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Annexins, targets and calcium-binding proteins in pathology

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Abstracts

Invited Lectures

New Ca^{2+}-buffers for enhanced performance

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Diastolic heart failure (DHF) is a clinical syndrome characterized by depressed myocardial relaxation performance and poor ventricular refilling. Defective intracellular calcium (Ca^{2+}) handling underlies one of the fundamental mechanisms of DHF. Manipulating the content and function of Ca^{2+} handling proteins in the heart has been the focus of intense study to develop effective therapies for DHF patients. Parvalbumin (Parv), a skeletal muscle Ca^{2+} binding protein, has been shown to facilitate myocardial relaxation both in vitro and in vivo. Parv acts as a unique "delayed" Ca^{2+} buffer and facilitates Ca^{2+} sequestration from cytosol. Here, we summarize studies employing gene transfer of Parv in cultured adult cardiac myocytes and in vivo to redress depressed diastolic function. By targeting defects in cardiac Ca^{2+} handling, Parv represents a promising therapeutic candidate for alleviating diastolic dysfunction in DHF.

Family ties: annexins, copine, and sorcin

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Annexin A1 and Copine I. Atomic force microscopy was used to monitor the assemblies formed in vitro on lipid bilayers by annexin A1 and copine I. When applied to supported bilayers composed of 25% brain phosphatidylserine and 75% dioleyl phosphatidylcholine in the presence of 1 mM Ca^{2+} both human annexin A1 and human copine I bound only to specialized domains that appeared to be 0.5 to 1.0 nm lower than the rest of the bilayer. These domains may be enriched in phosphatidylserine and have a more disordered structure allowing probe penetration. The binding of the annexin promoted the growth of the domains and created additional binding space for the copine. This may reflect a general ability of annexins to alter membrane structure in such a way that C2 domain-containing proteins like copine can bind.

Novel protein ligands for the annexin A7 N-terminal region. The N-terminal regions of annexins A7 (synexin) and A11 consist of an extended series of short sequence repeats rich in tyrosine, proline, and glycine that provide binding sites for other proteins, including the EF-hand protein sorcin. We recently identified two new potential ligands for the annexin A7 N terminal region by yeast two hybrid screening: the TNFα receptor regulatory protein SODD (Suppressor Of Death Domains) and KIAA0280, a protein of unknown function. Strikingly, the sites of interaction of these proteins with the annexin also contain sequence repeats similar to those present in the N-termini of annexins A7 and A11, and in the annexin A7-binding domain of sorcin. It was also found that the annexin A7 N-terminal region interacts with itself in the two hybrid assay. These results suggest that sequence repeats of this nature form novel structures, called YP pro-β helices, that are characterized by an ability to interact with one another. Specificity of interactions between the pro-β helices in different proteins may be encoded by the variations of residues and lengths of the sequence repeats.

Structure and function of annexin-S100 complexes

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Annexins are a family of Ca^{2+}-regulated membrane binding proteins involved in different membrane related events that include the regulation of membrane-cytoskeleton interfaces and certain membrane transport processes. Several annexins form specific and high affinity complexes with EF hand type Ca^{2+}-binding proteins, in particular those of the S100 protein family. We have characterized complex formation of annexin A1 with S100A11 and annexin A2 with S100A10. The latter appears to participate in the regulation of $PI(4,5)P_2$ rich domains in the plasma membrane and the surface delivery of a number of ion channels and receptors, which bind to the S100A10 protein. Structural aspects annexin-S100 complexes and the role of S100A10 interactions with the Ca^{2+} channel TRPV5 will be discussed.

Annexin A2 in endosome biogenesis

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During endocytic membrane transport, cell surface molecules destined for late endosomes and lysosomes, e.g. epidermal growth factor (EGF) receptor and other downregulated signaling receptors, are sequentially incorporated into membrane invaginations with opposite topology. After internalization via clathrin-coated vesicles (or caveolae), which bud into the cytoplasm, and delivery to early endosomes, ubiqitinated receptors are sorted into intralumenal vesicles that form within multivesicular regions of the early endosome. These regions then detach — or mature — from early endosomes, giving rise to a multivesicular endosome (or multivesicular body, MVB). Eventually, lumenal vesicles and their protein cargo are delivered to late endosomes and lysosomes for degradation. The biogenesis of multvesicular endosomes thus involves the concomitant deformation of early endosomal membranes towards the cytosol (to form the endosome itself) and towards the endosome lumen (to form intralumenal vesicles). We find that the formation of intralumenal vesicles is controlled by the PI3P-binding protein SNX3, while EGF receptor sorting depends, as expected, on Hrs — another PI3P-binding protein. By contrast, our data suggest that the biogenesis of the endosome itself — but not the intralumenal invagination process — is regulated by actin patches, presumably because these patches drive the membrane remodeling process. We find that this mechanism depends on AnxA2, an actin binding protein, the actin nucleation factor Spire1, and Arp2/3, which is necessary for filaments branching. Similarly, we find that AnxA2 itself is also required, much like actin, for endosome biogenesis — but not for intralumenal invagination. This activity of AnxA2 in endosome biogenesis is regulated by phosphorylation of AnxA2 Tyr23, and does not seem to depend on the AnxA2 light chain p11/S100A10. Our data indicate that, during multivesicular endosome formation, SNX3 and AnxA2/actin regulate the deformation of early endosome membranes towards the cytosol and the lumen, respectively.

Regulation of CFTR function by annexin A2-S100A10 complex in health and disease

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Annexin A2 and S100A10 proteins form a heterotetrameric complex and belong to different families of Ca^{2+}-binding proteins. Annexins are non-EF-hand-type Ca^{2+}-binding proteins that exhibit Ca^{2+}-dependent binding to phospholipids and membranes in various tissues. They have been implicated in many Ca^{2+}-regulated processes, including regulation of membrane organization, trafficking and interact with many targets such as ion channels. S100 proteins comprise a family of small proteins characterised by the presence of two consecutive EF-hand type Ca^{2+}-binding motifs, interact with ion channels and regulate diverse processes and play a role as Ca^{2+} sensors. Several annexin–S100 complexes have been characterized and require calcium. In this regard, S100A10 binding to annexin A2 is an exception in that it is regulated by a post-translational modification of annexin A2 and occurs independently of calcium concentration. This review focuses on the regulatory mechanism behind annexin A2–S100A10 complex formation, its role in regulating chloride transport in health and cystic fibrosis and the potential of this mechanism to integrate calcium and cAMP signalling in airway epithelia. We propose that cAMP/PKA-dependent activation of chloride flux (through CFTR and ORCC) requires the mobilisation of a multi-protein complex involving calcium binding proteins from three different families (annexin 2, S100A10 and Calcineurin A).

Regulation of annexin A2 and S100A10 expression on the endothelial cell surface

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Annexin A2 (A2) and its binding partner S100A10 (p11) form a heterotetrameric complex on the surface of vascular endothelial cells. This complex interacts with plasminogen and its endothelial cell-derived activator, tissue plasminogen activator (tPA), enhancing the catalytic efficiency of plasmin generation. Complete absence of A2 leads to a prothrombotic diathesis and angiogenic defects in mice. We have hypothesized that translocation of A2 from the cytoplasmic compartment to the endothelial cell surface is a dynamic process that regulates plasmin activation during periods of vascular stress or injury. Translocation of this "signal-less" protein follows brief temperature stress or stimulation with thrombin, and does not require an intact endoplasmic reticulum. The process involves src kinase mediated phosphorylation of A2 at tyrosine 23, and absolutely requires expression of p11. Interestingly, p11 is expressed at very low levels in A2-null cells both *in vivo* and *in vitro*. In the absence of A2 in the endothelial cell, unpartnered p11 becomes polyubiquitinated and rapidly degraded via a proteasome-dependent mechanism. A2 stabilizes intracellular p11 through direct binding, thus masking an autonomous polyubiquitination signal on p11 that triggers its destruction. This mechanism prevents accumulation of free p11 within the endothelial cell and suggests that regulation of tPA-dependent cell surface fibrinolytic activity is precisely tuned to the intracellular level of p11. These data suggest that A2 and p11 fulfill distinct, but related roles in regulating activation of plasmin and associated proteolytic cascades.

