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ABSTRACTS

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Fibrinogen gene mutations and intracellular, circulating and interstitial polymerization of the mutant fibrinogen

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Fibrinogen is synthesized in the liver as a dimer molecule with each half consisting of three polypeptide chains (A-alpha, B-beta, gamma). The symmetrical molecule consists of a central E domain joined to two peripheral D domain. After activation by thrombin, the fibrinogen monomer spontaneously polymerizes through D:E interaction to form a half-straggered bimolecular array that is stabilized by non covalent end-to-end D:D interaction. This process occurs in the intravascular compartment and is responsible for hemostasis. Mutations affecting the thrombin active sites of fibrinogen cause coagulation problems, either thrombosis or bleeding.

Fibrinogen can undergo other mutations not related with the thrombin active sites. These mutations lead to abnormal conformation of the fibrinogen molecule that aggregates either within the RER of hepatocytes or in the extracellular space (interstitial tissue) in the form of amyloid.

The former mutation affects the gamma chain gene (284 Gly-Arg – Fibrinogen Brescia – and 375 Arg-Trp - Fibrinogen Aguadilla -) and results in hepatocytic storage of the protein, hereditary non-dysfibinogenemic hypofibrinogenemia and severe chronic liver disease. In this hereditary form the mutant fibrinogen is in a metastable state because its D:D polymerization sites pre-exist and do not require thrombin activation.

The second group of mutation affects the Fibrinogen A-alpha chain and leads to extracellular (interstitial) deposition of amyloid that can result in fatal renal disease.

Light and Electron Microscopic studies, crystallographic analysis and tridimensional reconstruction have displayed a close similarity between the morphogenesis of fibrille formation (polymerized fibrinogen) in the three involved body compartments, i.e. intracellular, intravascular, and extracellular space. The three conditions are being now included in a novel group of diseases, the s.c. conformational diseases. However, despite a similar pathomorphogenesis, both biological and clinical manifestations appear to be completely different both in terms of cell/tissue damage and organ pathology.

Quantifying oncogenic signalling by mass spectrometry

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Cancer is often caused by aberrant activation of signalling enzymes. Protein kinases are prominent examples of signalling enzymes frequently over-activated in cancer, thus making this group of molecules attractive targets for therapeutic intervention. However, cancers are heterogeneous entities from the molecular standpoint and different tumours, even those of the same pathology, deregulate different signalling routes with roles in cell proliferation. In other words, cancer cells can proliferate using different signalling routes. In order to provide insights into the different signalling pathways that can contribute to cancer, we have developed mass spectrometry-based phosphoproteomics techniques for in-depth quantification of signalling. These methods are based on targeting the quantification of phosphorylation sites that provide a readout of protein kinase activity. Examples will be given of the application of this novel methodology for the analysis of signalling in leukaemia cells of different genotype and of different sensitivity to cell signalling inhibitors.

Diversity of proteasome complexes in the myocardium and shifts in response to the development of cardiac hypertrophy

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Proteasomes recognize and degrade proteins targeted for degradation by ubiquitination. Thus, the ubiquitin proteasome pathway participates in major cellular events and protein quality control. We identified that the myocardium contains the full complement of constitutive and inducible proteasome subunits, which are fully assembled in complexes, including a heterogenic group of proteolytic core complexes differing in their functionality. During the development of pathologic hypertrophy of the myocardium, protein quality control is highly engaged. We have analyzed the capacity of protein degradation by proteasomes at different stages of pharmacologically induced hypertrophy by sympathetic stimulation. Interestingly, degradation by proteasomes was regulated at multiple levels. Proteasome activities are divided into ATP-dependent 26S and ATP-independent 20S activities, which are further subdivided in caspase-, trypsin- and chymotrypsin-like activities. The study demonstrated that 26S activities were up-, whereas 20S activities except the chymotrypsin-like activity were down-regulated. Molecular analyses of proteasome composition revealed that increased 26S assembly and reorganization of 20S assembly are part of the mechanisms for proteasome regulation during hypertrophy development. Multiple pathways have been analyzed in context of myocardial hypertrophy, including PKA signaling, which increasingly remains latent in the chronic phase of the utilized model. In vitro studies indicate that PKA is potentially modulating proteasome activities. In our model, down-regulated 20S activities were largely recovered by endogenous PKA activation. Other proteasome activities as well as those in control hearts were unaffected by PKA activation. In conclusion, our studies provide insight into the molecular mechanisms of proteasome regulation in the hypertrophic heart, which potentially participate in the pathology of the disease.

