in freely moving mice. Thus, docosanoid-mediated signaling rescues neuronal network disruptions.
2) Since protein misfolding and proteotoxic stress are involved from early stages of neurodegenerative disease, we have explored these events as a possible NPD1 target in cell culture models (human RPE cells and primary neuronal mix cultures). We have studied Ataxin-1 PolyQ and to some extent huntingtin 72 Q. We found that NPD1 decreased phospho-Ser-776 in Ataxin-1. We speculate that in agreement with our previous findings NPD1 may work by increasing PP2A activity. Thus the lipid mediator may counteract PP2A inhibition, allowing the 82Q form to be de-phosphorylated and cleared or relocated into the spliceosome. The fact that Anp32 was proposed to have a stronger interaction with the expanded form rather than with the wild type Ataxin-1 makes this protein an excellent target candidate for NPD1 signaling. Impairment of neuronal circuitry likely also involves overexpression of the normal part of the misfolded protein. Thus in addition to the expansions in the poly-glutamine tract, AXH has an important role in the functionality of Ataxin-1. AXH, a self-folding domain present in Ataxin-1, is responsible for the protein-protein interactions between Ataxin-1 and other transcription factors, such as the capicua homolog CIC protein. The sequestration of the complex partners formed by Ataxin-1 by its inactive counterpart may be involved in the loss of function observed in neurodegeneration. Brother of Ataxin-1 (Boat), another member of the AXH domain-containing protein family, is an example of the proposed loss of function. Boat is an *in vivo* binding partner of Ataxin-1 that is also affected by the malfunction of Ataxin-1 82Q. Thus the expression of AXH alone in our cells resulted in increased apoptosis. Furthermore, it aggravated the cytotoxicity induced by Ataxin-1 82Q. Unlike the sequestration scenario, in which the complexes are formed but are inactive, AXH induces toxicity in this case by increasing disassembly of the complex, thus promoting inactivation of its partners. NPD1 signaling promotes survival by modulating a set of genes that homeostatically control cell fate. NPD1 reversed the toxicity of both AXH and Ataxin-1 82Q in our cells (Calandria *et al*., J Biol Chem 2012).
3) We found that NPD1 is drastically reduced in CA1 areas from Alzheimer's patients (Lukiw *et al*., J Clin

Inv 2005). Therefore we have explored the significance of NPD1 in cellular models that recapitulate part of the Alzheimer's pathology. Human neurons and astrocytes challenged by amyloid-β or by overexpressing APPsw (double Swedish mutation that causes familial forms of the disease) show that NPD1 downregulates amyloidogenic processing of amyloid-β precursor protein, switches off pro-inflammatory gene expression (TNF-α, COX-2 and B-94-TNF-α inducible pro-inflammatory element), and promotes neural cell survival. Moreover, anti-amyloidogenic processing by NPD1 targets α- and β-secretases and PPARγ receptor activation (Zhao *et al*. PLOsOne, 2011). Currently we are also using MALDI/TOF-MS imaging to further unravel the lipidome in specific brain regions. The availability of anti-apoptotic BCL-2 proteins is positively modulated by NPD1, whereas pro-apoptotic BCL-2 proteins are negatively regulated, as is microglial activation. The cell survival cascade and the events that sustain neuronal network homeostatic integrity involve multiple checkpoints and signaling networks. NPD1 regulation targets upstream events of cell survival as well as neuroinflammatory signaling, in turn promoting homeostatic regulation of synaptic and neural circuitry integrity.

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Molecular and ultrastructural alterations in the brain after peripheral stimulation of innate immune system. The role of PARP and CDK5 in regulation of gene expression

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The proper activity of innate immunity plays a critical role in the physiology and pathology of the brain. Induction of neuroinflammation is an integral component of virtually all disorders of the central nervous system (CNS). However, the association between the systemic inflammatory response (SIR) and pathophysiological changes in the brain is not fully understood. In our studies, the effects of SIR on biochemical processes, ultrastructure of brain cells and on cognitive functions were analyzed.

Our data demonstrated that systemic administration of lipopolysaccharide (LPS; ip) evoked molecular and ultrastructural alterations in the brain of adult male mice C57BL6. Rapid increase in the mRNA level of a number of inflammation-related pro-oxidative genes (cyclooxygenase-2, inducible isoform of nitric oxide synthase, 5- and 12-lipoxygenases, NADPH oxygenase) was observed. Free radical-dependent damage of macromolecules evoked the failure of mitochondria and activation of DNA-bound enzyme poly (ADP-ribose) polymerase (PARP). In parallel, we detected enhancement of expression of genes for PARP-1 and several other isoforms of PARP and an increase in PARP activity in the hippocampus of LPS-treated animals. Accumulation of poly(ADP) ribose evoked release of apoptosis inducing factor (AIF) from mitochondria and activation of apoptosis. Additionally, we found an increase in expression of the cyclin dependent kinase 5 (CDK5) activating protein p35. Our results indicated the essential role of PARP and CDK5 in controlling expression of pro-inflammatory proteins in the brain during SIR. Finally, we showed significant changes in the ultrastructure of brain cells, including neurons, astrocytes and microglia, and cognitive impairment after SIR. Moreover, we demonstrated the evident effect of PARPs on the course of SIR-evoked molecular alterations and cognitive impairment. Our data suggest that modulation of the individual isoforms of PARP and CDK5 can create a good opportunity for a neuroprotective strategy in conditions associated with the inflammatory response.

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Targeting mitochondrial dysfunction and ER stress for neuroprotection

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Impaired mitochondrial dynamics and function are considered as a major feature of the underlying neuronal cell death in many neurodegenerative diseases. In neurons, the pro-apoptotic bcl-2 family proteins such as Bid and Bax trigger mitochondrial fragmentation and the detrimental release of death-promoting factors, such as apoptosis inducing factor (AIF). Our previous studies revealed Bid-dependent mitochondrial fission and a direct interaction between dynamin-related protein-1 (Drp1) and Bax in immortalized hippocampal neurons (HT-22) exposed to glutamate. Based on this pivotal role of Bid and Drp-1 in mitochondrial death pathways, we validated the pharmacological inhibition of Bid and Drp1 as therapeutic strategies in models of neurological diseases.

Further, altered calcium homeostasis and increased endoplasmic reticulum (ER) stress may contribute upstream to impaired mitochondrial function in neurodegenerative diseases. Our recent findings demonstrated that small-conductance calcium-activated potassium (SK) channels counteract NMDA receptor-mediated deregulation of intracellular calcium homeostasis and may therefore serve as promising therapeutic targets for a variety of neurological disorders. Further, we demonstrated protective roles of SK channel activation in a model of glutamate toxicity and brefeldin A-induced ER stress using the MTT assay and real time impedance-measurements. Further, the SK-channel modulator CyPPA prevented mitochondrial fragmentation, and preserved mitochondrial membrane potential and mitochondrial maximum respiratory capacity in damaged cells, pointing to a novel role of SK channels for mitoprotection. In fact, we identified functional SK channel expression at the inner mitochondrial membrane as detected by fluorescence confocal microscopy and Western blot analysis of isolated mitochondria and mitoplasts. In summary, we showed that targeting proteins involved in mitochondrial dysfunction may serve as a promising strategy of neuroprotection.

Relationship between mitochondria and neurosteroids: implications for Alzheimer's disease

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Neurodegenerative diseases are common disorders of the nervous system. In fact, they exhibit deregulation of the processes controlling the protection and the survival of the nerve cells. The crucial role of bioenergetic dysfunction is becoming increasingly apparent in a broad range of metabolic and neurodegenerative diseases. Several studies indicate that mitochondria are a convergence point in neurodegenerative mechanisms due to their pivotal role in neuronal cell survival and death.

Furthermore, it has been shown that neurosteroids are involved in neuroprotection. Neurosteroids are neuroactive steroids that are synthesized within the nervous system, independently of peripheral endocrine glands. Enzymatic activities of proteins involved in steroidogenesis were found in many regions of the central and peripheral nervous systems, in neurons as well as in glial cells. The process of neurosteroid biosynthesis is a pivotal mechanism intervening in the protection or viability of nerve cells. Many studies have focused on the endogenous neurosteroid allopregnanolone demonstrating protective effects in different models such as the model of cranial trauma, transection of the spinal cord and AD transgenic mouse brain. So far effects of neurosteroids on mitochondrial functions have been analyzed only for estradiol but not for the other neurosteroids. Thus, we are currently studying the effects of different neurosteroids including allopregnanolone (and its chemical analogues) as well as estradiol on bioenergetics in SH-SY5Y neuroblastoma cells (stably transfected with vector or human wtAPP) to address the current pharmacolog-

ical concept focusing on stabilization/amelioration of mitochondria for prevention of AD. Moreover, we investigated the mitochondrial enzyme ABAD (Aβ binding alcohol dehydrogenase, which is also known under the name of 17β-hydroxysteroid dehydrogenase type 10: 17βHSD10) which exacerbates mitochondrial dysfunction induced by amyloid-beta (Aβ) and used estradiol levels as read-out of ABAD activity. The specific ABAD inhibitor AG18051 normalized Aβ-induced impairment of mitochondrial respiration and prevented down-regulation of ABAD activity.

In total, our research will help to better understand the complex mode of action of neurosteroids and the multimodal interaction between them on bioenergetics and neuroprotection in AD.

MotoRater – novel kinematic evaluation of animal movements

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The TSE MotoRater represents a novel set-up for standardized quantitative and objective kinematic evaluation of animal movements. A mirror system allows the investigation of rodents and the tracking of unlimited numbers of joints in a high-speed video showing the animals from below as well as the left and right site, not only restricted to foot prints.

The unique modular design of the TSE MotoRater allows the investigation of rats and mice during skilled walking on a ladder or beam, over ground walking, wading and swimming.

Objective, sensitive and specific readouts of locomotor impairments, recovery or improvement can be obtained.

Moreover, movements of all relevant body parts, i.e. forelimbs, hindlimbs, trunk and tail, can be assessed and correlated, leading to a complete profile of the animal's motor abilities.

As a consequence, the TSE MotoRater is an improvement in the standardization of behavioral assessment of rodent locomotor skills by replacing traditional scoring methods.

Intracerebroventricular streptozotocin injections as a model of Alzheimer's disease

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Streptozotocin (STZ), a glucosamine-nitrosourea compound derived from soil bacteria and originally developed as an anticancer agent, in 1963, has been found to induce diabetes in experimental animals. Since then systemic application of STZ has become the most frequently studied experimental model of insulin-dependent (type 1) diabetes. The compound is selectively toxic towards insulin-producing pancreatic beta cells, which is explained as the result of its cellular uptake by the low-affinity GLUT-2 glucose transporter protein located in the plasma membrane. Streptozotocin cytotoxicity is mainly due to DNA alkylation which results in cellular necrosis. Besides pancreatic beta-cells, STZ applied systemically also damages other organs expressing GLUT-2, namely the kidneys and liver, whereas the brain is not directly affected. However, intracerebroventricular (icv) STZ injections have been found to chronically decrease cerebral glucose uptake and produce multiple other effects that resemble molecular, pathological and behavioral features of Alzheimer's disease (AD), including for example hyperphosphorylation of tau protein and development of perivascular amyloid deposits. Taking into consideration that glucose hypometabolism is an early and persistent sign of AD, and that Alzheimer's brains present features of impaired insulin signaling, icv STZ injections have been recently exploited as a non-transgenic model of this disease, used also for preclinical testing of pharmacological therapies for Alzheimer's disease. While it has been assumed that STZ applied into cerebral ventricles produces hypometabolic and other effects through desensitizing insulin receptors throughout the brain, the evidence for such a mechanism is rather weak. Moreover, it has been reported that brain neuron-specific insulin receptor knockout mice exhibit substantially increased phosphorylation of tau protein but have neither depression of basal brain glucose metabolism, nor memory alteration. On the other hand, it should be noted that there are some GLUT-2 expressing cells in certain

brain areas, in particular in the hypothalamus. Early data on insulin immunoreactivity showed intense insulin expression in the rodent brain, and the possibility of local production of insulin in the brain has never been conclusively excluded. A mechanistic explanation of the mode of action of streptozotocin administered intracerebroventricularly is a prerequisite for validation of this experimental paradigm as a model of Alzheimer's disease.