Annexin A2 – friend or foe

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Annexin A2 knock-out mice are essentially healthy and viable, a feature of most annexin knock-out mice. If one assumes all genes to be essential (otherwise why would they be conserved in evolution), then the lack of overt phenotypes in annexin knock-out mice demands explanation. One possibility is functional redundancy between individual annexin family members, but in general there is little evidence i) that annexins have sufficiently overlapping activities that would permit such complementation of function, or ii) that targeted gene disruption of one annexin leads to changes in expression that might be expected in other annexins. Our studies in the annexin A2 knock-out mouse have revealed a role for this protein in retinal phagocytosis, such that the mutant mouse exhibits a delay in the uptake of shed photoreceptor outer segments following diurnal shedding. Despite this abnormality, the retina is not adversely affected in these mice, and indeed in the long term we observed that as these mice age their visual function is actually better than that of similarly aged littermates. This would indicate, surprisingly, that annexin A2 somehow contributes to the decline in visual function that accompanies ageing in all mammals. In a second study, we examined the effects of inducing diabetes in the annexin A2 knock-out mouse. Annexin A2 mice, when diabetic, develop a much more severe pathology than normal diabetic mice, attributable to defective kidney function and most likely linked to a role for annexin A2 in water reabsorption in the epithelial cells of the loop of Henlé. These mice succumb rapidly to the pathological consequence of diabetic nephropathy while their genetically normal diabetic littermates remain almost unaffected. Taken together, these studies suggest that in the mouse annexin A2 is actually both friend and foe, acting in a protective manner in the disease setting (diabetes), but contributing to the decline in sensory function in normal healthy ageing.

Approaches to unique S100 target protein interactions

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The S100 proteins are dimeric, calcium-binding molecules with 25 members in humans. Most members of this family undergo a calcium-induced conformational change allowing the S100 protein to interact with range of possible biological targets. Although a wealth of data exists from *in vitro* experimentation, the calcium-sensitivity for most of the S100 proteins makes traditional *in vivo* identification of target proteins and their interactions with S100 proteins quite difficult to address. In contrast, S100A10 does not bind calcium, and retains a calcium-like conformation having a structure nearly identical to other calcium-bound S100 proteins such as S100A11. In the absence of calcium S100A10 responds well to two-hybrid, co-immunoprecipitation and other *in vivo* methods to identify targets such as annexin A2, AHNAK and several membrane channel proteins. Using S100A10 as a template, our lab has initiated efforts to design a calcium-insensitive S100 protein to use for *in vivo* experiments. The initial results show that minor changes in the S100 sequence can have dramatic changes in the overall hydrophobicity of the protein. We have also engineered hybrid S100A10 and S100B proteins comprised of the S100 protein and a target protein arranged in tandem. These proteins effectively mimick an S100-target complex and are being used to identify the interactions and structures of multiprotein S100 complexes that include the annexins. The design of these proteins and initial experiments will be discussed.

The calcineurin/NFAT pathway in lymphoid malignancies

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Calcineurin is a calcium-activated serine/threonine phosphatase critical to pre-TCR signaling during thymocyte development, to TCR-mediated positive selection of thymocytes into mature T cells and to many aspects of the immune response. The critical role of calcineurin in the immune response is underscored by the fact that calcineurin inhibitors, such as cyclosporine A (CsA) and FK506 are powerful immunosuppressants of wide clinical use. We have observed sustained calcineurin activation in human B- and T-cell lymphomas and in mouse models of lymphoid malignancies. In these models, inhibition of calcineurin activity by CsA or FK506 induced apoptosis of leukemic cells, rapid tumor clearance and significantly prolonged mouse survival. Conversely, ectopic expression of a constitutively activated mutant of calcineurin favored leukemia progression [1][2]. Ongoing loss- and gain-of-function genetic approaches aimed at dissecting the function of calcineurin in mouse models of T-ALL/lymphomas will be discussed.

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Calpain inhibition improves synaptic function and memory in a mouse model of Alzheimer's disease

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Alzheimer's Disease (AD) is a devastating disorder that affects a large percentage of the senile population. The disease is thought to start with subtle synaptic changes leading to memory loss, probably due to amyloid-beta (Aβ) species, a group of peptides that is elevated in AD. Activation of the calpain system is likely to contribute to these changes [1-3]. Calpains regulate the function of many proteins by limited proteolysis and initiate the degradation of other proteins. In particular, they modulate processes that control the function and metabolism of proteins key to the pathogenesis of AD, including tau [4, 5] and Aβ precursor protein [6-8]. During the last 10 years we have investigated whether and how Aβ interferes with both memory formation and the regulation of hippocampal synaptic function. I will now present data showing that inhibition of calpains rescues the defect of synaptic transmission both in mouse models of amyloid elevation such as the APP/PS1 mouse and the APP mouse as well as after elevation of Aβ. I will also discuss our studies on the beneficial effect of calpain inhibition against the reduction of spatial-working memory and associative fear memory both in double transgenic APP/PS1 mice and single transgenic APP animals. Finally, I will present data showing that Aβ-induced down-regulation of phosphorylation of the memory-related molecule CREB is rescued by calpain inhibition. Taken together, these findings suggest that calpain inhibitors may prove useful in the alleviation of memory loss in AD.

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Structural bioinformatics of calcium-binding proteins associated with allergic diseases

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Calcium binding proteins are involved in allergic diseases as allergens or in regulatory pathways. The increasing number of structurally resolved proteins facilitates the application of structure based bioinformatics (BI) approaches to study antigen recognition and processing or to support the development of therapeutic allergen derivatives.

The most relevant BI methods are structure comparison, structure modelling and prediction and In-Silico mutagenesis. For structure alignment we developed a new method which can deal with structural variability and permutation in sequence order. For structure modelling we apply standard methods such as MODELLER or SwissModel. In-Silico mutagenesis is based on a statistical potential approach.

Here we concentrate on allergens. We give a short introduction to the BI methods and present several examples how they have been used to investigate relations between allergens and for the development of hypoallergenic proteins potentially applicable for desensitization.

Inhibition of S100A4-induced tumor growth and metastasis by intervening β-catenin signaling

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Colon cancer metastasis is often associated with activation of the Wnt/β-catenin signaling pathway and high expression of the metastasis-inducing gene S100A4. We identified previously S100A4 as a transcriptional target of β-catenin and thus highlighted the importance of the interconnection of these cellular programs for colon cancer metastasis [1]. Intervention strategies targeting Wnt/β-catenin signaling might represent promising approaches to inhibit S100A4-induced tumor growth and metastasis in vivo. Here we report the effects of nonsteroidal anti-inflammatory drug (NSAID) sulindac sulphide, a known inhibitor of the Wnt/ β-catenin signaling pathway, on S100A4-induced tumor growth and metastasis on human colon cancer cells. We demostrate the knock down of S100A4 expression and S100A4 promoter activity by sulindac, associated with reduced S100A4-induced cell motility and invasiveness in vitro, as well as with inhibition of tumor growth and liver metastasis in vivo. Our data provide evidence that modulators of β-catenin signaling such as NSAIDs may offer potential as antimetastatic agents by interdicting S100A4 expression [2].

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S100B in psychiatric disorders

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Recent research has supported a potential role of immune pathology in the etiopathogenesis of schizophrenic and affective psychoses. For schizophrenia specific and unspecific evidence has been obtained. In the CNS various viruses (e.g. HERV, BDV) were identified in the brains of schizophrenic patients. Pro-inflammatory cytokines were found to be associated with the stage of disease. Microglial cells were reported to be activated in a subgroup of schizophrenic patients in post mortem as well as imaging studies. For affective disorders, especially depression, evidence has also emerged that there is an activation of the innate inflammatory immune response including alterations in the ability of immune cells to express proinflammatory cytokines. It has been shown that peripheral immune signals can lead to an exacerbation of sickness and the development of symptoms of depression in vulnerable individuals. This illustrates that inflammation is an important biological event that might increase the risk of major depression.

Until recently, astrocytes were regarded as mere supporters of neurons regulating the environmental milieu. New research, however, has demonstrated that astrocytes together with microglial cells are the major immunocompetent cells of the brain and play an important role in the regulation of neuronal proliferation and differentiation. Since neuronal remodelling appears to be a relevant pathogenic factor in various psychiatric disorders the role of astrocytes needs to be evaluated. S100B, a calcium binding astrocyte-specific cytokine, presents a marker of astrocytic activation.

Several independent studies showed increased S100B levels in medicated acutely psychotic patients with schizophrenia and drug naïve schizophrenics. A positive correlation between negative symptoms and S100B was described. In a longitudinal approach over 24 weeks a continuously increased S100B concentration was associated with continuity of negative symptoms and deceleration of therapeutic

response. Cognitive deficits are observed primarily in patients with persistently elevated concentrations of S100B. Increased S100B concentrations are associated with increased myo-inositol, another astrocytic marker measured by MRSpectroscopy.