Brain development needs sugar: a view on the role of polysialic acid

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The neural cell adhesion molecule NCAM and its posttranslational modification polysialic acid are broadly implicated in neural

development. Mice lacking the polysialyltransferases ST8SiaII and ST8SiaIV are devoid of polysialic acid and show severe malformation of major brain axon tracts. Here we demonstrate how allelic variation of three interacting gene products (NCAM, ST8SiaII, and ST8SiaIV) translates into various degrees of anterior commissure, corpus callosum and internal capsule hypoplasia. Loss of ST8SiaII alone caused mild but distinct defects and the severity of the pathological phenotype found in mice lacking both polysialyltransferases could be stepwise attenuated by reducing NCAM expression. Analysis of mice with overall nine selected combinations of mutant NCAM and polysialyltransferase alleles revealed that the extent of the fibre tract deficiencies was not linked to the total amount of polysialic acid or NCAM, but correlated strictly with the level of NCAM erroneously devoid of polysialic acid during brain development. The defects implemented by the gain of polysialic acid-free NCAM were reminiscent to abnormalities found in patients with schizophrenia. Since variations in *NCAM1* and *ST8SIA2* have been implicated in schizophrenia, these findings provide a mechanism how genetic interference with the complex coordination of NCAM polysialylation may lead to neurodevelopmental predisposition to schizophrenia.

Organization and dynamics of the bacterial Ser/Thr/Tyr phosphoproteome

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The vital roles of protein phosphorylation on serine, threonine and tyrosine (Ser/Thr/Tyr) in cell signaling and regulation of protein activity are traditionally connected to eukaryotic cells. However, there is increasing evidence at the genome and proteome level that this modification is present and functional in prokaryotes.

We have recently reported phosphorylation site-resolved phosphoproteomes of model bacteria *Bacillus subtilis*, *Escherichia coli* and *Lactococcus lactis* showing that, despite of its low abundance, Ser/Thr/Tyr phosphorylation is present on many essential bacterial proteins. We detected phosphorylation sites on almost all glycolytic and TCA cycle enzymes, several kinases and the key members of the phosphoenolpyruvate-dependent phosphotransferase system. In addition, we demonstrated that bacterial phosphoproteins and phosphorylation sites show significantly higher degree of conservation than non-phosphorylated proteins, with a number of sites conserved from Archaea to humans.

Here we applied stable isotope labeling by amino acids in cell culture (SILAC) of the *B. subtilis* cells to investigate whether the Ser/Thr/Tyr phosphorylation is regulated upon exchange of carbon source (glucose vs. succinate), and under phosphate starvation. In a global proteomics experiment we measured dynamics of 1928 proteins and 33 phosphoproteins and used the MaxQuant algorithm for dependent peptide analysis and unbiased detection of post-translational modifications. We show that most of the detected phosphorylation sites are indeed different in the two analyzed conditions, suggesting that Ser/Thr/Tyr phosphorylation is a regulatory PTM in Eubacteria. Selected examples of phosphorylation changes on key metabolic and signaling proteins, such as the Hpr protein and fructose bisphosphate aldolase, will be presented and discussed.