Nucleolar stress: new insights into the molecular mechanisms of Parkinson's disease

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Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting dopaminergic neurons of the substantia nigra (SN) and ventral tegmental area (VTA). It is believed that PD is associated with mitochondrial and proteasomal dysfunction, oxidative stress and alteration of cellular defense mechanisms. The inhibition of protein synthesis represents a basic response to cope with stressful conditions. The nucleolus – being a center of ribosomal RNA (rRNA) synthesis – also plays a role as an essential stress sensor maintaining cell homeostasis. However, the possible function of the nucleolus in neurodegenerative processes has not yet been widely explored. We have engineered mouse models based on genetic ablation of the transcription initiation factor IA (TIF-IA) essential for synthesis of rRNA. We applied the conditional inactivation of the gene encoding TIF-IA by the Cre-loxP system to induce selective loss of dopaminergic cells, which was achieved by expressing Cre recombinase under the dopamine transporter (DAT) promoter. Disruption of the nucleoli in dopaminergic neurons resulted in generation of transgenic mice revealing the typical phenotype of PD: preferential degeneration of neurons in SN vs. VTA, striatal depletion of dopamine and typical motor dysfunctions. Moreover, our study indicated that cellular changes associated with nucleolar disruption may recapitulate some events associated with dopaminergic neurodegeneration. These mutant mice represent a valuable tool to gain understanding of the neuronal response to ribosomal stress and underscore a new role for nucleolar activity in neurodegeneration. However, it is well known that PD is associated not only with dopaminergic transmission affecting other neurotransmitter systems, in particular the noradrenergic system. Thus, the further attempt of our study was to determine whether genetically evoked, selective loss of noradrenergic neurons may negatively influence the dopaminergic system, not directly affected by the mutation. The selective ablation of TIF-IA within noradrenergic cells was achieved by expressing Cre recombinase under dopamine beta-hydroxylase (DBH) promoter. Our preliminary data indicate that lack of noradrenergic transmission in these mice may lead to enhanced expression of selected markers associated with neurodegeneration in dopaminergic neurons of SN/VTA.

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Role of toll-like receptors in the function of the neurovascular unit

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The functional integrity of the blood-brain barrier may be affected by systemic inflammations caused by different pathogens. Toll-like receptors (TLRs) play an important role in mediating the systemic effect of pathogens. These receptors recognize a broad range of exogenous and endogenous molecules and initiate inflammatory reactions. Toll-like receptors are mainly distributed on cells of the immune system but there is also growing experimental evidence suggesting TLR expression on different non-immune type cells as well, such as epithelial and endothelial cells. The goal of our study was to investigate the role of Toll-like receptors in the pathophysiology of the blood-brain barrier.

Our results showed that cells of a human brain endothelial cell line (hCMEC/D3) express TLR2, 3, 4

and 6 while primary rat brain endothelial cells express TLR2, 3 and 6 under basal conditions. The mRNA expression of all identified human endothelial Toll-like receptors was induced by oxidative stress caused by DMNQ whereas TNF-alpha upregulated the expression of TLR2 and TLR3. Zymosan, a TLR2/6 agonist, elevated the expression of these two receptors while having no effect on the expression of TLR3 and 4. Moreover, zymosan treatment increased the permeability of the endothelial cell cultures accompanied by a decrease in the expression of occludin and claudin-5 and loss of membrane staining of these two transmembrane components of the tight junctions. U0126, an ERK1/2 kinase inhibitor, was able to prevent the changes of occludin caused by zymosan. Furthermore, we have shown that besides astrocytes pericytes are also able to express different TLRs.

Our results suggest a significant role of the cells of the neurovascular unit in mediation of the CNS effects of different inflammatory processes.

Blood-brain barrier maintenance in epilepsy

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Blood-brain barrier dysfunction seems to be involved in epileptogenesis and in propagation of seizures. Experimental studies have shown that endothelium of the blood-brain barrier is activated after seizures and that leukocytes traffic to the brain. Blocking leukocyte influx or endothelium activation may result in less seizure activity and may break status epilepticus induced by pilocarpine injection in a mouse model of epilepsy. The aim of the present study was to explore the changes in the expression of the markers of blood-brain barrier activation in epileptic patients. As a marker of blood-brain barrier we chose the endothelial adhesion molecule ICAM-1, indicating activation of endothelial cells, and metalloproteinase-9, indicating a dysfunction of the basal lamina.

A group of 50 patients with epilepsy, hospitalized after seizure, was enrolled in the study. The participants had the blood-brain markers checked 3 times – 1 hour after seizure, 24 hours after seizure, and 3 days after seizure – to follow the variations of blood-brain barrier markers in time. The control group was selected from age-matched healthy people and non-epileptic patients. We found that the level of MMP-9 was clearly elevated 1 hour and 24 hours after seizure and then dropped to the control level on the 3rd day. The level of ICAM-1 was also higher than in the control group. This preliminary study showed that the blood-brain barrier was activated in patients after seizures. More investigations are necessary to discover the role and significance of blood-brain barrier dysfunction. Identifying serum biomarkers of brain inflammation may provide valuable tools for prognostic and diagnostic purposes and possibly to recognize those patients who might benefit from anti-inflammatory or immunomodulatory therapies.

Functional compensation of dopaminergic nigrostriatal system degeneration. Role of mitochondrial supercomplex function and assembly

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The first movement disorder signs of Parkinson's disease (PD) are observed after the irreversible loss of at least 70% of dopaminergic neurons in the substantia nigra (SN). The compensatory mechanisms prevent appearance of the symptoms at the early, preclinical stages of the disease. The surviving neurons increase their activity in order to compensate for the degeneration, so their energy demand is probably higher; therefore the functional adaptation of mitochondria is especially interesting in this aspect.

In the model of selective degeneration of the dopaminergic system, followed by spontaneous functional compensation of movement disability, we explored the role of mitochondrial assemblies of dif-

ferent complexes of the oxidative phosphorylation chain, called supercomplexes.

The study was focused on activity and stoichiometry of mitochondrial complexes and their assembly into supercomplexes, which influences their stability and energy production efficacy.

We have identified changes in complex I performance, specific activity and amount within different supercomplexes due to the processes correlating with the compensatory timeline. Those changes depended partially on the numbers of other complexes assembled together with complex I. Especially important is the role of complex IV. We show that differential supercomplexes arrangement can be adjusted to compensate for the neuronal function deficits. The influence of mitochondrial respiratory chain supercomplex assembly on activity of individual complexes will be discussed.

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Is MS an inflammatory or primary degenerative disease?

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Multiple sclerosis (MS) is characterized by multiple areas of inflammation, demyelination and neurodegeneration. Multiple molecular and cellular components mediate neuroinflammation in MS. They involve adhesion molecules, chemokines, cytokines, metalloproteases and the following cells: CD4+ T cells, CD8+ T cells, B cells, microglia and macrophages. Infiltrating Th1 CD4+ T cells secrete proinflammatory cytokines. They stimulate the release of chemokines, expression of adhesion molecules and can be factors that cause damage to the myelin sheath and axons. Chemokines stimulate integrin activation, mediate leukocyte locomotion on endothelial cells and participate in transendothelial migration. CD8+ cells can directly damage axons. B cells are involved in the production of antibodies which can participate in demyelination. B cells can also function as antigen-presenting cells and contribute to T cell activation.

Neuroinflammation is present not only in relapsing remitting MS, but also in the secondary and primary progressive forms of the disease. The association between inflammation consisting of T cells, B cells, plasma cells and macrophages and axonal injury exists in MS patients including the progressive forms of the disease. The above association does not exclude the possibility that neurodegeneration can exist independently from inflammation. Very little inflammation is seen in cortical MS plaques. Anti-inflammatory therapies with different modes of action change the course of MS. Anti-inflammatory and immunomodulatory treatments are beneficial in the early relapsing stage of MS, but these treatments are ineffective in secondary progressive and primary progressive MS.

Baclofen or NNLA suppresses nNOS-IR in α-motoneurons after spinal cord transection

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Spinal cord injury (SCI) may interrupt important neuronal pathways between the supraspinal centers and the spinal cord and very often leads to the loss of neurons providing presynaptic inhibition of α-motoneurons. The loss of presynaptic inhibition and permanent stimulation of inputs from the muscles and tendons into the spinal cord increases the excitability of motoneurons and causes the hyperexcitability of spinal reflexes. Clinical studies have shown that baclofen (a $GABA_B$ agonist), while effective in modulating spasticity, is associated with side-effects and the development of tolerance. We studied whether nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) plays a role in setting the excitability of the α-motoneurons after thoracic

SCI. The animals after Th9 transection were treated with 1) repeated 6 days administration of baclofen (30 mg/b.w., p.o.), starting the 1st week and then the 4th week after SCI, 2) baclofen (3 µg/2 × per day/i.t.) applied 3 days from the 7th day after transection, 3) NNLA (nNOS inhibitor), applied the first 3 days after injury (20 mg/kg per day, i.m), 4) NNLA/baclofen, or with 5) NNLA (60 mg/kg/day, single dose) applied the 10th day after transection. Strong nNOS-immunoreactivity (IR) was seen in spinal motoneurons from 1 to 8 weeks after SCI. Repeated treatment with $GABA_B$ agonist reduced nNOS-IR to control values and improved the BBB-locomotor rating scale 3-6 weeks postoperatively. In addition our results indicate that baclofen therapy is more effective than combined NNLA/baclofen treatment in both the inhibition of nNOS-IR in motoneurons and the reduction of pain-related symptoms. NNLA applied for the first 3 days after injury, or NNLA/baclofen therapy caused a strong decrease of nNOS-IR in dorsal horn neurons but a subtle change was found in motoneurons. The application of NNLA on the 10th day after SCI reduced α-motoneuronal nNOS expression and suppressed symptoms of increased reflex activity. Changes of nNOS expression in lumbar motoneurons after SCI and subsequent treatment clearly indicate an important role of NO in initiation and in maintenance of muscle spasticity.

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MicroRNA (miRNA): sequence and stability, viroid-like properties, and disease transmission in the central nervous system (CNS)

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MicroRNAs (miRNAs) are ~21-to-24-nucleotide small non-coding RNAs (sncRNAs) involved in the post-transcriptional regulation of eukaryotic gene expression. While our perceptions on the neurobiological mechanism and relevance of miRNA signaling continue to evolve, it is now widely accepted that the primary mode of miRNA action is to recognize and bind to specific complementary ribonucleotide sequences in the 3' prime un-translated region (3'-UTR) of target messenger RNAs (mRNAs), and in doing so, down-regulate their expression. Although miRNAs are considered to be vitally important epigenetic regulators of gene expression in human development, aging and disease, it is not often appreciated that these single stranded RNAs (ssRNAs): (i) are very highly selected in their ribonucleic acid (RNA) sequence and cell and tissue specificity; (ii) represent a genetic signaling system that is evolutionarily ancient (for example the miRNA-854-heterogeneous nuclear RNA [hnRNA] transcription regulatory system is conserved since the plant and animal kingdoms diverged about 1.5×10^9 years ago); (iii) are the smallest yet identified RNA carriers of genetic regulatory information, possessing viroid-like properties; (iv) are the most abundant nucleic acids contained in human extracellular fluid (ECF) and cerebrospinal fluid (CSF); and (v) as abundant constituents of the ECF, CSF and blood serum, may spread both homeostatic and pathological signaling amongst neighboring cells, tissues and perhaps even between individual organisms or species. This presentation will examine some of the more salient aspects on the structure, function and mechanism of these fascinating ssRNAs with specific emphasis on human central nervous system (CNS) disease, and relevance to Alzheimer's disease (AD) and prion disease neurobiology wherever possible.