In acute major depression S100B has been found to be significantly increased directing towards astrocyte activation. Obviously, this phenomenon is limited to the more biologically determined types of depression such as the melancholic subtype. In these patients a moderately elevated S100B concentration seems to be beneficial since patients with higher S100B showed better response and remission rates. Antidepressant treatment appears to normalize S100B concentrations. On a functional level it could be shown that depressed patients with increased S100B experience a better normalization of initially pathological evoked potential (ERP) patterns than patients with unchanged S100B. Even three months after psychopathological remission only those patients with primarily higher S100B showed normal ERP patterns while in patients with initially normal S100B the pathological patterns remained.

These findings suggest that the activation of astrocytes is an important pathogenic factor for the development of schizophrenia and depression. Astrocytic activation is associated with course of disease, treatment response, and functional outcome. This exemplarily illustrates the importance of immunological mechanisms in the etiopathogenesis of major psychiatric disorders.

Interplay between P-glycoprotein mediated multidrug resistance and intracellular calcium homeostasis

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Multidrug resistance (MDR) of neoplastic tissue represents real obstacle in effective chemotherapy of cancer. Several mechanisms of MDR were identified, from which overexpression and efflux activity of P-glycoprotein (P-gp) – plasma membrane ATPase (ABCB1 member of ABC transporter family) – represent the most common observed reason of neoplastic diseases chemotherapy misfunction.

Process of P-gp mediated MDR seems to be related to intracellular calcium homeostasis at least indirectly because: i. substances blocking calcium influx through L-type of calcium channels like verapamil were often found to antagonize P-gp mediated MDR; ii. calcium signal abnormalities were observed in cells overexpressing P-gp; iii. cells with P-gp mediated MDR were often resistant to thapsigargin; iv. several differences in intracellular calcium localization were observed when P-gp negative and P-gp positive cells were compared; v. differences in contents of several proteins of endoplasmic reticulum involved in calcium homeostasis were observed to be associated with P-gp overexpression. The current study represents an attempt to summarize knowledge about possible relations between P-gp mediated MRD and intracellular calcium homeostasis.

T-type calcium channels in health and disease

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T-type or low voltage-activated calcium channels are ubiquitously expressed in mammalian excitable cells. They are distinguished from high voltage-activated channels by low voltage threshold for activation. Therefore, these channels are able to initiate so-called low voltage calcium spikes and to contribute to specific types of excitability in neurons or in sinoatrial nodus cells. In health these channels regulate cardiac pacemaking or thalamocortical sleep rhythm. Their mutations underlie certain channelopathies, e.g., various types of epilepsy or bradycardia. The knowledge of the precise mechanisms that control the gating of LVA channels will potentially open new possibilities for the treatment of frequent disorders.

Voltage-gated calcium channels (VGCC) including LVA channels are composed of four homologous domains, each containing six transmembrane segments S1-S6 and a pore loop (P) between segments S5 and S6. Four voltage-sensing domains each composed of the transmembrane segments S1 - S4 are placed peripheral to a central pore domain formed by four sets of S5-P-S6. Most important role in Ca_v3 channel activation and inactivation has the voltage sensor in the domain III while than in the domain IV lacks a crucial role. The voltage sensors in domains I and II contributed intermediately to channel gating.

In addition to the low voltage activation threshold of Ca_v3 channels, the nonlinear charge movement reflecting conformational changes of the S4 segment in the putative voltage sensor of the channel i) precedes the ion current reflecting pore opening by less than 10 mV; and ii) only about 20% of the total charge is moved when inward current activation is already saturated. These peculiar features lead to a hypothesis that the pore of T-type calcium channel may be partly open before its voltage sensor is fully transferred into an activated state. Such interpretation is supported by recent discovery of the gating brake in the I-II loop of the $Ca_v3.2$ channel. This putative gating brake may prevent Ca_v3 channels from opening at membrane voltages close to cell resting potential as minimal movements of voltage sensor are sufficient for full opening of the conductive pore.

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Calcium binding chaperones of the endoplasmic reticulum

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The endoplasmic reticulum is a major Ca^{2+} store of the cell that impacts many cellular processes within the cell. The endoplasmic reticulum has roles in lipid and sterol synthesis, protein folding, post-translational modification and secretion and these functions are affected by intraluminal endoplasmic reticulum Ca^{2+}. In the endoplasmic reticulum there are several Ca^{2+} buffering chaperones including calreticulin, Grp94, BiP and protein disulfide isomerase. Calreticulin is one of the major Ca^{2+} binding/buffering chaperons in the endoplasmic reticulum. The protein has a critical role in Ca^{2+} signalling in the endoplasmic reticulum lumen and this has significant impacts on many Ca^{2+}-dependent pathways including control of transcription during embryonic development. In addition to Ca^{2+} buffering the calreticulin plays important role in correct folding and quality control of newly synthesized glycoproteins.

Cross-talk of intracellular calcium stores in the response to neuronal ischemia and ischemic tolerance

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Ischemic/reperfusion brain injury (IRI) is very severe event with the multiple etiopathogenesis. Ischemic preconditioning (IPC) is the phenomenon of adaptation of CNS to ischemic insult. An altered cross-talk between intracellular Ca^{2+} stores is presumed in the mechanisms of IRI. We show here that IRI leads to the inhibition of mitochondrial respiratory complexes I and IV, however Ca^{2+} uptake rate is not significantly depressed. IPC acts at the level of initiation and execution of mitochondrial apoptosis and activates inhibition of p53 translocation to mitochondria. [1] In addition, IRI initiates a time dependent differences in endoplasmic reticular (ER) gene expression of the key UPR proteins which is affected by preischemic treatment by the expression of Ca^{2+} binding GRP78 and ATF6 proteins. The expression pattern of the secretory pathways Ca^{2+} pump (SPCA1) after IRI is remarkably affected by IPC and IPC leads to partial recovery of depressed SPCA activity. [3] Functional alterations of mitochondria, ER and SP contribute to the understanding of cross-talk between neuronal Ca^{2+} stores in ischemia and ischemic tolerance and might suggest for targets of therapeutic interventions to enhance recovery after stroke.

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Transcriptional regulation and role of S100P calcium-binding protein in cancer cells

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S100P is an EF-hand calcium-binding protein that was originally identified in placenta and subsequently linked to cancer. It is a member of S100 family of proteins functioning as extracellular and/or intracellular regulators of diverse cellular processes and contributing to various human pathologies. S100P expression was detected in a range of human tumor cell lines and tissues, particularly those derived from breast, prostate, pancreas and colon, where it was connected with malignant phenotype, hormone independence and resistance to chemotherapy. In line with these observations, forced overexpression of S100P was shown to promote tumorigenesis in prostate, breast and cervical cancer models. Functional studies of S100P indicate that it operates either via intracellular interaction with ezrin, leading to increased cell migration, or via extracellular signaling through RAGE receptor, resulting in increased proliferation and survival. Molecular mechanisms regulating expression of S100P in cancer cells are just starting to emerge. Besides earlier described DNA methylation, recent studies revealed involvement of bone morphogenic protein and non-steroidal anti-inflammatory drugs in control of S100P expression during tumor progression. We performed functional analysis of S100P promoter and identified SMAD, STAT/CREB and SP/KLF binding sites as key regulatory elements participating in transcriptional activation of S100P gene in cancer cells. Moreover, our latest data reveal that expression of S100P is up-regulated by activation of glucocorticoid receptor suggesting that S100P could play a role in therapy resistance mediated by glucocorticoids in solid tumors.

Posters

The effects of ischemia/reperfusion on matrix metalloproteinases in rat hearts

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Matrix metalloproteinases (MMPs) are a family of Zn^{2+} and Ca^{2+}-dependent endopeptidases involved in remodeling of extracellular matrix. MMP-2 has been also shown as a primary mediator of the acute mechanical dysfunction of the heart after ischemia/reperfusion (I/R). The aims of the study were to investigate the influence of I/R on MMP-2 and to study the effects of wortmannin (WT) and LY294,002 (LY), specific inhibitors of PI3K/Akt kinase pathway, on modulation of MMP-2 activities after ischemic preconditioning (IP, cycles of short I/R). In the study isolated Langendorff-perfused rat hearts subjected to protocols of test I/R and/or IP were used. WT or LY were infused before and during the reperfusion phases of IP. The levels and activation of proteins were determined by immunoblot assay. The MMP activities were measured by zymography. We found that ischemia induced activation of tissue pro-MMP-2 with maximum reached after 15 min of ischemia. During prolonged ischemia and the following reperfusion the activities of this form of MMP-2 declined. Short ischemia and reperfusion that led to increased cardiac tolerance against prolonged I/R reduced MMP-2 activities and induced also an activation of Akt kinase. The application of both WT and LY was connected with inhibition of IP-mediated Akt kinase activation and modulation of MMP-2 activities. Our results suggest that MMP-2 may be involved in the responses of rat hearts to ischemia and point to possible relationship between Akt kinase and modulation of MMP-2 activities in rat hearts.