Molecular determinants of vesicular storage and release of the gliotransmitter D-serine from cortical astrocytes

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Neuron-astrocyte reciprocal communication at synapses has emerged as a novel signalling pathway in brain function. Astrocytes sense the level of synaptic activity and, in turn, influence its efficacy through the regulated release of 'gliotransmitters' such as glutamate, ATP or D-serine. A calcium-dependent exocytosis is proposed to drive the release of gliotransmitters but its existence is still debated. Over the last years, we have been studying the molecular determinants governing D-serine release from glia using different approaches. Using a novel bioassay for D-serine, we have been able to show that D-serine release occurs mainly through a calcium- and SNARE protein-dependent mechanism just supporting the idea that this amino acid is released by exocytosis from glia. We next have pursued our exploration by confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission and have shown that D-serine releasable pools are confined to synaptobrevin2/cellubrevin-bearing vesicles. To shed light onto the mechanisms controlling the storage and the release of gliotransmitters and namely D-serine, we have developed a new method for the immunoisolation of synaptobrevin 2-positive vesicles from rat cortical astrocytes in culture while preserving their content in gliotransmitters. The purified organelles are clear round shape vesicles of excellent purity with homogeneous size (40 nm) as judged by electron microscopy. Immunoblotting analysis revealed that isolated vesicles contain most of the major proteins already described for neuron-derived vesicles like synaptic vesicle protein 2 (SV2) and the proton pump H^+-ATPase. In addition, we have analyzed the content for various amino acids of these vesicles by means of chiral capillary electrophoresis coupled to laser-induced fluorescence detection. The purified vesicles contain large amount of D-serine. We also detect peaks corresponding to unidentified compounds that may correspond to others amino acids. Postembedding immunogold labelling of the rat neocortex further revealed the expression of D-serine in astrocytes processes contacting excitatory synapses. Finally, we have examined the uptake properties for D-serine and glutamate inside the isolated glial vesicles. Our results provide significant support for the existence of an uptake system for D-serine in secretory glial vesicles and for the storage of chemical substances like D-serine and glutamate.

Comparative studies on conventional and microwave synthesis of peptide nucleic acid monomers and peptide conjugates

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Peptide nucleic acids (PNA) are oligonucleotide mimics in which the sugar-phosphate backbone has been replaced by a pseudopeptide chain of N-aminoethylglycine monomers. PNAs were shown to bind to complementary DNA or RNA sequences with high affinity and specificity. On account of these properties, they are widely used in molecular biology and biotechnology, as tools in genetic diagnosis, for specific regulation of gene expression, and are currently being investigated as potential antiviral and anticancer drugs. However, the lack of charge and polar groups in the backbone decrease their solubility in aqueous environment and their ability to cross cell membranes, reducing their performance in in vivo applications. In order to overcome these problems—to improve solubility, increase affinity and specificity of binding, a number of analogues were synthesized.

Therefore, we focused our attention on the synthesis of PNA-monomers on the base of non-protein amino acids analogues of basic amino acids Lys and Arg.

Here, we describe synthesis of the PNA-monomers utilizing both conventional and microwave methods. The performance of two methods was also compared.

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Comparative study of the interaction between Met-enkephalin analogues and monolayers of zwitterionic and negatively charged phospholipids

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The capabilities of synthetic analogues of endogenous neuropeptide Met-enkephalin (Met-enk) to induce transformations in model lipid membranes were investigated. Using Langmuir's monolayer technique, surface behavior and the interaction between Met-enk amide analogue and zwitterionic dimyristoylphosphatidylcholine (DMPC), and the negatively charged dimyristoylphosphatidylglycerol (DMPG) at air/water interface are studied.

By using the Wilhelmy method, surface tension (γ, mN/m) changes (after injection of the peptide under surface) of DMPG and DMPC monolayers as a function of time at given molecular area are observed. The decrease in γ values shows that there is a penetration effect of Met-enk molecules into the monolayers.

The comparison of the interaction between the hydrophobic pentapeptide and both phospholipid monolayers shows that the enkephalin interacts similarly with DMPG and DMPC molecules, and that the ability of Met-enk to penetrate correlates with the given surface area per lipid molecule.

Based on the results obtained we suggest that both the phospholipids polar head size and charge do not play a significant role in the process of penetration, and that for the latter effect the hydrophobic molecular interactions are dominated.