Astrocytic NMDA receptors in the pathogenesis of hepatic encephalopathy

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Studies of the last decade have established that not only neurons but also astrocytes are in possession of a functional NMDA receptor (NMDAr); however, the role of the latter in brain physiology or pathology remains elusive. Hepatic encephalopathy (HE) is a neuropsychiatric disorder related to hyperammonemia and increased extrasynaptic accumula-

tion of glutamate (Glu). Brain edema resulting from astrocytic swelling (AS) is a major manifestation of acute HE. Previous *in vitro* studies have provided evidence that AS evoked by ammonia in brain slices or cultured astrocytes can be attenuated by NMDAr antagonists but stopped short of identifying specific molecular targets for this response. This presentation summarizes our own recent data which link ammonia-, or HE-induced changes in the expression/function of crucial astrocytic proteins to astrocytic NMDAr. Kir4.1 is the astrocytic inward rectifying potassium channel crucial for the maintenance of brain ion and water homeostasis and down-regulation of Kir4.1 is a pathogenic event in HE. Decrease of Kir4.1 mRNA expression in the cerebral cortex of HE rats was attenuated by administration of an NMDAr antagonist, memantine. Treatment of cultured rat cortical astrocytes with Glu or NMDA decreased the expression of Kir4.1 and the decrease was ameliorated in cells co-incubated with the NMDAr antagonists MK-801 and AP-5. Changes in Glu-ergic neurotransmission are the primary manifestation of neuronal dysfunction in HE and the endogenous NMDAr antagonist kynurenic acid (KYNA) is the only known astroglia-derived modulator of Glu-ergic tone. Kynurenine aminotransferase II (KAT-II), the astrocytic enzyme catalyzing KYNA synthesis, is increased in HE and in cultured cerebral cortical astrocytes treated with ammonia, and KAT-II expression was found to be increased by NMDAr antagonists in rat cerebral cortex and in astrocytes in culture. The results suggest that stimulation of KAT-II expression in HE-affected brain may be associated with partial inactivation of astrocytic NMDAr by excess of Glu. Taken together, the results underscore the role of excessive Glu acting at the astrocytic NMDAr as a partner of ammonia in inducing glial impairment involved in HE.

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Beneficial effects of dietary EGCG and voluntary exercise on behavior in an Alzheimer's disease mouse model

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Alzheimer's disease (AD) is one of the most debilitating age-related disorders and is the sixth leading cause of death, with an estimated global prevalence of 30 million, and more than 5 million currently in the United States. Preventing or postponing the onset of AD, as well as delaying or slowing its progression, would lead to an improvement of health status and quality of life for the elderly. AD is a progressive, age-dependent neurodegenerative disorder affecting specific brain regions that control memory and cognitive functions. AD is characterized by the presence of amyloid plaques, and neurofibrillary tangles in these regions. Other features commonly seen include oxidative damage and inflammation. Since the neuropathology is likely to occur at least decades prior to the emergence of clinical symptoms, prevention can be effective. Studies to explore the influence of psychological fitness, physical fitness, diet and environment to ameliorate the progression of AD are emerging. Epidemiological studies suggest that exercise and dietary antioxidants are beneficial in reducing AD risk. To date, botanical flavonoids are most consistently associated with prevention of age-related diseases. Research from our laboratory has shown that both EGCG from green tea and voluntary exercise are able to improve learning and memory performance in an AD animal model. The TgCRND8 mouse model of AD displays significant cerebral amyloid-beta plaque deposition as well as high levels of Aβ peptide by 3 months of age. These mice also show altered behavioral activity patterns, and spatial learning deficits. Our studies have found age-dependent changes in cognitive and non-cognitive behaviors in these mice, and demonstrated that EGCG/exercise prevented or reversed some of these changes. These results, together with epidemiological and clinical studies in humans, suggest that dietary polyphenols and exercise may have beneficial effects on brain health and slow the progression of AD.

Pharmacogenetics of stroke treatment

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Botanical polyphenols as potential therapeutic agents for mitigating oxidative/nitrosative and inflammatory responses in microglial cells

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Microglial cells represent a unique class of immune cells in the central nervous system and are known to play important multi-functional roles in interacting with neurons and other glial cells. Activation of microglial cells has been implicated in a number of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and stroke. Many studies, including those in our laboratory, have focused on elucidating the mechanisms responsible for the oxidative/nitrosative and inflammatory responses during microglial activation. Immortalized microglial cells (BV-2 from mouse and HAPI cells from rat) actively respond to bacterial lipopolysaccharides (LPS) and pro-inflammatory cytokines such as interferon gamma (IFNgamma) by releasing TNFalpha and IL-1beta, as well as products of reactive oxygen and nitrogen species (ROS and RNS). Besides the canonical transcriptional pathways associated with NF-kappaB and JAK-STAT, LPS and IFNgamma can also activate kinases in the MAPK family. Our recent studies unveiled the ability for IFNgamma to stimulate pERK1/2, which in turn further activates cytoplasmic reactions for the production of ROS from NADPH oxidase, NO from iNOS, phosphorylation of STAT1alpha, activation of cytosolic phospholipase A2, and production of filopodia. Many botanical phenolic compounds, including epigallocatechin-gallate (EGCG) from green tea, honokiol from Magnolia bark, and quercetin from berries, have been shown to inhibit IFNgamma-induced ERK1/2 phosphorylation, as well as the oxidative/nitrosative and inflammatory responses in microglial cells. In an *in vivo* study, mice given a diet supplemented with elderberries (rich in anthocyanins) could protect the brain against cerebral ischemic damage through suppressing p-ERK1/2 and microglial activation. Taken together, these studies unveil the role for ERK1/2 in mediating oxidative/nitrosative and inflammatory responses in microglial cells, and provide new insights for the therapeutic potential of botanical polyphenols against neurodegenerative diseases.

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Mitochondrial acetyl-CoA, signal for death and survival for cholinergic neurons. Locus minoris resistentiae in Alzheimer's pathology?

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Intramitochondrial decarboxylation of glucose-derived pyruvate by pyruvate dehydrogenase complex (PDHC) is a principal source of acetyl-CoA, for energy production in all types of brain cells. Deficits of PDHC and energy metabolism in the brain were found to correlate with losses of cholinergic markers and cognitive functions, in the course of Alzheimer's disease (AD), and other cholinergic encephalopathies. This indicates that preferential susceptibility of cholinergic neurons to degeneration might result from relative shortages of acetyl-CoA for energy production due to its utilization for acetylcholine synthesis. In contrast, noncholinergic cells/neurons should be less susceptible to neurodegeneration. This thesis has been verified in direct inhibition as well as acute and chronic cytotoxicity studies using nondifferentiated (NC) and differentiated (DC) cholinergic SN56 cell lines, origi-

nating from mouse septum. Most common AD neurotoxins such as Zn, Al, NO, at pathophysiologically relevant concentrations, but not amyloid-β, caused direct inhibition of PDHC, ketoglutarate dehydrogenase and aconitase activities, similar in NC or DC homogenates. On the other hand, the same compounds, including amyloid-β, when applied to cell culture media caused preferential loss of DC over NC viability, accompanied by differential respective decreases in intramitochondrial levels of acetyl-CoA, both in short- and long-term neurotoxicity studies. These neurotoxin-evoked alterations in mitochondrial enzyme activities resembled those seen in pathology affected areas of human AD cortex. Neurotoxic effects could be prevented in part by lipoic acid, acetyl-L-carnitine or TPP, which improved both acetyl-CoA and ATP status in the DC mitochondria. Secondary shortages of acetyl-CoA in the cytoplasmic compartment down-regulated ACh metabolism. Hence, survival of cholinergic neurons under exposure to AD neurotoxins correlated with intramitochondrial level of acetyl-CoA. On the other hand, disturbances in acetylcholine metabolism in these pathologic conditions correlated with levels of this precursor in the cytoplasmic compartment. For this reason therapeutic strategies aiming to preserve proper rates of acetyl-CoA synthesis in the encephalopathic brain should attenuate high susceptibility of cholinergic neurons to AD-like neurotoxic conditions.

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HIV and amyloid interplay 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 infection from an acute to a chronic disorder, there is a sharp increase in infected patients 50 years old and older. This increase in age of the HIV infected population constitutes a new challenge in the HIV epidemic in affluent countries. Importantly, older HIV infected patients are more susceptible to neurocognitive impairments associated with the disease. HIV infected brains are characterized by increased deposition of amyloid beta (Aβ) in the perivascular space, indicating the importance of brain microvessels in amyloid accumulation. Our research focused on the mechanisms of HIV-1 interaction with Aβ at the blood-brain barrier level. Exposure to HIV resulted in accumulation of Aβ in brain endothelial cells, with prominent accumulation in the nucleus. We demonstrated that these effects are dependent on functional caveolae and can be prevented by inhibition of Ras and the receptor for advanced glycation end products (RAGE). In addition, HIV-induced nuclear entry of Aβ involves activation of the dynamin-dependent EEA1 and TGF-β/Smad signaling. Using transgenic mice that express a chimeric mouse/human amyloid precursor protein and a mutant human presenilin 1, we next demonstrated that cerebrovascular toxicity of HIV-specific protein Tat is enhanced in mice with amyloid deposits in the brain. Indeed, exposure to Tat increased permeability across cerebral capillaries, enhanced disruption of ZO-1 tight junction protein, and elevated brain expression of MMP-9 in transgenic mice as compared to age-matched littermate controls. These changes were associated with increased leukocyte attachment and their transcapillary migration.

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Extracellular α-synuclein and its role in nitrosative stress, mitochondrial dysfunction and neurodegeneration

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Alpha-synuclein (ASN) oligomerization and accumulation are the key molecular processes involved in the etiopathology of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and other synucleinopathies. Mutations in the gene encoding ASN were shown to be responsible for causing familial form of PD. Alterations of ASN expres-

sion and impairment of its degradation can lead to the formation of intracellular deposits of this protein known as Lewy bodies. Recent studies, including our own, indicated that oxidative/nitrosative modifications of ASN are responsible for its oligomerization and release to the extracellular space by an exocytotic mechanism. Release of ASN into the extracellular space might contribute to the spread of ASN oligomers between neurons. This event can accelerate and propagate alteration of ASN molecules in neighboring cells that may cause progression of neurodegeneration in the brain. Studying the molecular mechanisms responsible for extracellular ASN toxicity, it was shown that this protein induces the Ca^{2+} influx indirectly via activation of VOCC as well as NMDA receptor or directly by forming Ca^{2+} channels in the plasma membrane. Enhancement of cytoplasmic Ca^{2+} concentration leads to excessive liberation of nitric oxide (NO). Subsequently, NO is responsible for ASN-evoked disturbances of dopaminergic neurotransmission and for acceleration of oxidative stress, mitochondria failure, caspase/calpain activation and apoptotic cell death. The latest results showed the significant role of ASN in Gsk3-beta related Tau hyperphosphorylation. Moreover, it was demonstrated that this protein increases the release of Abeta peptides, and through interaction with them it causes irreversible mitochondrial dysfunction and neurodegeneration.

In summary, the understanding of the molecular mechanisms of ASN cytotoxicity could be useful in developing a novel strategy for the treatment of neurodegenerative diseases.

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Perturbation of the cholesterol transport proteins caveolin-1 and apoE by amyloid beta-protein in astrocytes

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Cholesterol has attracted much attention in the Alzheimer disease (AD) research community. It has been suggested that high serum cholesterol levels are a risk factor for AD. Some reports, mostly retrospective epidemiological studies, have observed decreased prevalence of AD in patients taking the cholesterol-lowering drugs statins. The strongest evidence linking cholesterol as a causative factor in AD comes from experimental studies showing that adding/reducing cholesterol alters amyloid precursor protein (APP) processing and amyloid beta-protein (Aβ) levels. However, there are problems with the cholesterol-AD hypothesis. Data on cholesterol levels in serum and brain of AD patients do not support cholesterol being a causative factor in AD. Prospective studies on statins and AD have largely failed to show efficacy. Even the experimental data are challenging, given that it is well established that modification of cholesterol levels has effects on multiple proteins, not only APP. An alternative hypothesis directly linking cholesterol to AD is that cholesterol homeostasis is altered by Aβ. Specifically, we propose that Aβ disrupts cholesterol trafficking and regulation and function of the cholesterol transport proteins caveolin-1 and apoE. Data will be presented showing that Aβ causes a redistribution of cholesterol within the Golgi complex and drives movement of cholesterol from the plasma membrane to the Golgi complex, which is caused by caveolin-1. At the same time, Aβ stimulates transcription of apoE occurring via the β_2-adrenergic receptor and increases abundance of apoE protein levels, a key cholesterol transporter in the brain. Consequences of changes in caveolin-1 and apoE may include (but are not limited to) reduction of cholesterol asymmetry in membranes, impaired caveolae and Golgi functions, and accumulation of apoE in the ER, inducing the unfolded protein response.