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Structures and dynamics of S100 proteins

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The dynamical properties of S100A2, S100A5, S100A16 and S100P have been investigated by NMR. The analysis of the R_1, R_2 and NOE values indicates for each protein the regions experiencing a large flexibility. In particular the hinge loop region is characterized by a mobility faster than protein tumbling. The interaction of members of the S100 family with RAGE, and the structural and dynamic properties of the complex have been characterized to obtain information on the molecular species responsible for RAGE activation by extracellular S100 proteins.

Targeting calcium channels specifically expressed by Th2 cells prevents experimental allergic asthma

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Allergic asthma is a chronic inflammatory disease of the lungs which prevalence and severity are both increasing. T helper (Th) lymphocytes include T-cell subsets that exert different functions. Among them, Th1 and Th2-cells produce interferon gamma and interleukin (IL-)4 respectively. Asthma arises from aberrant $CD4^+$ T-cell-mediated immune responses, with a Th2-cell phenotype. Ca^{2+} signalling in Th2-cells is responsible for the production of IL-4, IL-5 and IL-13 that are all deleterious in asthma. Signalling pathways and especially calcium regulation differ between Th1 and Th2-cells with little being known in Th2-cells. Our aim was to investigate the steps controlling the calcium response in Th2 lymphocytes. We demonstrated that dihydropyridine receptors (DHPR) were expressed in Th2 but not in Th1 murine cells, and that they were involved in TCR-dependent calcium response. Moreover, DHPR controlled Th2-cytokine production (IL-4, IL-5, IL-10 and IL-13). Th2 cell differentiation was associated with an up-regulation of DHPR and these channels were identified at the molecular level as calcium channels related to those expressed by excitable cells. A DHPR antagonist or antisense oligonucleotides directed against these channels suppressed both calcium response and Th2 cytokine production without any effect on Th1 cells. In a murine model of allergic asthma, we showed that lung-infiltrating $CD4^+$ T-cells expressed DHPR. DHPR antagonist or specific antisense oligonucleotides prevented all the features of allergic asthma by abrogating Th2-mediated inflammation in an active model of allergic asthma and following adoptive transfer of effector Th2-cells. However, nicardipine did not prevent lung inflammation consecutive to Th1-cell transfer, emphasizing Th2-cell specific targeting. Th2-cells transfected with antisense oligonucleotides also showed an impaired capacity to trigger asthma when compared to control transfected Th2-cells. Altogether these data indicate that these Ca^{2+} channels are crucial in Th2 but not in Th1-cell signalling and that they could be a target to prevent allergic asthma.

Evaluation of RAGE dependent S100A4 signaling for intervention strategies against metastasis formation in colorectal cancer

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Colon carcinoma, due to its high potential of metastasis formation, is still a major cause of death even after the excision of the primary tumor. A search for metastasis related marker proteins showed the involvement of the small calcium binding protein S100A4 in metastatic progression and the expression level of S100A4 in tumor cells is positively correlated to the metastatic potential of human colon cancer and predictive for metachronous metastasis formation [1]. It promotes cell motility and invasion, as well as angiogenesis, and has been shown to interact both with cytosolic proteins and extracellular factors. The cell surface receptor RAGE is controversially discussed as a mediator of extracellular S100A4 signaling; but, although the physical interaction of both proteins has been demonstrated, S100A4 signaling can also occur independently of RAGE. In this study we analyze the effects of extracellular S100A4 in colorectal cancer cell lines on cancer specific signaling pathways, cell motility and invasion with respect to forced expression or RNAi mediated knock down of RAGE and examine therapeutic strategies to prevent S100A4 induced metastasis formation in colon carcinoma.

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The function of external domain in the first repeat of the $Ca_v3.1$ channel

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A sequence comparison between low and high voltage activated calcium channels revealed an extremely large external domain within the first repeat of all Ca_v3 subunits located between the IS5 transmembrane segment and the pore loop (P), which encompasses nearly 100 residues. The function of this domain containing 6 conserved cysteine residues is unknown. The aim of our study was to analyze role of cysteines in external IS5-P linker of the $Ca_v3.1$ channel. Each of cysteines was exchanged for an alanine. Resulting mutants (C241A, C271A, C282A, C323A, C298A and C313A) were transfected into HEK 293 cells. Whole-cell patch-clamp was used for current analysis. Four of channel mutants did not form functional channels: C241A, C271A, C282A and C323A, possibly because of incorrect protein folding. Two remaining channels C298A and C313A were capable of carrying inward calcium current. Application of reducing agent DTT altered current amplitude: $Ca_v3.1$: 77 ± 4 %, C298A: 40 ± 4 %, C313A: 115 ± 2 %. DTNB (Ellman's reagent) inhibited current through all three channels: $Ca_v3.1$: 1 ± 1 %, C298A: < 1 %, C313A: 48 ± 4 %. All values refer to current amplitude in presence of each reagent expressed as percentage of current amplitude measured under the control conditions.

We concluded that all cysteines in IS5-P linker of the $Ca_v3.1$ channel are essential for channel function. However, nature of their contribution is different.

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Characteristics of the S100P-IQGAP1 interaction

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S100P is an EF-hand calcium-binding protein initially identified in placenta that interacts with the cortical actin regulator ezrin. Here we identified the multi-domain protein IQGAP1 as another specific interaction partner of S100P. By association with numerous binding partners IQGAP1 is involved in different cellular activities such as modulation of the actin cytoskeleton, microtubule dynamics, cell-cell-adhesion, gene transcription and signaling pathways. To further investigate the S100P-IQGAP1 interaction and to map the respective binding motives we performed affinity chromatography approaches with mutant forms of the two proteins. Our results indicate that the CHD- and the IQ-domains of IQGAP1 are crucially involved in the interaction that occurs in a direct and calcium-dependent manner. Using a Surface Plasmon Resonance approach the binding affinities of S100P with the respective IQGAP1 domains were calculated. Our analyses also reveal that amino acids 21-25 of S100P are involved in the calcium-dependent binding of IQGAP1. This suggests a novel type of S100-target protein interaction since these typically involve residues at the C-terminus.

Synaptic plasticity in NCS-1 knock-out and NCS-1-EGFP overexpressing mice

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Neuronal calcium sensor 1 (NCS-1) has an important role in synaptic plasticity. For example, increases in NCS-1 protein concentration in cultured hippocampal neurons can switch short-term plasticity from paired-pulse depression (PPD) to paired pulse facilitation (PPF). Recently it was shown that functional knock-down of NCS-1 impairs metabotropic glutamate receptor dependent long-term depression (mGluR-LTD) in perirhinal cortex from newborn rats [1]. This form of mGluR-LTD is thought to promote AMPA receptor internalization involving a PICK-1 and NCS-1 dependent Ca^{2+}-signaling mechanism.

In order to better define the physiological role of NCS-1 in mGluR regulated vesicular trafficking of ion channel protein we have generated NCS-1 knock-out (KO) and transgenic mouse lines expressing NCS-1-EGFP fusion protein (TG). We have begun to investigate effects of loss or gain of NCS-1 function on synaptic plasticity in mouse brain. The NCS-1 KO as well as TG animals show no gross behavioral phenotype and have normal appearance, body weight and life expectancy.

Electrophysiological recordings of field excitatory postsynaptic potentials (fEPSP) in hippocampal slices showed altered short term plasticity at hippocampal synapes of mice with altered NCS-1 protein expression. We recorded PPF at perforant path synapses in dentate gyrus as well as in the CA1 region of hippocampus. The results showed markedly reduced PPF in TG animals in comparison to wildtype controls. This data will be compared to results obtained from hippocampal slices of NCS-1 KO mice.

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Secretagogin (SCGN) expression and its biological relevance in renal cell carcinoma

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Clear cell renal cell carcinomas (CCRCCs) represent 70% of all neoplasms of the kidney. The introduction of novel treatment strategies such as kinase inhibitors provide hope that metastasis and progression could be stabilised. In this context, the central role of calcium signalling in a diverse range of intracellular processes including oncogenesis has to be stressed. The present study aimed at the evaluation of the recently defined neuroendocrine marker calcium binding protein SCGN in CCRCCs. Immunohistochemical analysis revealed considerable SCGN expression in 28 of 96 tested CCRCCs (=29%). Notably, none of the tested non-CCRCC (n=91) exhibited expression of SCGN. The immunohistochemical results were confirmed by immunoblotting of CCRCC derived tissue samples. Moreover the SCGN expressing subgroup of CCRCCs associated with a high metastasis rate. Reported gene array data on 9 CCRCC and their normal tissue counter-parts revealed similar results [1]. Using bioinformatics, we found 300 genes strongly correlated with SCGN. By pathway analysis involvement of these 300 genes in replication as well as neuronal differentiation processes was identified. SCGN and its functional relevance in CCRCCs is currently under investigation in stable transfectant clones of the kidney cell line Hek-293. In conclusion, we demonstrate expression of the neuroendocrine marker SCGN in a subgroup of CCRCCs, which correlates in its expression level with important proteins involved in cell replication.