Design, synthesis, analysis and pharmacological evaluation of new neuropeptide mimetics: analogues of MIF-1 and endormorhin

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The problem of the efficient therapy of pain and epilepsy are important not only from clinical but from social and economic point of view. The search of highly effective analgesic of the central action with minimal side effects and devoid of narcogenic activity is of a particular interest for applied medicine. Creation of highly effective analgesics based on the structure of endogenous opioid peptides (OPs) seems to be perspective due to a number of reasons: there is a target in an organism for them; there are systems of peptide degradation that decrease their possible toxicity; peptides finely control the modulation of physiological processes; and peptides are active at a low dozes.

However, natural peptides are often excreted rapidly or do not pass biological membranes resulting in a poor bioavailability. In respect to overcome the problems associated with their low bioavailability, rapid metabolism, and lack of oral activity our special interest was focused on designed synthesis and screening of new neuropeptide mimetics: in particular MIF-1 and endormorhin related compounds

Here, we describe the synthesis of a series of tetrapeptide mimetics containing non-protein amino acids, utilizing both conventional and solid phase methods. The performance of two methods was also compared.

We studied analgesic effects of analogues of MIF-1 and endormorhin, modified at position 2 with unnatural amino acids Cav, NCav, sLys, and NsArg. Antinociceptive effects were evaluated using the paw pressure (PP) test.

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Astrocytic glutamate induces long-term potentiation of synaptic transmission

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Accumulating evidence indicates the existence of bidirectional communication between astrocytes and neurons. However, the effects of astrocytes on action potential-evoked synaptic transmission at single synapse level are largely unknown.

We investigated the neuromodulatory role of astrocytes on synaptic physiology at single hippocampal synapses. Using electrophysiological and Ca^{2+} imaging techniques on rat hippocampal slices,

we performed paired recordings from CA1 pyramidal neurons and single astrocytes. Astrocytes were loaded with the Ca^{2+} indicator Fluo 4 (50 M) and the Ca^{2+}-cage NP-EGTA (5 mM) and were selectively stimulated by UV-flash photolysis (2 Hz, 5 s). Single synapses of Schaffer collaterals were stimulated at 0.5 Hz.

We found that:

The selective elevation of Ca^{2+} in single astrocytes transiently increased the synaptic efficacy due to the potentiation of the probability of transmitter release, without affecting the amplitude of synaptic currents.

This form of short-term plasticity was due to SNARE protein- and Ca^{2+}-dependent release of glutamate from astrocytes, that activates presynaptic type I metabotropic glutamate receptors (mGluRs).

The concurrent activity of astrocyte Ca^{2+} elevation and postsynaptic neuron caused the persistent potentiation of synaptic transmission. Therefore, the temporal coincidence of neuronal and astrocytic signals induced the long-term potentiation (LTP) of hippocampal synaptic transmission.

LTP was independent of NMDA receptor activation and postsynaptic intracellular Ca^{2+}. However, LTP was prevented by blockage of presynaptic type I of mGluRs and synthesis of nitric oxide.

We conclude that astrocytes potentiate synaptic transmission playing an active role in the transfer and storage of synaptic information by the nervous system.

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Phosphorylation stoichiometry: the missing dimension in quantitative phosphoproteomics

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Once a protein phosphorylation site is identified and localized, quantification of this site is paramount to determine the regulatory significance of the named site. Mass spectrometry is the method of choice for such analysis as it can provide protein identity, phosphorylation site localization and quantitative information. When performing quantitative protein phosphorylation analysis two aspects have to be addressed: i) how does the abundance of a particular phosphopeptide change over time, location, state, etc., and ii) what is the degree of phosphorylation. The most currently available quantitative phosphoproteomics methods, irrespective of whether they use metabolic labeling or derivatization strategies address the first aspect of quantitative phosphoproteomics, but not the second aspect: the quantification of phosphorylation stoichiometry. The importance of this 'missing dimension in quantitative phosphoproteomics' is obvious when considering that very low degrees of phosphorylation are sufficient for activating phosphorylation sites, but high phosphorylation stoichiometries are necessary for inhibiting sites. Here, currently available, and novel label-free and stable isotope-assisted mass spectrometric methods for the quantification of protein phosphorylation stoichiometries will be discussed.