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Different vital stains used to track human mesenchymal stem cells for their detection *in vivo*: limitations and challenges

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Recently, the therapeutic properties of mesenchymal stem cells (MSC) have been studied extensively and it is now clear that these cells stimulate repair and regeneration of damaged tissue. In order to gain insights into MSC migration, tissue localization, and the level of engraftment, cells require labeling and subsequent tracking. However, before *in vivo* use it is imperative to determine that the incorporation of dyes into MSC are not inducing any cellular changes that could affect their function. In this study, we examined the effect of cell labeling on the *in vitro* functionality of human bone marrow-derived mesenchymal stem cells (hBM-MSC). In particular, we investigated the effect of CMFDA and GFP, and iron (SPIO) labeling on hBM-MSC viability, phenotype, proliferation rate and differentiation potential. Materials and Methods: hBM-MSC (Lonza) cultured in MSCBM medium were labeled with CMFDA, transfected with mRNA GFP, or tagged with SPIO nanoparticles. Vital observation of hBM-MSC morphology and intracellular persistence of different stains was performed for 2 weeks at various time points. Proliferation potential of hBM-MSC was assessed by CCK-8 assay. The expression of different cell markers was defined by immunocytochemistry. Results: CMFDA, GFP or SPIO labeling did not affect the morphology and baseline expression of CD-90, CD44, SSEA-4, CXCR-4, or VLA-4 molecules of hBM-MSC. No influence of any vital stains on hBM-MSC adipogenic differentiation potential was detected, whereas in comparison with unlabelled cells CMFDA and GFP, but not SPIO, tracking decreased the proliferation rate of hBM-MSC. Moreover, CMFDA and GFP are unsuitable for long-term detection of hBM-MSC. The intracellular persistence of different agents was demonstrated for 7 days after CMFDA labeling, up to 14 days after GFP transfection but more than 21 days after SPIO tracking. Conclusions: SPIO particles provided long lasting labeling of hBM-MSC and did not modify their viability, phenotype or function. This indicates that they are biocompatible with hBM-MSC and they would be suitable labels for tracking these cells to evaluate cell fate and distribution after transplantation using noninvasive MRI.

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Ventilatory effects of acute intermittent hypoxia in conscious rats following striatal lesion caused by 6-hydroxydopamine

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Severe intermittent hypoxia elicits attenuated reflex hyperventilation in conscious patients suffering from Parkinson's disease (PD). In an animal model of PD, anesthetized, paralyzed and mechanically ventilated rats exposed to acute intermittent hypoxia demonstrate an enhanced response of the neural upper airways output and a similar response in the phrenic motor output compared to sham operated animals. Distinct respiratory effects of intermittent hypoxia in spontaneously breathing humans and artificially ventilated animals might suggest a muscular dysfunction in PD patients. The purpose of the present study was to determine whether in conscious animals following striatal lesion produced by 6-hydroxydopamine (6-OHDA) the hypoxic ventilatory response to acute intermittent hypoxia is altered and whether this effect may depend on severity of the hypoxic stimulus. Adult rats received a microinjection of 6-OHDA or vehicle into the right striatum stereotaxically. Before and 14 days after sham or 6-OHDA striatal injection conscious rats were placed in a whole body plethysmograph. Ventilatory parameters (tidal volume, frequency of breathing and minute ventilation) were measured at baseline and during two trials of intermittent hypoxia with two levels of oxygen. Each trial consisted of five 3-min hypoxic episodes (inspired O_2 fraction, FI_{O_2}, = 0.11 or 0.09)

interspersed with 5-min returns to baseline breathing with air. The exposure to intermittent hypoxia of both intensities elicited augmentation of the hypoxic ventilatory response in 6-OHDA treated animals compared to the sham group. This effect became statistically significant with more severe intermittent hypoxia. Ventilatory enhancement during hypoxia was attributable to increased tidal volume while changes in frequency of breathing in both groups were comparable, suggesting a lack of respiratory motor impairment in this PD model. Since dopamine has an inhibitory effect on breathing an enhancement of the hypoxic ventilatory response following 6-OHDA striatal lesion may manifest a reduced inhibition. The discrepancy between hypoxic respiratory responses to intermittent hypoxia of PD patients and conscious animals with 6-OHDA striatal lesions suggests that respiratory disturbances in PD may not be linked with depletion of dopamine in the nigrostriatal system but result from other, non-dopaminergic neurotransmission and nervous structures.

Mutations in *C9ORF72*, *PGRN* and *MAPT* genes associated with frontotemporal lobar dementia in the Polish population

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Frontotemporal lobar degeneration (frontotemporal dementia, FTD), the second most common form of presenile dementia, is a clinically, pathologically and genetically heterogeneous disorder. Up to 40% of FTD patients report family members with FTD, supporting the important contribution of genetic factors to these diseases.

The aim of the study was to establish genetic backgrounds and frequencies of mutation causing FTD in the Polish population.

The Polish patient sample consisted of 120 FTD patients (mean age of onset 63.6 ± 7.8 years). All *PGRN* and *MAPT* exons with flanking intronic regions were sequenced. Analysis of the hexanucleotide repeat expansion in *C9ORF72* was performed.

The sequencing of *PGRN* and *MAPT* in 120 FTD samples allowed identification of six different non-synonymous changes. From these latter, three are new changes, and six are previously reported as pathogenic. All coding non-synonymous variants were observed in familial cases of FTD. The frequencies of mutations in examined genes are similar (ca. 4%), and are responsible for 12% of FTD cases.

We identified mutations in 14 probands. The frequencies of mutations in individual genes are consistent with recent estimates in the European population. In conclusion, this study provides convincing evidence that mutations in *PGRN*, *MAPT* and *C9ORF72* are an important cause of frontotemporal dementia, but there are probably other genetic factors causing the disease.

Sphingosine kinase 1 and its role in extracellular amyloid β peptides cytotoxicity

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Sphingosine kinases (SphKs) are conserved lipid kinases, which are responsible for biosynthesis of sphingosine-1-phosphate (S1P). There are two forms of these enzymes, SphK1 and SphK2, displaying 80% homology. SphK1 is found in the cytosol and migrates to the plasma membrane upon activation by oxidative/genotoxic stress. SphK2 is mainly localized in the nucleus. SphK1 activity regulates cell growth and proliferation and exerts anti-apoptotic effects, while the action of SphK2 has been implicated in pro-apoptotic processes. These enzymes are responsible for maintaining the sphingolipid biostat and the cell's fate. Sphingolipids are significant constituents of lipid rafts where amyloid precursor protein (APP) is degraded and amyloid beta (Aβ) peptides are liberated. There arose the question through what kind of mechanism extracellular Aβ peptides

may affect SphK1, a key enzyme in S1P synthesis and cell survival.

In this study rat pheochromocytoma PC12 cells were subjected to Alzheimer's $A\beta_{1-42}$ oligomers, and the experiments were performed using biochemical, spectrometric and RT-PCR methods.

Our data indicated that short exposure to exogenous Aβ peptides induced a decrease in Sphk1 gene expression. However, the prolonged 24-hour exposure to $A\beta_{1-42}$ resulted in SphK1 up-regulation. In these conditions, $A\beta_{1-42}$ increased the level of free radicals and the content of mitochondrial apoptosis inducing factor (AIF). This flavoprotein with NADH oxidase/reductase activity and antioxidant properties may protect mitochondria function against $A\beta_{1-42}$ toxicity. However, these changes are not able to protect cells against death. Aβ evoked oxidative stress decreases the Gsk-3β level and activity that may affect glycogen, protein synthesis and cell viability. Our results demonstrated that $A\beta_{1-42}$ evoked death of 32% of the cell population. A similar effect was observed by inhibition of SphK1. Inhibitor of SphK1 (SKI II) had no effect on $A\beta_{1-42}$-evoked Gsk-3β alteration or on cell viability, suggesting that modification of the sphingolipid biostat may play a crucial role in $A\beta_{1-42}$-evoked cell death.

Our data suggest that the free radical cascade mediated by $A\beta_{1-42}$ induced SphK alteration. These changes may be the early and crucial event responsible for the activation of molecular processes leading to cell death.

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Increased expression of HAP1, CacyBP/SIP, CALB2, PSEN2 and CIB1 in the striatum of a transgenic mouse model of Huntington's disease

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Huntington's disease (HD) is a neurodegenerative disease caused by the expansion of a polyglutamine stretch in the huntingtin (HTT) protein and characterized by dysregulated calcium homeostasis. We investigated whether these disturbances are correlated with changes in the mRNA level of the genes that encode proteins involved in calcium homeostasis and signaling (i.e., the calciosome). Using custom-made TaqMan low-density arrays containing probes for 96 genes, we quantified mRNA in the striatum in YAC128 mice, a model of HD, and wildtype mice. HTT mutation caused the increased expression of some components of the calcium signalosome, including calretinin (CALB2), presenilin 2 (PSEN2), and calmyrin 1 (CIB1), and the increased expression of genes indirectly involved in calcium homeostasis, such as huntingtin-associated protein 1 (HAP1) and calcyclin-binding protein (CacyBP/SIP). We confirmed these mRNA data by western blotting, showing that in most cases increases at the protein level are also observed. To verify these findings in a different model, we used PC12 cells with inducible expression of mutated full-length HTT. Using single-cell imaging with Fura-2AM, we found that store-operated Ca^{2+} entry, but not endoplasmic reticulum store content, was changed as a result of the expression of mutant HTT. Statistically significant downregulation of the Orai calcium channel subunit 2, calmodulin 3, and septin 4 was detected in these cells. Our data indicate that the dysregulation of calcium homeostasis correlates with changes in the gene expression of members of the calciosome. These changes, however, differed in the two models of HD used in this study. Our results indicate that each HD mod-

el exhibits distinct features that may only partially resemble the human disease.

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Poly(ADP-ribose) polymerase-1 inhibition protects neuronal cells against ceramide toxicity

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Ceramides play an important role in regulation of the fluidity and structure of the lipid bilayer. Ceramides are recently recognized as very important second messengers involved in regulation of cell proliferation, differentiation, growth arrest and apoptosis. Our previous data indicated that ceramide increased the free radical level in a concentration-dependent manner. Generation of reactive oxygen species causes DNA damage that activates poly(ADP-ribose) polymerase-1 (PARP-1). The aim of this study was to examine the role of PARP-1 in ROS signaling and cell death induced by C2-ceramide in a human *neuroblastoma* cell line (SH-SY5Y). Our study indicated that ceramide induced PARP-1 activation and accumulation of poly(ADP-ribose) PAR, a signaling molecule involved in mitochondria-nucleus cross-talk and in alteration of mitochondria integrity. The ceramide treatment significantly decreased the level of apoptosis inducing factor (AIF) in mitochondria. The PARP-1 inhibitor (PJ-34) prevented AIF release from mitochondria and protected cells against death. Moreover, it was revealed that ceramide decreased the anti-apoptotic Bcl-2 mRNA level and up-regulated the pro-apoptotic Bax and Hrk gene expression. PARP-1 inhibition significantly enhanced the Bcl-2 mRNA/protein level and decreased the Hrk mRNA level. PJ-34 had no effect on Bax gene expression. However, it was also observed that C2-ceramide induced dephosphorylation of pro-apoptotic Bad protein on Ser136, which was reversed by PARP-1 inhibition. In summary, our data demonstrate that neuronal cell death evoked by ceramide is regulated by PARP/PAR/AIF. PARP-1 inhibitor through enhancement of anti-apoptotic Bcl-2 protected neuronal cells against death evoked by ceramide.