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Structural basis of RAGE S100B interaction

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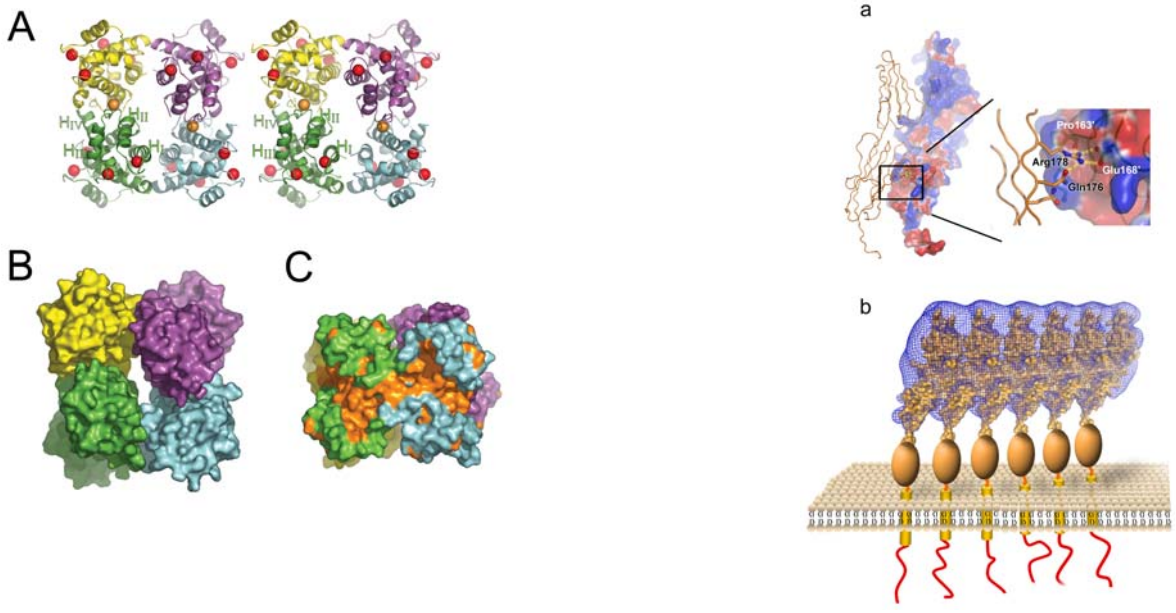
RAGE (Receptor for Advanced Glycation Endproducts) is a multi ligand receptor which binds proteins glycan-modified proteins, amyloid ß and several S100 proteins, among them S100B. Our aim was to resolve the structural basis for the ability of RAGE to recognize structurally divergent molecules and in particular for its interaction with S100 proteins. We recently identified that S100B like S100A8/A9 [1] and S100A12 [2] forms non-covalent multimers, and characterized their binding properties and structural properties [3]. Several S100 proteins bind next to Ca(II) also Zn(II) with rather high affinity. We found that Zn(II) which occurs extracellularly in µM concentrations modulates strongly S100-RAGE interactions, suggesting a further level of regulation. To characterize the counterpart of S100-RAGE interaction we determined the X-ray structure of RAGE ligand-binding domain at 1.85 Å resolution. The arrangement of the molecules in the crystal suggests a pre-assembly of the receptor during activation which is supported by our biochemical studies.



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Hippocampal response to ischemic preconditioning: gene expression of Ca^{2+} ATPase (SPCA) and ERK pathways after cerebral ischemia/reperfusion in rats

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Stroke is the third leading cause of death in humans. Ischemic preconditioning (IPC) is phenomenon of CNS adaptation which results in increased tolerance. The Golgi apparatus, as a part of secretory pathways (SP), is Ca^{2+} store with secretory Ca^{2+}ATPases (SPCA1). SP are involved in stress sensing and transduction of apoptotic signals. ERK protein is part of the mechanisms leading to survival of neurons. We have determined the effect of ICP on alterations of mRNA and protein levels of SPCA1 and level of ERK and pERK in hippocampus. RT-PCR and Western blot analysis detected expression of SPCA1 gene in injured area after ischemia/reperfusion. In injured area, an increase of mRNA was maximal in the reperfusion period. IPC did not change significantly the expression profile, however the tissue response was elevated. Similar pattern was observed on the translational level by Western blot analysis. Protein level of SPCA1 and pERK was highest in the reperfusion time and IPC initiated elevation of tissue response. In addition, both the *in vitro* oxidative stress and IRI induced lipo- and protein oxidation as well as depression of SPCA activity which were partially reversed by IPC [1].

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Xenopus a vertebrate model to study Ca^{2+} signalling involved in genetic diseases

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In recent years the field of organ engineering has advanced through the use of embryonic stem cells (ES). ES are pluripotent cells ie they have the potentiality to differentiate into multiple cell types depending on the culture conditions. This potentiality is confined to cell differentiation and does not extend to the differentiation of organs that have three dimensional shapes.

Amphibians such as *Xenopus* have several advantages over mammals as experimental models: a) They lay a large number of eggs at a time and the embryos develop outside the maternal body b) In vitro fertilizations are easy to perform c) According to the size of the embryos, tissues can be dissected, isolated, or transplanted with high precision and ease in these embryos; d) In addition, it is easy to manipulate the expression of gene products by injecting in-vitro transcribed RNAs into developing embryos, e) The recently sequenced genome of *X. tropicalis* affords unique opportunities for genomic studies, e) transgenesis on Xenopus is fully controlled and high throughput methods for transgenesis are available.

At blastula stage (7 hours post fertilization) the ectoderm of the early amphibian embryo is constituted of multipotent cell groups (animal caps) and can be considered as stem cells.

Animal caps are easily dissected from blastula stage embryo using fine needles. The animal caps can be kept several days in physiological saline for several days in absence of any extrinsic factors. In these conditions it gives rise to a mass of epidermal cells. However the animal caps exhibit plasticity and can differentiate into a variety of neural tissues (central nervous system organs, sensory organs such as eye and ear vesicle), mesoderm tissues (blood cells muscles, and functional organs such as heart, kidney) and/or endoderm derivatives (liver, pancreas) by addition of appropriate inducers in the culture medium.

Animal caps treated with activin and retinoic acid differentiates in vitro into a functional pronephros completed within 2 days (Moriya et al. 1993). Most of the genes necessary for the formation of the *Xenopus* pronephros are also crucial for the formation of the more complex mammalian metanephros.

We have recently provided direct evidences showing that an increase in intracellular Ca^{2+} concentration is a necessary signal in the process of pronephric tubule differentiation. This increase occurs in response to RA via calcium channels located at plasma membrane (Leclerc et al. 2008). Our hypothesis is that this calcium increase may occur via the complex polycystin 1 (Pc1) and polycystin 2 (Pc2). Pc2 also called TRPP2 belongs to the TRP family of cation channels. Pc1 would play a role of regulatory unit of this Ca^{2+} channel. These proteins are respectively encoded by 2 genes PKD1 and PKD2. Autosomal dominant polycystic kidney (ADPKD) (the most frequent human monogenic disorder) is caused by mutations in PKD1 and PKD2 and is characterized by progressive renal enlargement due to the formation of cysts leading to terminally kidney failure which characterize disorders in tubule differentiation. By identifying mechanisms involved in Ca^{2+} signalling in pronephros development we propose to transfer this knowledge to higher vertebrate systems. This work may provide understanding of the molecular basis of kidney diseases which is an important problem in public health. Animal caps may constitute a powerful model in high throughput assay for screening new therapeutics agents for ADPKD.

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RAGE and S100 proteins transcription levels in melanoma tumors

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It has been suggested that the Receptor for Advanced Glycation Endproducts (RAGE) may play an important role in melanoma. Animal studies with anti-RAGE antibodies have shown that RAGE blockade leads to reduced tumor growth and metastasis formation. RAGE is a multiligand receptor and among its ligands are S100 proteins, which can be highly expressed in melanoma. Indeed, S100B is clinically used as a biomarker for melanoma diagnosis and prognosis.