Quantifying phosphorylation in cytokinesis

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Animal cells undergo cytokinesis during a brief window in the cell cycle; this phase is called C-phase, and starts shortly after anaphase onset and lasts ~30–60 min. Cytokinesis requires coordinated reorganization of microtubules, actin and membranes, which implies global regulation of cellular biochemistry. Unlike all other cell stages, we have little knowledge about how C-phase is regulated, and how it compares to other phases. One likely locus of C-phase regulation is microtubule binding proteins. Microtubules change dramatically in organization from the highly dynamic mitotic spindle in M-phase to the less dynamic midzone and astral arrays in C-phase. Another likely locus of regulation is proteins that interact with mitotic kinases, and direct changes in their localization and substrate specificities. We used mass spectrometry-based proteomics to characterize changes in protein biochemistry in C-phase. We analyzed changes in abundance and phosphorylation of microtubule binding partners as cells enter C-phase. More than 1000 proteins were identified with 2 or more quantified peptides in microtubule sedimentation assays; 25 proteins selectively bind to microtubules in C-phase, including known midzone proteins such as NUSAP. Analysis of C-phase-specific phosphorylation sites on these proteins suggested that Aurora B plays a central role in promoting their microtubule binding during C-phase. Perturbation experiments using inhibitors of Aurora kinases showed this to be the case. We identified ~250 candidate microtubule binding proteins that were affected by the VX680 treatment cells including many microtubule binding proteins we previously identified as being C-phase selective interactors. Aurora B binding partners switched dramatically between M-phase to C-phase, and we identified several novel C-phase binding partners include PRC1, KIF4 and the Anaphase Promoting Complex/cyclosome (APC/C), suggesting targeting of the kinase to its substrates changes dramatically between M- and C-phase. We conclude that dramatic switching of binding partners by Aurora B is an important regulatory mechanism in M-phase to C-phase transition.

Trafficking of excoytic vesicles to and from the fusion site

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We study the secretory activity of astrocytes in a number of ways. By the patch-clamp technique membrane capacitance (C_m), a parameter reflecting surface area changes, is monitored. Fluctuations in C_m reflect the activity of exo- and endocytosis. The results have shown that a calcium-dependent process operates in astrocytes, however, although similar to neurons, the process is at least one order of magnitude slower. Calcium dependent increase in C_m may result from the fusion of a number of vesicle-containing structures and the slow kinetics may be in part due to the slow vesicle delivery to the fusion site. Therefore, we also monitored single vesicles by fluorescently tagged peptides, such as the atrial natriuretic peptide (ANP) by confocal microscopy. We observed that these structures exhibit remarkable rectilinear mobility, suggesting a role for cytoskeleton in vesicle traffic and in secretory activity. To study the role of the cytoskeleton in vesicle trafficking we labelled different types of vesicles and characterized their mobility with confocal microscopy and quantitative image analysis in different experimental conditions. Pre-fusion transport (vesicle transport to the fusion site

for exocytosis) of peptidergic vesicles was studied by fluorescently labeled ANP; and post-fusion (post-exocytosis) transport of glutamatergic and peptidergic vesicles was studied by labeling vesicles with antibodies against specific membrane or lumenal vesicle proteins. Studies revealed that both pre-fusion and post-fusion traffic strongly depended on cytoskeleton, actin and microtubules. Interestingly, directional mobility of peptidergic vesicles to the exocytotic site required intact intermediate filaments. Unlike the pre-fusion vesicle traffic, which is insensitive to increases in cytosolic calcium $[Ca^{2+}]_i$ increases, the mobility of post-fusion vesicles was sensitive to cell stimulation. Peptidergic vesicles and lysotracker-positive vesicles exhibited stimulation-dependent mobility inhibition, whereas vesicles taking the anti-VGLUT1 antibody exhibited an increased mobility after cell stimulation by ionomycin, which increases cytosolic calcium. These results show that distinct vesicle groups respond to stimulation in a distinct manner. Moreover, intermediate filements mediate stimulation-dependent fusion in all vesicle types studied, however in a distinct manner. Since intermediate filaments get overexpressed in a number of CNS pathologies we propose that vesicle traffic is affected selectively, contributing to changes in astrocytes vesicle dynamics which is a key element of communication with the neighbouring cells.