Neuroprotective potential of group III mGlu receptor agonist ACPT-1 in ischemic neurodegeneration: *in vitro* studies

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Ischemic stroke is the second most common cause of death worldwide. Although a large number of substances have been studied and found to be neuroprotective in animal stroke models, the results of clinical trials have been unsuccessful. Our earlier studies showed that the group III mGlu receptor agonist (1*S*, 3*R*,4*S*)-1-aminocyclopentane-1,2,4-tricarboxylic acid (ACPT-1) was neuroprotective against kainate-induced excitotoxicity *in vitro* and *in vivo*. In the present study we investigated the protective potential of ACPT-1 in an ischemic *in vitro* model (oxygen-glucose deprivation; OGD). In the *in vitro* experiment, mouse primary cortical neuronal cultures were exposed to OGD for 3 hours, which evoked toxic effects. ACPT-1, at concentration of 1, 10, 100 or 200 μM, was applied in two ways: twice, before the start of OGD and just after the end of OGD, or once, immediately or 30 min after the termination of OGD. We evaluated the effects of ACPT-1 on the ischemia-induced LDH release and MTT reduction 24 h after the end of OGD. In addition, some cultures were stained with propidium iodide for identification of necrotic cells. It was found that a double application of ACPT-1 significantly decreased ischemic-induced LDH release and increased cell viability by 25-56%. The neuroprotective effects of ACPT-1 were reversed by (*RS*)-alpha-cyclopropyl-4-phosphonophenyl glycine (CPPG), the group III mGluR antagonist. Of particular importance is the finding that ACPT-1 (100 or 200 μM) given only once 30 min after OGD also significantly decreased ischemic neurodegeneration by 31-39%. Those data were confirmed by the propidium iodide staining of degenerated cells. We also observed significant induction of calpains after OGD,

evidenced by an increase in spectrin alpha II 145 kDa cleavage product, and that effect was diminished by double application of ACPT-1 at concentrations of 100 or 200 μM. In conclusion, our results indicate that ACPT-1 may be neuroprotective against ischemic neuronal damage.

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The protective effect of Selol against sodium nitroprusside-induced PC12 cell death

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An imbalance between oxidative stress and antioxidative defense has been implicated in the pathophysiology of neurodegenerative diseases, leading to functional and structural alterations of neurons. Selenium, an essential micronutrient required for cellular antioxidant systems, was previously shown to protect neurons against oxidative stress and degeneration in Alzheimer's and Parkinson's disease. However, higher doses of inorganic Se compounds could be harmful for neuronal cells. Selol is a new semi-synthetic organic mixture of selenotriglycerides with reduced cellular toxicity. In this study we investigated the *in vitro* efficacy of the supplementation of Selol in cytoprotection against sodium nitroprusside (SNP)-induced oxidative stress. PC12 cell cultures were exposed to 0.5 mM SNP and the concentration dependent effect of Selol (5-50 μM) was evaluated. The cells were preincubated with Selol for different times (0 h, 1 h or 2 h) before exposure to SNP for 24 h. The effect of Selol was compared with sodium selenite (5 μM) – an inorganic donor of Se. The study was carried out using spectrophotometric and spectrofluorometric methods and fluorescent microscopy. Our data indicated that 24 h exposure to SNP resulted in mixed apoptotic and necrotic cell death, as well as the significant formation of free radicals. Selol supplementation prevented SNP-induced cytotoxicity in a concentration dependent manner and attenuated the elevation of oxidative stress. In contrast, sodium selenite had no protective effect in cells treated with SNP. The antioxidative effect of Selol was mainly mediated by the enhancement of activity of selenoenzymes: glutathione peroxidase and S-transferase. Taken together, these studies provide direct evidence that Selol supplementation can stimulate endogenous antioxidant systems and protect neuronal cells from oxidative stress. Selol could be considered as a solid basis for the improvement of neurodegenerative disorders therapy.

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Mutations of PARK genes and alpha-synuclein, and Parkin concentrations in Parkinson's disease

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Parkinson's disease (PD) is a chronic and progressive neurological disorder characterized by resting tremor, rigidity, and bradykinesia, affecting at least 2% of individuals above the age of 65 years. The causes of PD include both environmental and genetic factors. It is known that the genetic background of PD is in mutations of a number of pathogenic PARK genes, e.g. *SNCA*, *PRKN*, *UCHL1*, *DJ-1*, *PINK1*, *ATP13A2*, *LRRK2*. It seems that in PD protein phenotypes of these genes (e.g. alpha-synuclein, ASN; Parkin) affect the degenerative process. It has been shown that the configuration of increased plasma level of ASN and decreased Parkin concentration was associated with earlier onset of PD.

The aim of the study was to analyze ASN and Parkin concentrations in PD patients and controls with/without PARK gene mutations.

The studies were performed on 32 patients with PD, including 18 women and 14 men aged 35-82 years. The control groups included 24 individuals, 20 women and 4 men aged 40-69 years. The mutations of the *PRKN* gene and NACP-Rep1 promoter region of *SNCA* were investigated using PCR and DNA sequencing, and capillary electrophoresis. The ASN and Parkin concentrations in the plasma were determined by ELISA.

In PD patients *PRKN* mutations (in exons 4, 8, 11) were more than four times more frequent as compared to controls. Moreover, in PD patients genotypes +2/+2 and +2/+3 of the promoter region of the *SNCA* gene occurred more frequently than in controls. Patients with PD showed higher concentration of ASN while a higher Parkin level was observed in controls. In PD patients, the highest concentration of ASN and Parkin occurred in the first two stages of disease progress (Hoehn and Yahr scale) and in the first ten years of the disease. However, only a PD patient with a mutation in exon 11 of the *PRKN* gene showed presence of Parkin without ASN after ten years of disease duration.

Analysis of the variations of the PARK genes as well as plasma levels of ASN and Parkin may be an additional diagnostic factor for PD.

Association study of the 2-bp deletion polymorphism in exon 6 of the *CHRFAM7A* gene with idiopathic generalized epilepsy

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There is evidence of linkage between the 15q13–q14 locus, containing the gene encoding the α7 subunit (*CHRNA7*) of the neuronal nicotinic acetylcholine receptor (nAChR) and its partially duplicated isoform (*CHRFAM7A*), and epilepsy. Additionally, a 2-bp deletion polymorphism (c.497-498delTG; rs67158670) in *CHRFAM7A*, resulting in a frame shift and truncation of the protein product, is associated with some neurological diseases.

This study was designed to explore the possibility of an association of the c.497-498delTG polymorphism of *CHRFAM7A* with idiopathic generalized epilepsies (IGEs) in Polish children and young patients.

The study included 197 IGE patients and 258 unrelated healthy individuals. The frequency of the *CHRFAM7A* c.497-498delTG polymorphism was determined in each group using heteroduplex analysis. An association between the c.497-498delTG polymorphism of *CHRFAM7A* and IGE was evidenced. It was demonstrated that the frequency of the *CHRFAM7A* 2-bp deletion carriers was significantly lower in the IGE patients than in the control group. The observed frequency of 2-bp deletion carriers was high in IGE subjects (64%) but significantly higher in control subjects (76%). Carriers of at least one copy of the -2bp allele had halved the risk of IGE susceptibility (delTG/delTG and delTG/wild-type versus wild-type/wild-type: OR = 0.55; 95% CI = 0.365-0.827; $p = 0.004$). Moreover, it has been demonstrated that this polymorphic variant is associated with the c.524-12_524-11insGTT variation (rs10649395) in intron 7 of *CHRFAM7A*. Our study substantiates the involvement of the α7 subunit of nAChR in the pathophysiology of IGEs and indicates that the *CHRFAM7A* c.497-498TG deletion or a nearby polymorphism may play a role in the pathogenesis of IGE. Further work should concentrate on ascertaining the exact mechanism of this polymorphism's effect and its relationship with IGE.

Neural differentiation and pluripotency signature of neonatal and adult human MSCs

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Mesenchymal stem cells (MSCs) possess a large paracrine secretion and multi-lineage-differentiation

capacity which helps maintain tissue homeostatic and regenerative potential. Therefore MSCs have already entered the clinical arena; however, their therapeutic effect varies in dependence on the tissue of cell origin, age of cell donors and numerous other but still undefined factors. Looking for determinants of this variability we isolated MSCs from neonatal Wharton jelly (WJ-MSC) or adult bone marrow (aBM-MSC) and compared them in terms of cell phenotypes, optimal growth rate and differentiation. Special attention was paid to expression of cell pluripotency markers and their positive correlation with proliferation and capacity for neural differentiation. We found that both isolated cell types exhibit typical mesenchymal markers (CD73, CD90, CD105, CD166) and multilineage differentiation abilities but with sharp quantitative differences between them. WJ-MSC appeared to be less prone to adipogenesis than to osteogenic and especially neural differentiation. Also only WJ-MSC revealed spontaneous induction of markers typical for early and late stages of neural lineage: Nestin, NF200, GFAP. In addition WJ-MSCs proliferate much faster during increasing numbers of passages than aBM-MSC cultures. Moreover, when WJ-MSCs were grown under 5%O_2 low oxygen conditions, significant enhancement of cell proliferation and pluripotency markers expression was induced, especially in 3D cell proliferation centers. This was followed by induction of "stemness" genes such as Oct4, Nanog, CXCR4 and HIF-1α. Concomitantly to appearance of these signatures of cell pluripotency, rapid regression of neural differentiation ability was observed. In parallel experiments BM-MSC cultures were found much less sensitive to all of the investigated plastic changes under the influence of low oxygen.

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Hyperbaric treatment reduces ROS production and antioxidative enzyme activities in a neonatal hypoxia-ischemia rat model

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Hypoxic-ischemic encephalopathy (HIE) is a serious condition that causes significant mortality and long-term morbidity. The aim of the present study was to evaluate the effect of hyperbaric oxygen (HBO) on antioxidative enzyme activities – catalase (CAT) and glutathione peroxidase (Gpx) – and on reactive oxygen species (ROS) production in 7-day old rat brain after hypoxia-ischemia (HI).

An experimental model of hypoxia-ischemia was used. In this widely used model left (ipsilateral) common carotid artery ligation is followed by 75 min hypoxia. HBO (2,5 ATA) was applied 1, 3 or 6 h after HI for 60 min. Treatment was repeated for 3 consecutive days.

In our previous experiments we showed that HBO reduces brain damage by almost 60%.

The DCF test showed that HI causes an almost 4-fold increase in ROS production in ipsilateral hemispheres, but not in contralateral hemispheres. HBO reduced ROS levels by 40%, 24% and 18%, used respectively 1, 3 and 6 h after HI.

The obtained results show almost 2.5-fold increase in glutathione peroxidase activity and 41% increase in catalase activity after HI, probably as a compensation for high ROS concentration. HBO used after HI reduces activity of those enzymes by 30, 12 and 11% in the case of GPx, and by 25, 24 and 16% in the case of CAT, used respectively 1, 3 and 6 h after HI. This decrease is probably a consequence of self-regulation caused by reduced oxygen radical production.

Our results suggest that HBO has a beneficial effect on ROS production, which manifests in decreased DCF fluorescence and decreased antioxidative enzyme activity. This may be one of the mechanisms suggesting how HBO reduces brain damage after HI.

Sphingosine kinases/sphingosine-1-phosphate and death signaling in an experimental model of Alzheimer disease

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In recent studies it has become evident that impaired sphingolipid metabolism, especially the alterations of sphingosine kinases 1 and 2 (SphK1/2) and sphingosine-1-phosphate level (S1P), are fundamental events in the neurodegeneration occurring in Alzheimer's disease (AD). AD-related amyloid beta peptides (Aβ) were previously shown to play a crucial role in regulating sphingolipid metabolism but the effect of Aβ on SphK1/2 expression and the role of S1P in toxicity evoked by Aβ are not fully elucidated. The aim of our study was to investigate the relationship between the level of Aβ and SphK1/2 gene expression. Moreover, the role of SphK(s)/S1P in cell survival and death was analyzed. PC12 cells transfected with human Aβ precursor protein wild type (APP_{wt}), involved in sporadic form of AD, or bearing double Swedish mutation (APP_{sw}), that causes familial form of AD, and control PC12 cells transfected with an empty vector were used. Our results indicated that endogenously liberated Aβ significantly decreased the expression of SphK1/2. Inhibition of SphK(s) activity by SKI II (10 μM) reduced the viability of APP transacted as well as control PC12 cells. Moreover, we showed that expression of S1P receptor-1 (S1PR1) was significantly decreased in APP transfected cells. The product of SphK(s), S1P (1 μM), enhanced the survival of APP_{wt} and control cells, affected by inhibition of SphK1, and had no effect in non-treated cells. However, S1P alone induced death of some populations of APP_{sw} cells and had no protective effect on these cells treated with SKI II. Using an S1P1 agonist (SEW2871, 5 μM) and antagonist (W123, 20 μM), we confirmed that the cytoprotective effect of S1P was receptor independent. All these results indicate that Aβ peptides evoke down-regulation of gene expression for SphK(s) and S1P1. Inhibition of SphK(s) significantly decreased cell survival. The effect of exogenous S1P depends on the genetic and molecular events occurring in the cells. Our data suggest that S1P may exert a protective effect in sporadic but not in familial form of AD.