We have surveyed 40 melanoma tumor samples for transcription of RAGE and four S100 proteins and compared them to normal skin tissue. We found tremendous differences between tumors. On average RAGE and S100A6 expression was moderately increased in melanoma. S100B was highly over-expressed whereas S100A2 was greatly under-expressed in melanoma tissue. Because tumor tissue is intrinsically heterogeneous we next analyzed three well characterized melanoma cell lines. A primary tumor cell line showed generally higher transcription levels for RAGE and S100 proteins compared to two metastatic cell lines. In all cell lines S100B was by far the dominant S100 protein exceeding S100A2 transcription levels by three orders of magnitude. Detailed profiling of S100 transcription in melanoma tumors, together with clinical data, may facilitate improved molecular diagnostic and treatment in the future.

Interaction between tau and secretagogin in insulinoma cells

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There is evidence from epidemiological data that Alzheimer's disease is associated with type 2 diabetes. Motivated by results from another research group (Miklossy et al. 1999) who found that a similar tauopathy occurs in pancreatic β-cells such as in brains of Alzheimer's patients, we evaluated tau protein expression in the rat insulinoma cell lines Rin-5F and INS-1E as well as in human insulinomas. In order to investigate this subject we demonstrated in the rodent insulinoma cell lines presence of all six tau isoforms at high quantity. Hyperphosphorylation is detected in neuronal tauopathies. In pancreatic β-cells phosporylation of cellular proteins is tightly controlled and regulated by intracellular calcium spikes. In this direction we investigated the interaction of the recently cloned neuron- and neuroendocrine-specific calcium sensor protein secretagogin (SCGN) with tau proteins. Using SCGN-GST pull down assays and insulinoma cell lysates we demonstrated a specific interaction of one tau isoform with SCGN. This interaction is most probably calcium dependent as inclusion of EDTA in the cell lysate buffer abrogated this complex formation. Immunological data suggest that tau isoform 5 a four-repeat variant represents the SCGN interaction partner. In addition, we demonstrated presence of not only tau but also SCGN in the Sarkosyl-insoluble pellets of Rin-5F cell lysate.

In conclusion, these data might suggest that SCGN is involved in regulating the calcium spike mediated molecular tau dynamics of tubule stabilization in β-cells.

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Injection of neural stem cells in a rat model of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a progressive and terminal neuroinflammatory and neurodegenerative disease specifically affecting motoneurons. Death caused by paralysis occurs only 3 to 4 years after the first observed symptom. Although genetic background of 90% of the cases is not defined, uniformity of pathological features in familial superoxide dismutase 1 (SOD1) mutation yielded high interest for this mutation and corresponding animal model. As recent publications suggested that several molecules (cytokines) which are involved in inflammatory process attract neural stem cells, we decided to test whether cells injected in the rat blood stream will be attracted by pathological process within CNS. Moreover, as undifferentiated neural stem cells express only tumor necrosis factor receptor type 2 (TNF-R2), which protects them from induction of apoptosis by TNFα, we wanted to test whether injection of TNFα will increase their migration to CNS. Rational was the well known permeabilization of blood brain barrier by TNFα. After injection of 10 millions of wild type nestin/GFP positive cells, some were found scattered in the brain cortex. More cells were found in both disease affected animals and TNFα treated wild type animals than in untreated wild type animals. This suggests that increased levels of cytokines in the CNS of ALS affected animal might increase chemo-attraction of neural stem cells towards the inflamed neural tissue.

RAGE expression in motoneurons from human ALS

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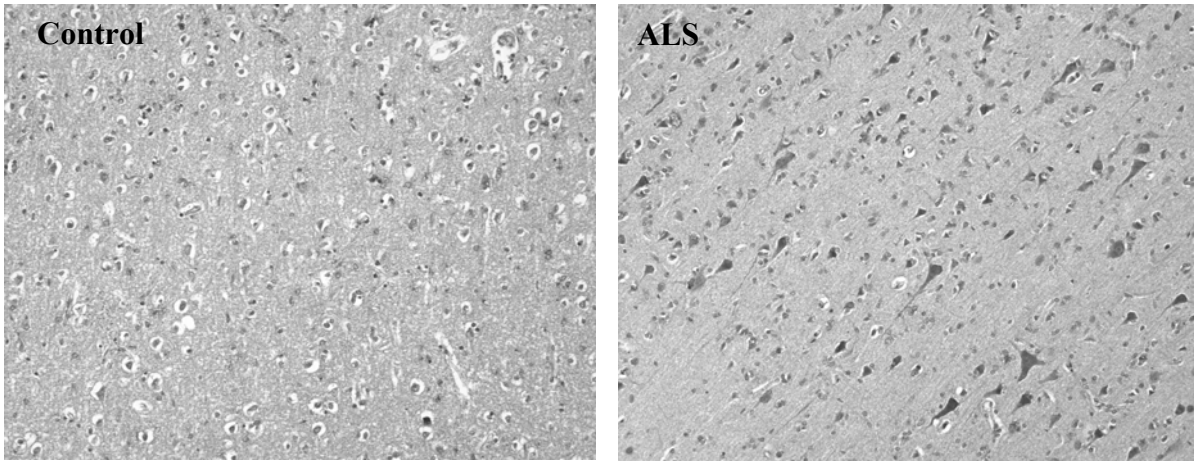
Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder resulting in the progressive loss of motoneurons and during which neuroinflammation occurs. RAGE (Receptor for Advanced Glycation Endproducts) is a multiligand member of the immunoglobulin superfamily of cell surface molecules which binds S100A6 and S100B [1]. S100A6 and S100B differentially modulate cell survival by interacting with distinct RAGE Ig domains [2]. RAGE signalling induces, through $NF_{\kappa}B$, the production of pro-inflammatory cytokines. In this work we have looked at the RAGE expression within the human spinal cord of ALS patients. In human frontal cortex, RAGE is not or weakly expressed on normal human motoneurons but highly expressed in motoneurons from ALS patients. Considering that S100A6 and S100B are also overexpressed in the CNS from ALS patients [3], our results reinforce the concept that neuroinflammation and RAGE are playing an important role during ALS ethiopathology.



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What can we learn from honeybees? The role of calcium in long-term memory formation

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Synaptic plasticity associated with an important wave of gene transcription and protein synthesis underlies long-term memory (LTM) processes. Calcium (Ca^{2+}) plays an important role in a variety of neuronal functions and indirect evidences suggest that it may be involved in synaptic plasticity and in the regulation of gene expression correlated to LTM formation. The aim of this study was to determine whether Ca^{2+} plays a role in LTM formation and which Ca^{2+}-dependent genes are involved in this process. To address this question we used the honeybee *Apis mellifera,* which is a well-established model to study the molecular basis of memory formation. It presents important learning and memory capacities[1] and genomic analysis is now possible since its genome has been sequenced[2]. Experiments are possible on a large number of animals, allowing the screening of new potential therapeutics. We used the Pavlovian appetitive conditioning of the proboscis extension reflex in the honeybee to establish that Ca^{2+} is necessary and sufficient to induce LTM formation. Furthermore, microarray analysis has been done to determine which Ca^{2+}-dependent genes are involved in LTM formation.

Decline in memory and cognitive performances marks neurodegenerative diseases such as Alzheimer's disease[3]. Some studies have shown the importance of Ca^{2+} in neurodegenerative deseases[4], it is thus crucial to better understand the link between Ca^{2+} and LTM processes to find new therapeutics.

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S100A4 is a target of relaxin-like peptide signaling and mediates some of the tumor promoting effects of relaxin and INSL3 in human carcinoma cells

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The heterodimeric peptide hormones relaxin (RLN) and INSL3 and their cognate receptors RXFP1 and RXFP2 (relaxin-family-peptide receptors 1 and 2) are expressed in carcinoma tissues of the human thyroid. We have established RLN and INSL3 expressing transfectant cell clones of the human follicular thyroid carcinoma cell line FTC-133 (FTC-133/RLN and FTC-133/INSL3) and investigated their ability for migration and in-vivo tumor growth. Relaxin and INSL3 transfectants showed an increased cellular motility and in-vitro invasiveness compared to vector controls. This was associated with increased S100A4 protein levels and S100A4 secretion. Relaxin and INSL3 failed to increase cell motility after S100A4 siRNA knock-down suggesting S100A4 to be a downstream target of relaxin/ relaxin receptor (RXFP1) signaling and INSL3/RXFP2 signaling in thyroid carcinoma cells. In addition, the in-vivo tumor growth of FTC-133/RLN and FTC-133/INSL3 was increased and correlated with S100A4 protein expression. Microvessel density in RLN- and INSL3-expressing xenograft tumors was higher than in control tumors. We show that S100A4 initiates capillary tube formation in a Matrigel tube formation assay using human umbilical vein endothelial cells and suggest that extracellular S100A4 contributes to the increased vascularization of RLN- and INSL3 expressing tumors.