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Blood-brain barrier during experimental autoimmune encephalomyelitis: possible involvement of microvessel-located P2X7R

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Multiple sclerosis (MS) is a serious and unsolved problem of contemporary medicine. It is an inflammatory and neurodegenerative disorder which affects young adults and inevitably leads to serious disabilities. In the course of MS, without known reason, immunological cells flow in through the blood-brain barrier (BBB) into brain tissue and attack myelin sheath. BBB fails to protect homeostasis of the central nervous system (CNS). Mechanisms of this disease are still unknown. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS where inoculum containing guinea pig spinal cord homogenate is injected into the hind limb of female Lewis rats. In 10 days rats develop symptoms that reflect MS in humans. P2X7R is a receptor that is involved in the inflammatory process and apoptosis. In this study we show existence of P2X7R in microvessels and the correlation of P2X7R expression with BBB state.

During the development of EAE BBB breakdown occurs. We analyzed BBB tightness in the early phase of EAE. Expression of claudin-5 (part of tight junctions) decreased in the microvessel fraction. These data suggest BBB dysfunction 4 day post immunization (dpi). These data were confirmed by immunofluorescence staining against claudin-5. To provide functional data about BBB state we carried out immunohistochemical staining against albumin. We observed outflow of albumin from microvessels

in the early phase of EAE at 4 dpi. We also observed colocalization of P2X7R with microvessels and noted a correlation between expression of P2X7R and BBB state.

The metabotropic glutamate receptor 8 (mGluR8) positive allosteric modulator AZ12216052 attenuates chemotherapeutics-induced cell death in human neuroblastoma SH-SY5Y cells

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Several experimental studies have demonstrated the potential role of mGluR group III stimulation in protecting neurons against various kinds of neuronal injury. Although the neuroprotective effects of mGluR III- and mGluR4-specific agonists and positive allosteric modulators (PAMs) are rather well recognized, the potential contribution of other subtypes of mGluR III (mGluR7 and mGluR8) to neuroprotection is less recognized. Thus in the present study we elucidated the effects of an mGluR8-specific agonist, (S)-3,4-DCPG (DCPG: 01-10 μM), and mGluR8 PAM, AZ12216052 (AZ: 0.001-10 μM), on chemotherapeutic-induced cell damage in undifferentiated (UN-) and retinoic acid-differentiated (RA-) human neuroblastoma SH-SY5Y cells. The chemotherapeutics used in the study (doxorubicin, irinotecan and cisplatin) in concentration- and time-dependent manner induced cell death in SH-SY5Y cells with their higher toxicity in undifferentiated cells. The mGluR8 PAM, AZ12216052 but not mGluR8 orthosteric agonist (S)-3,4-DCPG partially attenuated the cell death induced by all tested chemotherapeutics in UN-SH-SY5Y. No protection of tested mGluR8 ligands was found in RA-SH-SY5Y cells. The neuroprotective effects of AZ12216052 in UN-SH-SY5Y differed at the level of effective concentrations, which were for cisplatin AZ 0.001-1 μM, for irinotecan AZ 0.01-1 μM, and for doxorubicin AZ 0.1-1 μM. Moreover, the cell proliferation-stimulating effects of AZ (0.001-1 μM) but not (S)-3,4-DCPG in UN-SH-SY5Y were demonstrated. These *in vitro* data suggest the potential utility of mGluR8 PAM as a neuroprotective drug and encourage further studies in other cellular and animal models of neuronal injury.

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Cell proliferation in neurogenic regions of neonatal rat brain after hypoxic ischemia

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Birth asphyxia remains a frequent cause of perinatal morbidity and mortality. During perinatal hypoxic-ischemic (HI) brain injury neuronal cells are damaged and lose their function or die. In the past few years, it has become clear that ischemic brain injury stimulates neural stem cell proliferation and differentiation in the cerebral neurogenic area – dentate gyrus (DG) of the hippocampus – which is frequently injured after perinatal HI. There is considerable speculation that the induction of these progenitors after injury may represent an endogenous mechanism for brain regeneration. To study the response of hippocampal progenitors to neonatal HI brain damage, we utilized an established model of HI induced in rats of postnatal day 7 (PND7). The left common carotid artery was ligated and then, after 1 hour of recovery, the animals were exposed to hypoxia (7.6% oxygen for 60 min). The hypoxic undamaged hemisphere served as a control for developmental modification. In addition, age-matched sham-operated rats were also used as controls. To determine the proliferation profile, animals were injected with BrdU (50 mg/kg) at various days after HI (2, 5, 8, 10 and 13) and the next day perfused transcardially with PBS followed by 4% PFA. At 3-14 days after HI the presence of BrdU-positive cells was seen in both ipsi- and contralateral hemispheres, with the greatest number of dividing cells on the ischemic side and the strongest proliferation level occurring at days 3 and 6 after the injury. The labeling pattern also revealed

structure-dependent differences. At 3 days after the insult the highest density of cells incorporating BrdU was seen in the hilus, whereas at longer survival time the labeled cells started to change their localization towards the subgranular zone (SGZ) of the DG, where they were strictly observed at day 9, 11 and 14 after HI. To confirm that the BrdU-positive cells represent newly generated neuroblasts, we used double staining BrdU/DCX.

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Sphingosine kinase inhibition modulates the secretion of amyloid beta precursor protein and alpha-synuclein from human SH SY5Y neuroblastoma cells

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Sphingolipid deregulation may be an important factor of age-related neuronal stress vulnerability. Current data suggest links between sphingosine kinases (SphK1&2) and age-related protein conformation diseases. Recent results suggest that in addition to the toxicity of intracellular alpha-synuclein (ASN) its secretion may play a significant role in Parkinson's disease (PD) but also in Alzheimer's (AD) pathology, which classically has been associated with amyloid beta (ABeta). We have shown that ABeta precursor protein (APP) levels are dependent on ASN and that the secretion of both proteins is dependent on sphingolipid metabolism.

Our current aim was to further investigate the possible role of sphingosine kinases (SphKs) in the secretion of ASN and APP. The studies were carried out using a human SH-SY5Y neuroblastoma cell line stably transfected with the human wt ASN gene.

SphK inhibitor SKI had different effects on the secretion of both proteins. 3-day SKI treatment significantly elevated cellular APP level; APP secretion was reduced by about 50%. These results correspond with the previously shown pro-secretory effect of S1P.

However, SKI significantly elevated ASN secretion in a concentration-dependent manner without affecting its cellular level. We suggest that this effect could be mediated by ceramide formation.

Together our data further delineate the potentially important phenomenon of sphingolipid-mediated modulation of secretion of neurotoxic proteins.

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Amyloid β peptides and their role in alteration of the urea cycle in PC12 cells

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Amyloid beta peptide (Aβ) and its precursor protein (APP) are associated with the induction of oxidative/nitrosative stress and stimulation of nitric oxide (NO) synthase (NOS) activity. The enhancement of NO and other free radicals may influence Aβ oligomerization and toxicity. Our previous data demonstrate that pheochromocytoma (PC12) cells bearing the Swedish double mutation in the amyloid precursor protein gene (APPsw, K670M/N671L) have increased Aβ production and NO levels compared with human wild type APP-expressing (APPwt) and control (empty vector-transfected) PC12 cells. We observed higher expression of nNOS in APP transfected cells. Moreover, previous results indicated that the urea cycle that is associated with the citrulline-NO pathway may be altered in the Alzheimer's (AD) pathology. However, there are no data to support the significance of Aβ peptide in ornithine cycle alteration. Therefore, we investigated the gene expression of urea cycle enzymes in cells secreting different amounts of Aβ (control, APPwt, APPsw). We used real-time PCR and high-performance liquid chromatography (HPLC) methods. We compared the expression levels of genes involved in the urea cycle: arginase-1 (Arg-1) and arginase-2 (Arg-2), ornithine

carbamoilotransferase (OTC), argininosuccinate synthase (ASS), argininosuccinate lyase (ASL) and ornithine decarboxylase 1 (ODC) in PC12 control and APP transfected cells by directly quantifying the amounts of mRNA. In parallel we analyzed arginine, ornithine and citrulline levels by HPLC. Our results indicated that both APPwt and APPsw cells displayed significantly lower expression levels of Arg-1 and Arg-2 as well as of ASS1 compared to control PC12 cells. The ASL gene appeared to be up-regulated in APPsw PC12 cells in comparison with control PC12 cells. In conclusion, our results emphasize the important influence of Aβ peptide on the urea cycle.

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The neuroprotective properties of Wharton's jelly aggregates and mesenchymal stem cells isolated from the umbilical cord

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Ischemic stroke is one of the main causes of central nervous system damage which often results in disability or death. To date, the only medicine with proven therapeutic effect is tissue-plasminogen activator. Unfortunately, it has restricted application, for example it can be applied only 4.5 hours after ischemia. Because of that, researchers are still looking for new therapeutic solutions, one of which may be human mesenchymal stem cell (MSC) therapy aimed at reconstructing damaged tissue and inducing repair mechanisms. The mesenchymal stem cells are able to produce cytokines and growth factors which have anti-inflammatory, neuroprotective and neuroregenerative properties. Nowadays, there are many experiments trying to characterize MSC derived from different tissues such as adipose, bone marrow or umbilical cord.

The aim of the project was to compare the neuroprotective properties of mesenchymal stem cells derived from umbilical cord, depending either on the time of *in vitro* cultivation or on the type of culture (i.e. monolayer vs. Wharton's jelly aggregates). The Wharton's jelly aggregates and mesenchymal stem cells derived from them were co-cultured with intact or OGD (oxygen glucose deprivation)-injured hippocampal slices.

Results: Both Wharton's jelly aggregates and mesenchymal stem cells isolated from human umbilical cord had a neuroprotective effect on damaged tissue. The strongest neuroprotective potential was observed in co-cultures based on Wharton's jelly aggregates or freshly isolated MSC. The cultured Wharton's jelly/mesenchymal stem cells were also influenced by damaged tissue. The mesenchymal stem cells co-cultured with hippocampal slices after OGD proliferated faster and expressed neural markers.

Conclusion: There is a significant difference in neuroprotective properties between stem cells derived from the same source but cultured *in vitro* for different periods of time, as well as between stem cells cultured either as a selected fraction in a monolayer or as 3D aggregates containing stem cells and supporting tissue.

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Parkinson's disease related nitrosative stress affects the expression of Parkin and PC12 cell viability

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Parkin functions as an E3 ubiquitin ligase, and loss of this ubiquitin ligase activity is linked to death of dopaminergic neurons in sporadic and genetic Parkinson's disease (PD). Elevated oxidative/nitrosative stress induced by alpha-synuclein (ASN) oligomerization or by environmental factors was also shown to be responsible for selective death of dopaminergic neurons in PD. It was shown that nitro-

sative stress leads to S-nitrosylation of Parkin that affects its activity and the E3 ligase-ubiquitin-proteasome pathway. Our previous studies indicated that ASN activated neuronal nitric oxide synthase and induced excessive liberation of nitric oxide (NO). However, the relationship between Parkin, ASN and nitrosative stress in PD remains unclear. Therefore, in these studies, for better understanding of the mechanism of ASN-induced cytotoxicity we investigated the effect of nitrosative stress evoked by exogenous ASN oligomers and by the NO donor sodium nitroprusside (SNP) on expression of Parkin and cell viability. PC12 cells were treated with exogenous ASN oligomers (500 nM) and SNP (0.1 and 0.5 mM) for 24 h and 48 h. The experiments were performed using spectrophotometric, spectrofluorometric and immunochemical methods and qRT-PCR assay. The results indicated that oligomers of ASN and SNP induced activation of the stress response genes superoxide dismutase (SOD1) and gadd45, formation of free radicals, as well as apoptotic cell death. Our results indicated that nitrosative stress leads to changes in Parkin expression and protein level. The SNP treatment resulted in significant down-regulation of the Parkin mRNA level in PC12 cells. In summary, these data showed the relationship between Parkin expression and intensity of cellular nitrosative stress which would impair ubiquitination and clearance of Parkin substrates. These findings may thus provide a molecular link between ASN toxicity, free radical and protein accumulation in sporadic PD.

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Classical estrogen receptor signaling is not involved in apoptotic actions of nonylphenol in mouse embryonic neuronal cells

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Nonylphenol is an alkylphenol present in plastic wrappings, bottles, and textile paints, and through its extensive usage may easy affect living organisms. It belongs to a wide group of endocrine disrupting chemicals (EDCs) causing deleterious effects on human health. These substances, present in the environment, are known to mimic, antagonize or modify the endogenous hormonal activity. There is a growing body of evidence that exposure to EDCs during pregnancy or perinatally may cause strong and persistent developmental impairments including neuropsychiatric disorders such as learning disabilities or attention deficit and hyperactivity disorder (ADHD). Considering that nonylphenol may accumulate in the brain tissue in much higher concentrations than in other tissues, there are strong grounds for studying mechanisms of its negative action on the developing brain.

We studied apoptotic effects of nonylphenol in mouse embryonic neuronal cells and its possible interaction with classical estrogen receptor (ER) signaling. We demonstrated that nonylphenol induced loss of mitochondrial membrane potential in the first 3 hours of exposure, which was followed by activation of caspase-3. Caspase-8 inhibitor (Z-LETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) reversed the effects of nonylphenol with respect to caspase-3 activity as an endpoint, whereas calpain inhibitor (Acetyl-Calpastatin) was ineffective. These data were supported by Hoechst 33342 and calcein AM staining which visualized apoptotic, pycnotic nuclei with condensed chromatin and impaired cell survival of nonylphenol-treated hippocampal cells. We also demonstrated that nonylphenol caused a substantial increase in ERα and ERβ mRNA expression. However, silencing of ERα and ERβ with specific mRNAs did not inhibit nonylphenol-induced caspase-3 activity in neuronal cells.

This study demonstrated that apoptotic action of nonylphenol in mouse embryonic hippocampal cells was not only mediated by caspase-3, but was also a caspase-8, and caspase-9-dependent process. Furthermore, we found that nonylphenol-induced caspase activity was preceded by loss of mitochondrial membrane potential and the classical estrogen receptors ERα and ERβ were not involved in these effects.

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Differentiation of iPS cells derived from human cord blood on fibronectin and poly-L-lysine patterned domains

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Stem cell fate decisions are dependent upon signals coming from the microenvironment composed of extracellular matrix proteins, soluble factors and specificity of cell-cell contacts. We have developed an *in vitro* system to trace developmental processes of stem cells and to control their fate commitment. Nano/micro-fabrication techniques such as microcontact printing and piezoelectric microspotting of biomolecules on a plasma deposited cell repellent surface have been applied to obtain defined patterns of bioactive surface domains.

In this report we aimed to compare the developmental response of lineage related induced pluripotent stem cells (iPSC) and committed neural progenitors obtained in our laboratory from HUCB-NSC (human umbilical cord blood neural stem cell line) to different geometry of the patterned bioactive domains. Tested bioactive domains included 10 μm wide lines and 120 μm/120 μm squares microprinted with fibronectin or poly-L-lysine. The iPS cells and committed neural progenitors were seeded on designed patterns with density of 5 × 10 cells/cm^2 and incubated for a further 5 days in stable conditions of low serum (2%) medium, 5% CO_2 and 37°C. All samples were tested for the expression of genes typical for three germ layers (*BRACHYURY*, *MSX1*, *SOX2*), genes typical for pluripotency (*OCT* 3/4, *NANOG*, *REX1*, *hTERT*) as well as those related to neural commitment differentiation (*PAX6*, *GFAP*, β-*TUB3*, *MAP2* and *NEURO D*). In all tested experimental variants cells were negative for endodermal and mesodermal markers while revealing ectodermal commitment. Some pluripotency markers (*hTERT* and *OCT* 3/4) were present in all tested samples, but their expression was stronger in iPS cells as compared to neural progenitors. The expression of β-*TUB3* was at the same high level in all samples, but *MAP2*, an advanced marker of neuronal differentiation, revealed the strongest expression in iPS cells elongated on the 10 μm wide lines. In contrast, *GFAP* expression was the most abundant in iPS cells incubated on squares. This was confirmed by immunocytochemical analysis at the protein level. Such data revealed that the type of geometry of bioactive domains may influence neural differentiation of iPS cells.

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ACPT-1 diminishes brain damage and improves motor functions of rats subjected to transient focal ischemia

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ACPT-1 ((1*S*, 3*R*,4*S*)-1-aminocyclopentane-1,2,4-tricarboxylic acid) is a blood-brain-barrier permeable, unspecific agonist of group III metabotropic glutamate receptors (mGluR). This compound has been shown to protect neurons against excitotoxicity *in vitro* and *in vivo* as well as against ischemic neuronal damage *in vitro*. The aim of the present study was to assess the neuroprotective potential of ACPT-1 against ischemic neuronal damage *in vivo* using functional tests. Twenty-five male Sprague Dawley rats, weighing 260-280 g, were used for this study. Transient focal cerebral ischemia was induced for 90 min by intraluminal occlusion of the right middle cerebral artery (MCA). Microflow (LDF) in the brain cortex supplied by this artery was continuously monitored with the help of a laser-Doppler flowmeter starting before the occlusion of the vessel. ACPT-1 (30 mg/kg administered in a volume of 1 ml) or vehicle (1 ml) was injected intraperitoneally either 30 min after MCA occlusion (group 1) or 30 min after reperfusion (group 2). Behavioral tests (CatWalk and open field) were performed before ischemia and 72 hours after reperfusion. The area of infarction was evaluated after the completion of behavioral

tests on brain tissue slices stained with 2,3,5-triphenyltetrazolium chloride (TTC) using a computer based image analysis system (GIMP 2). Seventy-two hours after ischemia, the volume of infarction was smaller ($p < 0.05$) in the groups treated with ACPT-1 (group 1 – 26 ± 1%; group 2 – 25 ± 4%) compared with the vehicle-treated one (37 ± 3% of the hemisphere was damaged). In addition to less morphological damage, results of the analysis of behavioral tests demonstrated the improvement of selected gait parameters on CatWalk as well as improved mobility in an open field in the rats treated with ACPT-1. What is promising, the improvement was observed not only when ACPT-1 was administered during ischemia but also when it was given during reperfusion.

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MPP+ alters expression of sphingosine kinase 1/sphingosine 1-phosphate (S1P) receptor and Bcl-2 proteins in human neuronal SH-SY5Y cells. Effect of S1P

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Sphingosine-1-phosphate (S1P) is one of the most potent signaling molecules, which is synthesized by two isoforms of sphingosine kinases (Sphk1/2). S1P exerts an essential role in neurotransmission, synaptic function, neuron cell proliferation, inflammatory responses and senescence. S1P acts through five G protein-coupled receptors (S1P1-5) or intracellularly as a second messenger. The significance of the Sphk1/2 and S1P mediated signaling pathway in pathogenesis of Parkinson's diseases (PD) is unknown. In our study we investigated gene expression for Sphk1, S1P1 receptor and Bcl-2 pro-apoptotic proteins and the mechanism of S1P action in human dopaminergic cells (SH-SY5Y) under oxidative stress, evoked by 1-methyl-4-phenylpyridinium (MPP+). Human neuroblastoma SH-SY5Y cells were cultivated in the presence of 3 mM MPP+ for 3-24 h, or in the presence of S1P (1 μM) and MPP+. The effect of MPP+ and S1P on regulation of selective protein expression was analyzed using real-time PCR and immunochemical methods (Western blot). Analysis of cell survival and oxidative stress was determined using spectrophotometric and spectrofluorometric methods. We showed down-regulation of Sphk1 and S1P3 genes and enhanced expression of pro-apoptotic proteins: Bax and death protein 5, harakiri (DP5/Hrk) in stress conditions, evoked by MPP+. It was found that exogenous S1P (1 μM) exerted a neuroprotective effect by activation of Sphk1 and S1P1 receptor genes expression. Moreover, S1P down-regulated Bax and DP5/Hrk expression in MPP+ treated cells. Our study also indicated that S1P reduces the reactive oxygen species (ROS) level and percentage of apoptotic cells and protects a significant pool of SH-SY5Y cells against death evoked by MPP+. Moreover, in these conditions sphingosine analogue (P-FTY720) also has neuroprotective effects. Our data indicated by using specific agonists and antagonists of S1P receptors that the mechanism of S1P is mainly dependent on S1P1 receptor signaling. On the basis of these data we suggest that S1P receptor agonists and sphingosine analogue (P-FTY720) may offer a novel strategy for neuroprotection.

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Deficits of thiamine pyrophosphate in glial cells

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Thiamine deficits inhibit metabolic fluxes through pyruvate and ketoglutarate dehydrogenase steps yielding inhibition of acetyl-CoA and energy production in the brain. Therefore, we investigated whether thiamine antagonists, amprolium (AM) and pyrithiamine (PT), impair acetyl-CoA metabolism and neurotransmission in cholinergic SN56 cell line 48 h exposure of nondifferentiated (NC) and differentiated (DC) SN56 cholinergic cells, to 5 mM AM caused inhibition of thiazolyl blue reduction rate and increased their mortality, by 15 or 30% and 10 or 43%, respec-

tively. AM altered neither PDH and KDH nor choline acetyltransferase activities, but decreased cytoplasmic acetyl-CoA levels by about 50%, both in DC and NC. On the other hand, acetylcholine (Ach) content in DC was suppressed by 40%, whereas in NC it was not altered by AM. Nerve terminals isolated from brains of PT-TD displayed 40% inhibition of pyruvate metabolic flux through the PDH step, 40% decrease of citrate accumulation as well as 53% suppression of Ca-dependent Ach release, respectively. Acetyl-CoA levels in TD nerve terminal mitochondria and synaptoplasm were decreased by 40 and 30% respectively. These data demonstrate that deficits of cholinergic transmission in TD brains are caused by inhibition of acetyl-CoA provision to the cytoplasmic compartment through the indirect ATP-citrate lyase pathway. The next experimental model we used was astroglial C6 and microglial N9 cell line cultured in low thiamine medium. In such conditions microglial N9 cells displayed significantly greater loss of viability than the C6 ones. In both groups of cells the activity of the key energy enzymes such as PDHC, KDHC, and aconitase was inhibited when the cells were grown in low thiamine conditions. In parallel, the level of acetyl-CoA in the mitochondrial compartment was decreased in the cells.

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Oxidative stress and ultrastructural changes in brain of rats chronically exposed to silver nanoparticles

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Silver nanoparticles (AgNPs) are widely used in different applications, because they demonstrate strong antibacterial and antifungal activities. Over the last decades AgNPs have found applications in the food industry, textile engineering and silver-based consumer products (e.g. cosmetics, cleansers). Nanosilver is also used in a wide range of medical devices. For that reason human exposure to AgNPs is constantly increasing. However, the potentially negative health consequences of using materials which consist of silver nanoparticles have not been fully identified yet.

Presently, there are only a few studies dedicated to investigating their neurotoxic potential. Most of them have been conducted using *in vitro* models.