S100A4 is considered a molecular marker for the metastatic potential for thyroid and breast cancer with high prognostic significance. We show here that the relaxin/RXFP1 and INSL3/RXFP2 ligand-receptor signaling is a novel transcriptional regulator of S100A4 expression in human thyroid carcinoma cells and suggest that S100A4 mediates, in part, the relaxin- and INSL3-induced increase in cancer cell invasiveness, neovascularization and tumor growth.

A novel technique allows for structural characterization of high-affinity S100-peptide interactions

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S100 proteins belong to the EF-hand calcium-binding family of proteins. Other than S100A10 that is unable to bind calcium, the rest of the members accommodate one calcium ion per EF-hand. Calcium-binding to the C-terminal EF-hand causes a significant conformational change in S100s, such as S100B, leading to the exposure of hydrophobic residues for target-protein interactions. To determine the precise binding surface of the target proteins on the S100s, and to provide a template for larger S100 complex assemblies, a method for the generation of hybrid S100-target complexes was developed. In this method, peptides of annexin A2 (residues 1-14) and TRTK-12 (residues 1-12 of actin-capping protein) were expressed as S100 hybrid molecules. A linker between the S100 protein such as S100A10 and the binding peptide, annexin A2 was determined using molecular modeling. The linker residues were selected so that they were flexible enough to tolerate the interaction between the peptide and the S100 protein. A PreScission protease site was also engineered near the N-terminus of the peptide segment to facilitate its cleavage and purification. Cloning and high expression levels with no degradation of the S100A10-annexin A2 and S100B-TRTK-12 hybrid proteins are completed. NMR spectroscopy of the apo and Ca^{2+}-bound form of both complexes show that the structure and position of annexin A2 and TRTK-12 peptides in the hybrid proteins are very similar to those obtained for individual proteins. This work will allow these hybrid S100 proteins to be used to test *in vivo* and *in vitro* assembly of larger S100 complexes.

Knock down of S100A4 expression by small molecules restricts metastasis formation in colon cancer

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Metastasis formation is the main reason why colon carcinoma is still one of the leading causes of cancer deaths worldwide. Efforts made to identify the main molecular players in metastasis formation revealed the calcium binding protein S100A4. S100A4 is a Wnt/β-catenin target gene [1] which induces migration, invasion and angiogenesis and its overexpression leads to aggressive tumor growth and metastasis formation in many types of cancer. Hence, its suppression bears large potential for therapeutic intervention.

Here we report the identification of a small molecule inhibitor from a high throughput screening of 1,280 drugs, which significantly reduces S100A4 expression in colorectal carcinoma cells. S100A4 suppression is achieved by downregulation of the Wnt/β-catenin pathway. Functional assays revealed that proliferation rates are diminished upon treatment while cell viability is only slightly affected. More strikingly, migration and invasion rates of treated cells are significantly decreased, but rescued by overexpressing S100A4 cDNA. The impact of the small molecule on metastasis formation *in vivo* shows first promising results. In summary, our findings present a new strategy to restrict S100A4 induced metastasis formation in colon cancer.

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S100A6 deficiency induces senescence of mouse NIH 3T3 fibroblasts

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S100A6 (calcyclin) is a calcium binding protein of the S100 family expressed mostly in fibroblasts and epithelial cells. We have established a NIH 3T3 fibroblast cell line stably transfected with siRNA against S100A6 to examine the effect of S100A6 deficiency on non-transformed cell physiology. We report that S100A6 deficient fibroblasts reveal major phenotypic changes and proliferate at a much slower pace than control cells. Cell cycle analysis showed that a large population of these cells lost the ability to respond to serum and persisted in the G0/G1 phase. Furthermore, fibroblasts with diminished S100A6 level exhibited features of cellular senescence as revealed by β-galactosidase and gelatinase assays. Immunocytochemical examination showed changes in actin cytoskeleton and vinculin staining as well as formation of lamellipodial extensions. These features, in turn, induced a profound impact on adhesive and migratory properties of the S100A6 deficient fibroblasts. In conclusion, it appears that the S100A6 protein is indispensable for normal proliferation of mouse NIH 3T3 fibroblasts and that its deficiency may compel cells to proliferative senescence accompanied by changes in cell cytoskeleton and metabolism.

Interaction partners of neuronal calcium sensor-1 in the mouse

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The neuronal calcium sensor protein NCS-1 has been implicated in the modulation of synaptic efficacy and vesicular trafficking. The yeast NCS-1 orthologue Frq1 functions as a regulatory subunit of phosphatidylinositol 4-kinase Pik1, playing an important role in Golgi function and vesicle trafficking. Given the high sequence conservation of NCS-1 proteins across phyla, we hypothesized that mammalian NCS-1 also functions in vesicular trafficking.

To address this hypothesis, we generated transgenic mice expressing GFP-tagged NCS-1 (NCS-1-EGFP) in the forebrain. We prepared forebrain lysates and isolated NCS-1-EGFP containing protein complexes by size-exclusion chromatography followed by immunoprecipitation with anti-GFP antibodies. Immunoprecipitated proteins were separated by SDS-PAGE. For control, we used brain lysates from transgenic mice expressing only EGFP under control of the same promoter. Specific protein bands, not apparent in controls were isolated and characterized by mass spectrometry (LC-MS/MS).

The MS-analysis indicated several candidate interaction partners of NCS-1, but a mammalian Pik1 orthologue was not among them. Instead, we identified two novel candidate proteins, Bet3 and SNAP-47, which both have an albeit ill-defined role in vesicular trafficking in eukaryotic cells. Their interaction with NCS-1 could be confirmed by co-immunoprecipitation with anti-NCS-1 antibodies from brain lysate of wild type mice using NCS-1 knock out animals for control. Immunofluorescence analysis shows perinuclear co-localization of NCS-1 with Bet3 and SNAP-47. We propose that *in vivo* NCS-1 plays a role in Ca^{2+}-dependent regulation of intracellular vesicle trafficking.

AnxA6-regulated bone mineralization by Saos-2 osteoblasts is Src and rock kinase dependent

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In this study we used human osteosarcoma Saos-2 cells undergoing the osteoblastic differentiation program, producing the extracellular matrix and spontaneously releasing matrix vesicles (MVs) as a model to determine the role of AnxA6, the acto-myosin cortex as well as of Src and ROCK phosphorylation in bone mineralization. The mineralization process was stimulated with ascorbic acid/β-glycerophosphate and modulated using specific inhibitors. Calcium nodule detection by Alizarin Red-S staining showed that mineral formation is accompanied by changes in cell morphology. After stimulation, cells become round and release MVs with a mineral nodules inside. Stimulated cells produced a 5-fold higher amount of mineral phase within 12 days than control cells. SDS-PAGE analysis of whole cell lysates indicated that stimulation of the cells for mineralization induces also changes in their protein profile. We observed that mineralization index decreased in the presence of cytochalasin D (inhibitor of actin polymerization), PP2 (inhibitor of Src kinase activity) and Y-27632 (inhibitor of ROCK kinase activity) whereas it increased in the presence of blebbistatin (inhibitor of non-muscle myosin II). Immunofluorescence analysis showed that the enrichment in AnxA6 in MVs correlated with acto-myosin cortex rearrangements and release of MVs. In addition, localization of AnxA6 and non-muscle myosin IIA was affected in Saos-2 cells treated with Y-27632 and blebbistatin suggesting the role of ROCK phosphorylation and myosin IIA in mineral formation.

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Overexpression of P-glycoprotein in L1210/VCR is associated with changes of endoplasmatic reticulum

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A multidrug resistant L1210/VCR cell, which expresses an abundant amount of P-glycoprotein (Pgp), we found to be resistant to thapsigargin, an inhibitor of SERCA (sarco/endoplasmic reticulum calcium pump). We have studied the possible differences among L1210 and L1210/VCR cells in expression of endoplasmic reticulum proteins involved in calcium homeostasis. Thapsigargin influenced expression of calnexin (CNX) in L1210 and L1210/VCR cells. Amounts of mRNA encoding both calcium release channels (ryanodine receptor channels – RyR and inositol 1,4,5 triphosphate receptor channels – IP3R) were found to be at similar levels in sensitive and resistant cells. However, mRNAs encoding IP3R1 or 2 were decreased in resistant cells cultivated in the presence of vincristine (VCR), while mRNA encoding RyR remained unchanged. The amount of mRNA for SERCA2 was decreased in resistant cells when compared with sensitive cells. This decrease was more pronounced when resistant cells were cultivated in the presence of VCR. CNX was found to be less expressed at the protein level in resistant as in sensitive cells. The level of mRNA encoding CNX was decreased only when resistant cells were cultivated in the presence of VCR. CNX was found to be associated with immature Pgp in resistant cells.