The aim of the present study was to investigate whether commercially available small-sized nanosilver particles induce neurotoxic effects in male adult rats. Animals were exposed to oral application of 10 ± 4 nm nanosilver for 14 days. Using transmission electron microscopy (TEM) ultrastructural changes in cortical and hippocampal neurons were found mainly related to mitochondria and nuclei. The level of Bax and Bcl-2 protein which are involved in apoptosis was measured using Western blot. To assess oxidative stress in brain homogenates from exposed animals the level of membrane peroxidation was measured by the TBARs method. Additionally, the ratio of reduced to oxidized glutathione was investigated, as was the level of protein glutathionylation. The results indicate that the induction of oxidative stress under conditions of nanosilver exposure is one of the mechanisms of its neurotoxicity.

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The impact of prenatal stress on the insulin-like growth factor (IGF-1) level in primary microglial cells: the possible role of cytokines

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Growing evidence indicates that interactions between the endocrine, immune and nervous systems have an important role in pathogenesis of depression. It has also been suggested that weaker activity of growth factors, such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF), may lead to the development of this disorder. In peripheral tissues, proinflammatory cyto-

kines and glucocorticoids reduce IGF transmission; however, little is known about the IGF regulation in the central nervous system.

The aim of the present study was to find out whether the prenatal stress procedure – an animal model of depression – may influence IGF-1 level in primary microglial cells. Simultaneously we estimated the levels of cytokines, whose role in the regulation of IGF-1 has been suggested.

Pregnant Sprague-Dawley rats were subjected daily to three (at 9.00, 12.00, 17.00) stress sessions from 14th day of pregnancy until delivery. Control pregnant females were left undisturbed in their homecages. Primary cultures were prepared from the cerebral cortices of 1-2 day old offspring. After 8 days cells were plated onto 24-well or 96-well plates. Adherent cells were incubated for 48 h in culture medium before being used for the treatments. The cell viability was determined by the MTT test, and the cell death by the LDH test. Moreover, supernatants were collected and the levels of IGF-1 and cytokines were quantified by specific ELISA kits.

Prenatal stress decreased cell viability as evidenced by the MTT test and significantly enhanced cell death. Moreover, primary microglial cells obtained from prenatally stressed rats released less IGF-1. Also the cytokine levels were changed.

The results indicate that prenatal stress may have an important influence on primary microglial cells. It modifies the viability and death processes of these cells. Furthermore, maternal stress modulates cytokine production and in this way it can affect the insulin like growth factor level. All these data suggest the involvement of prenatal stress in the development of neuroinflammatory diseases by acting on microglial cells.

Effects of antagonists of glutamate receptors on glutamate transport and glutamate receptor binding in the brain cortex of rats subjected to EAE

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Recent studies have strongly indicated a role of glutamate receptors in glutamate homeostasis during the course of EAE, implying that excitotoxicity may be involved in the pathogenesis of multiple sclerosis (MS). The current investigations were undertaken to determine whether the use of uncompetitive NMDA receptor antagonists (amantadine, memantine) and antagonists of group I mGluR (MPEP, LY 367385) can modify the neurological course of EAE. Drugs were administered intraperitoneally once daily into EAE rats for 7 days, starting from day 5 to 12 post immunization. During the experiment the body weight and neurological deficits were monitored daily, as regards duration of the disease phases and the lethality. Rats showed a progressive loss of body weight (20-30%) starting from 8-10 days post immunization. The neurological decline also started from the 10^{th}-12^{th} day post immunization and peaked on the 13^{th}-14^{th} day. We investigated modulation of glutamate transport (uptake and release of glutamate), the expression of mRNA for glutamate transporters (EAATs), and changes in kinetic parameters of receptor binding after therapy of EAE rats with selected glutamate receptor antagonists. Amantadine and memantine significantly improved neurological deficits in rats suffering from EAE, and modulated transport of glutamate measured in synaptosomal fraction and MK-801 binding to the membrane fraction.

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Local tissue microenvironment governs OPC commitment and differentiation

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Oligodendrocyte progenitor cells (OPCs) are cycling cells scattered almost uniformly in the young and adult brain parenchyma. They are capable of myelinogenesis, but they are also among the first cells to react to CNS disorders. Over the last decade however these glia-committed progenitors have been the object of intensive research in the context of their presumed neural stem cell-like prop-

erties. In our studies, we addressed the question of the impact that the local tissue microenvironment exerts on the OPCs commitment and differentiation. Their susceptibility to external stimuli and their intrinsic neurogenic potential were investigated in co-culture models with organotypic slices derived from two distinct CNS regions (hippocampus and spinal cord). The hippocampal slice culture exposed to oxygen and glucose deprivation (OGD) was used to evaluate cell differentiation in a microenvironment conditioned by traumatized tissue. The results show that the local instructive clues not only trigger the neuronal commitment of oligodendrocyte progenitors, but also govern the oligodendroglial maturation. While trophic factors secreted by the hippocampal slices efficiently promoted neurogenesis, the effect nearly disappeared in co-cultures with the OGD-subjected tissue. Less pronounced susceptibility to adopting neuronal phenotype and a considerable slowdown in oligodendroglial differentiation was observed in the co-cultures with the spinal cord slices. The obtained results indicate that OPCs actually meet some of the neural stem cell criteria. They also imply that the specificity of the instructive clue cocktail might influence the fate choice of mobilized endogenous or transplanted cells, which should be taken into consideration when planning neurorepair strategies.

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Modifications in the expression of HDACs and DNMTs in human neural stem cells induced by reprogramming and low oxygen tension

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To better understand the reprogramming and differentiation process in human neural stem cells we aimed to look at the mutual relationship between the pluripotency regulatory network and epigenetic process in different oxygen conditions. Thus the expression of pluripotency genes, hypoxia inducible factors, histone deacetylases and DNA methyltransferases has been tested in a HUCB-NSC (human umbilical cord blood – neural stem cell) line at different developmental stages. Our previous data have shown that low oxygen tension promotes maintenance of the undifferentiated state of HUCB-NSC and is beneficial for their proliferation and activate OCT4 and NANOG genes in time of cultivation and developmental stage dependent manner. 5% oxygen also enhanced neural commitment of HUCB-NSC as shown by elevated MAP2 expression. Cells incubated in all tested oxygen conditions expressed HIF 1α and HIF 2α; however, expression of HIF 3α was not detected. In this study genes involved in epigenetic modulations, such as HDAC1, HDAC2, DNMT3a and DMNT3b, were tested for their expression at different developmental stages of HUCB-NSC: reprogrammed to pluripotency or kept in culture as a neurally committed population. The iPSC and neurally committed population were both incubated in low oxygen tension conditions. Our results show that in neurally committed HUCB-NSC expression of HDAC1, HDAC2, DNMT3a and DMNT3b was significantly lower than in induced pluripotent stem cells. Moreover, the highest level of expression of genes involved in histone deacetylation (HDAC1 and HDAC2) was observed in pluripotent cells obtained after a reprogramming procedure.

The studies indicated the important role of epigenetic factors and low, physiological oxygen tension in the induction of pluripotency and in the process of differentiation into neuronal lineage of neural progenitor cells derived from human cord blood.

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Interaction of nuclear matrix proteins with specific regions within the 30 kb genomic fragment containing the bovine tyrosine hydroxylase gene

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Tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step in catecholamine biosynthesis. Its expression is found mainly in adrenal chromaffin cells and catecholaminergic neurons of the central and peripheral nervous system. TH undergoes multilevel regulation, including both short-term modulation of the enzyme activity and long-term regulation of gene expression. TH gene expression is activated by many trans-acting factors that recognize regulatory elements located at the 5' promoter region, its coding sequence, and downstream of the coding region. We have postulated that some of the TH regulatory factors are the nuclear matrix (NM) residents. NM is defined as an insoluble nuclear scaffold engaged in the transcriptional regulation of gene expression. Chromatin loops are bound by the NM proteins through specific DNA sequences, called scaffold/matrix attachment regions (S/MARs), which act in cis to anchor the chromatin to the NM structure in a cell-type- and cell-cycle-specific manner. To determine the attachment regions within the 30 kb genomic fragment to the NM, we (i) examined DNase I sensitivity in the aforementioned region, (ii) mapped constitutive S/MAR in the TH first intron, and (iii) used Southwestern technique to characterize the molecular masses of the bovine or human NM proteins that anchor the bovine TH S/MAR. During experiments some different tissues/cell lines with differing TH activity were used to prepare the NM samples: the adrenal medulla, in which TH is expressed, and the liver, in which the TH gene is inactive, or TH-active (SH-SY5Y) and TH-inactive (HepG2) human cell lines. The obtained results indicate that four regions (-18457/-18021, -7676/-7054, +3200/+3641, +4581/+5129) within the 30 kb genomic fragment are DNase I sensitive in the chromatin isolated from the adrenal medulla. By contrast, chromatin obtained from bovine liver is less sensitive to digestion and two regions (+3200/+3641 and +4581/+5129) were released from the NM. Using two molecular probes encompassing fragments of the bovine TH first intron (+770/+1598 and +766/+1193), we were able to determine that both regions are sufficient for binding to NM. Moreover, a comparative analysis of the binding profiles revealed that both molecular probes were bound by both human specific proteins and proteins that are evolutionarily conserved between bovines and humans. All DNA fragments were mapped to the transcription start site of the TH gene.

TNFα gene G-308 polymorphism and the risk of ischemic stroke

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TNFα, a significant immune mediator, may contribute to the initiation and progression of ischemic stroke. Genetics of the TNFα molecule may have an important role in the risk of ischemic stroke. The most interesting aspects of the G-308 polymorphism remain unexplained; there are many discrepancies between the results. Differences in the ethnicity of the studied cohorts may be taken as one possibility. Our study material consisted of 101 patients with ischemic stroke. The diagnosis was based on the presence of rapidly developing neurological signs lasting longer than 24 hours and confirmed by neuroimaging methods. All patients were of Polish Caucasian origin. 100 randomly selected individuals without any signs of vascular disease of the central nervous system were taken as the control material. The frequency of polymorphism G-308 A in the TNFα gene was determined as described by Rubattu *et al.* (2005). The genotype distribution in our material was similar and statistically insignificant between patients and controls. The heterozygous G/A genotype was detected in 9% of patients and in 15% of control materials. Homozygous A/A was found in 5% of patients and only in one of control and G/G in 87% of patients and in 84% of control

individuals. Our results are negative with respect to the impact of 308 TNFα polymorphism on the risk of ischemic stroke in Caucasians living in Poland.

Regulatory effect of L-type voltage-gated calcium channel on acetyl-CoA metabolism in cholinergic neurons during acute Zn exposure

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Acetyl-CoA serves as a precursor of acetylcholine (ACh) in the cytoplasmic compartment and in the mitochondrial compartment is used for energy and N-acetylaspartate (NAA) synthesis. The first pathway, present only in cholinergic neurons, can cause, under neurotoxic conditions, temporary shortages of acetyl-CoA, which is an essential metabolite for energy and NAA production. Disturbances in Ca signaling and its handling protein can play a particular role in neuron susceptibility to neurodegenerative conditions. The aim of our study was to investigate how the L-type voltage-gated calcium channel (L-VGCC) moderates the phenotype-dependent susceptibility cholinergic neurons into degeneration. 10 μM nifedipine, a blocker of L-VGCC, decreased Ca levels in SN56 nondifferentiated cells (NC) and differentiated cells (DC) by 41 and 21%, respectively. Short exposure of SN56 cells to 0.15 mM Zn increased the Zn levels from 0.5 nmol/mg protein to 46.41 nmol/mg protein (NC) and from 0.58 nmol/mg protein to 59.08 nmol/mg protein (DC). However, in the presence of nifedipine accumulation of Zn was decreased by 60 and 67% in NC and DC, respectively. Zn caused a 59% increase of the nonviable cell fraction both in ND and DC SN56, whereas incubation of cells with nifedipine and Zn led to a 20% decline in the number of trypan blue positive cells in both the NC and DC. In Zn-exposed cells the level of acetyl-CoA in NC and DC was decreased by 40% and 57%, respectively. At the same time, levels of NAA and ACh were decreased by 50% both in NC and DC. Nifedipine prevented the Zn-evoked neurotoxic effects on acetyl-CoA metabolism in SN56 cholinergic cells. These results indicate that L-VGCC play an important role in neurotoxicity of Zn and show that disturbance of Ca homeostasis in this condition can be one of the factors which moderate acetyl-CoA metabolism in cholinergic neurons.

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