Thus, differences exist between sensitive and resistant cells in the expression of endoplasmic reticulum proteins involved in the control of intracellular calcium homeostasis or calcium-dependent processes.

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Annexin A2 and S100A10 in the mammalian oviduct epithelium

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In eutherian mammals, sperm-oviduct interaction provides the formation of a sperm storage and allows the selection of sperm with certain qualities. In sows, we proposed that the oviductal Sperm Binding Glycoprotein SBG is involved in sperm selection. As to reservoir formation, in bovine, essential proteins involved have been characterized as sperm adsorbed PDC109 and oviductal cell's Annexin (ANXA) 1, 2, 4 and 5. In porcine, AQN1 is the sperm component and ANXA2 the oviductal component most likely to be clue proteins responsible for sperm reservoir formation. We have previously proposed that ANXA2 may be part of a ubiquous mechanism of sperm-oviduct interaction. As S100A10 is usually associated to ANXA2, in this work we search for the presence of ANXA2 and S100A10 in the oviducts of several mammals by western blot. We find that ANXA2 and S100A10 co-exist in the oviductal cell extracts from pig, human, cow, cat, dog, mouse and rabbit. Immunohistochemistry of porcine isthmus and ampulla shows that ANXA2 and S100A10 localize to the same type of cells, cilliary isthmic cells surrounding the lumen. Different extraction procedures were performed on porcine oviductal cell membranes, showing ANXA2 liberation in every case, and S100A10 release increasing from Triton X100 to ionic strength to EGTA containing buffer, suggesting Ca^{++} may be involved in its attachment. Immunoprecipitation of epithelial cell extracts with anti-ANXA2 antibodies shows S100A10 co-precipitation.

We conclude that ANXA2-S100A10 complex is present in the mammalian oviduct and that its interaction with sperm proteins may be a part of the sperm-oviductal cell relation in several mammals.

Effect of H_2S donor, NaHS on rat blood pressure and possible involvement of calcium RyR2 channel

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Hydrogen sulfide, H_2S, is an endogenously formed gaseous transmitter. H_2S is involved in a regulation of cardiovascular functions. It affects several cardiac ion channels (i.e. L-type Ca^{2+} channel, K_{ATP} channel, Cl^- channel). Protective effects of H_2S were shown for both ischemia/reperfusion preconditioning and postconditioning. It might be also beneficial in treatment of hypertension. Therefore, we decided to study the effects of H_2S on blood pressure and heart rate of anaesthetized rats and on the activity of ryanodine receptor Ca^{2+} release channels (RyR2) derived from cardiac sarcoplasmic reticulum.

NaHS (up to 64 μmol/kg, i.v.), a donor of H_2S, decreased transiently the blood pressure to (84.5–46.5)%, followed by its increase to (107.8–170.8)%. Single channel properties of RyR2 channels were studied in bilayer lipid membrane. NaHS modulated RyR2 channel activity. It increased 3.6-fold open probability of the channel at 50 μmol/l. At higher concentrations (500 – 1000 μmol/l), in 6/11 experiments NaHS decreased P-open, while in 5/11 experiments, it did not have any effect.

The results indicate that H_2S and/or HS^- effects on blood pressure are concentration dependent (biphasis), and that modulation of RyR2 by H_2S might be responsible, at least in part, for the increase of blood pressure.

Vinflunine inhibits CaM-STOP interaction

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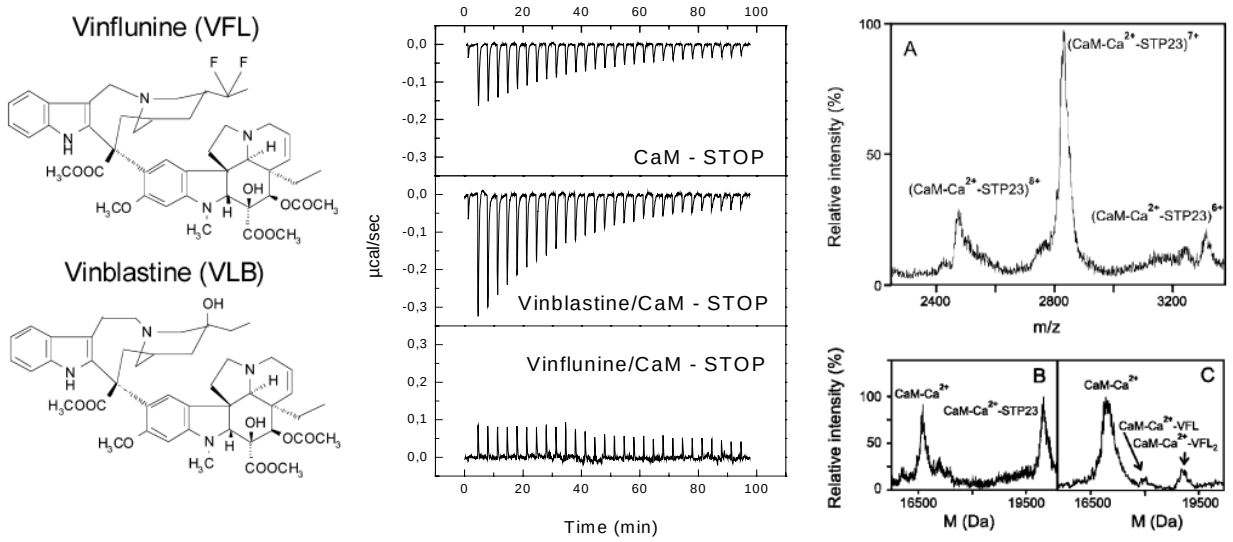
Vinca alkaloids vinblastine and vincristine are widely used in therapy of leukemia and solid tumors. Their action is associated with alterations of the mitotic spindle functions. A number of studies show that some Vinca alkaloids inhibit CaM target interaction. The newest microtubule inhibitor, vinflunine (Javlor), is remarkably more active and less toxic than vinblastine against a number of tumors. The high antitumor activity of this molecule is not well understood since it binds to tubulin with affinity several-fold lower than that of vinblastine. We characterized the influence of Vinca alkaloids on CaM STOP (stable tubule only polypeptide) complex formation by using a combination of thermodynamic and mass spectrometric approaches. Our results revealed different binding modes to CaM for vinflunine and vinblastine. We demonstrate that vinflunine is a better inhibitor for STOP binding to calmodulin than vinblastine. We suggest that vinflunine action on calmodulin can have an effect on microtubule dynamics. These data may contribute to a better understanding of the superior antitumor efficiency and lower toxicity of vinflunine.



Diversification of Ca^{2+}-mediated signal in a simple model system

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The most prominent feature of Ca^{2+}-mediated signalling is its pluripotency („Kalzium macht alles...“), which poses important question, how the calcium signal activates the specific pathway determined by the signalling molecule without activating other Ca^{2+}-mediated pathways activated by different signalling molecules. Spatial-temporal properties of Ca^{2+}-signal and Ca^{2+}-binding proteins could participate in the process of diversification of Ca^{2+} signal.

We studied the diversification of the Ca^{2+} signal in artificially induced Ca^{2+} influx in human red blood cells (RBC). Earlier we observed that the activation of the Ca^{2+}-influx by fluoride, or vanadate, led to the activation Na^{+}-permeability. Interestingly, vanadate activated the Na^{+}/H^{+} antiporter sensitive to amiloride, while fluoride activated Na^{+} channel sensitive to tetrodotoxin. This observation represents the example of the Ca^{2+}-signal diversification on the simplest level, where no participation of intracellular structures could be expected. Novel inhibitors of both modes of Ca^{2+}-influx activation were found. Fluoride-activated Ca^{2+} influx was inhibited by a phosphoprotein phosphatase 2B inhibitor – cyclosporin A, and tetrodotoxin, whereas Ca^{2+} influx activated by vanadate was sensitive to Li^{+} ions and dihydropyridine Ca^{2+} blockers.

The fate of fluoride in the RBC suspension was monitored by means of ^{19}F-NMR. Results showed two signals, which correspond to both extracellular and intracellular fluoride, but no signal, which could represent a protein-bound fluoride, was found. The possible role of phosphorylation/dephosphorylation mechanisms in the diversification of Ca^{2+} signal was studied by means of ProQ Diamond staining and by ^{32}P-phosphate incorporation. We could not find any changes in protein phosphorylation induced by both vanadate or fluoride and Ca^{2+} (and other additions), when proteins separated in SDS-PAGE were stained by the ProQ-Diamond dye. However, some changes, which remain to be characterized, were observed by radioactive labelling.

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