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THE ASPECTS OF CYANIDE INHIBITION AND PYRUVATE-INDUCED RECOVERY OF CYTOCHROME C OXIDASE ACTIVITY

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Mitochondrial cytochrome *c* oxidase (COX) is an essential member of cellular ATP-producing system under aerobic conditions. The mechanism of cyanide inhibitory effect on COX as well as the conditions for its reversal are not yet fully explained. With regard to the inhibition by KCN and its reversal by pyruvate, we investigated three parameters of COX function, namely the transport of electrons in the terms of oxygen consumption, the transport of protons evaluated as mitochondrial membrane potential ($\Delta\psi_m$), and the enzyme affinity to oxygen by means of p_{50} value calculation. We analyzed the function of COX in intact rat liver mitochondria, either within the respiratory chain or as a sole enzyme, using succinate or ascorbate + TMPD to fuel respiration. We found that 250 μM KCN completely inhibited both electron and proton transport function of COX, and this inhibition was reversible as proved with washing of mitochondria. The addition of 60 mM pyruvate induced the maximal recovery of both parameters to 60-80 % of original values. Using KCN in the low concentration range up to 5 μM, we observed a profound, 30-fold decrease of COX affinity to oxygen. Again, this decrease was completely reversed by washing of mitochondria while pyruvate induced only a partial yet still significant recovery of oxygen affinity. Our results demonstrate the reversible nature of inhibition of COX by cyanide and reveal the limited potential of pyruvate to act as a cyanide poisoning antidote. Importantly, we also show that the COX affinity to oxygen is the most sensitive indicator for the detection of toxic effect of cyanide.

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MITOCHONDRIAL ATP SYNTHASE DEFICIENCY CAUSED BY MUTATION OR DOWNREGULATION OF F1 EPSILON SUBUNIT

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F_1F_o-ATP synthase is a key enzyme of mammalian energy provision as it provides most of the cellular ATP. So far, mitochondrial disorders of ATP synthase of the nuclear origin have been shown to result from mutations in biogenesis factors TMEM70 and ATPAF2. In collaboration with Paracelsus Medical University in Salzburg we described for the first time that a mutation in a structural subunit of ATP synthase can cause ATP synthase deficiency (1). Analysis of patient fibroblasts revealed that the homozygous p.Tyr12Cys mutation in the epsilon subunit, encoded by the nuclear gene *ATP5E,* decreases both oligomycin-sensitive ATP synthase activity and mitochondrial ATP synthesis to 60-70 %. Electrophoretic analyses showed reduced amount of fully assembled ATP synthase with the normal size containing the mutated epsilon subunit. Also the content of all F_1 and F_o ATP syntase subunit was similarly reduced, except for F_o-c subunit which was found accumulated in a detergent-insoluble form. Moreover, when we down-regulated the expression of epsilon subunit in HEK293 cells by shRNA (2), silencing of *ATP5E* gene decreased activity and protein content of mitochondrial ATP synthase complex and ADP-stimulated respiration to approximately 40 % of control. In *ATP5E* silenced cell lines the decreased amount of ε subunit was accompanied by a decreased content of the F_1 subunits α and β and as well as of the F_o subunits a and d, while the content of F_o subunit c was not affected. We found the accumulated subunit c to be present in fully assembled ATP synthase complex or in subcomplexes of 200-400 kDa, which contained neither the F_1 subunits nor the F_o subunits. Our studies thus showed that the ε subunit is necessary for assembly and/or stability of the F_1 catalytic part of the mammalian ATP synthase and it is also important for incorporation of the hydrophobic subunit c into F_1-c oligomer during ATP synthase biogenesis.

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CHARACTERIZATION OF LOCATION AND PROCESSING OF THE TMEM70 PROTEIN

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TMEM70 protein represents a novel ancillary factor of mammalian ATP synthase (1). We have investigated import and processing of this factor in human cells using tagged forms of TMEM70 and specific antibodies. TMEM70 is synthesized as a 29 kDa precursor protein that is processed to a 21 kDa mature form. Colocalization of TMEM70-GFP, TMEM70-FLAG and TMEM70 with MitoTracker Red and ATP synthase indicates mitochondrial localization. Western blot of subcellular fractions revealed the highest signal of TMEM70 in isolated mitochondria and mitochondrial location was confirmed also by mass spectrometry analysis. Based on analysis of submitochondrial fractions, TMEM70 appears to be located in the inner mitochondrial membrane, in accordance with predicated transmembrane regions in the central part of the TMEM70 sequence. Two-dimensional electrophoretic analysis further indicated the presence of dimeric form of TMEM70. TMEM70 biosynthesis is prevented in the patients with c.317-2A>G mutation, because no TMEM70 protein could be found in cells and isolated mitochondria from patients with ATP synthase deficiency due to TMEM70 c.317-2A>G mutation.

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n-3 POLYUNSATURATED FATTY ACIDS AND CALORIE RESTRICTION CAUSE ADDITIVELY INCREASE IN MITOCHONDRIAL BIOGENESIS AND LIPID CATABOLISM IN WHITE ADIPOSE TISSUE

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n-3 polyunsaturated fatty acids are known as potent drug in prevention of insulin resistance (IR) and obesity. One of their most important target organs is white adipose tissue where n-3 PUFA cause increase in mitochondrial biogenesis and activate lipid catabolism. In our experiments, we are trying to potentiate the effects of n-3 PUFA by the mild (10 %) calorie restriction (CR), which is often a part of the treatment strategy for patients with obesity and type 2 diabetes mellitus. Male mice (C57BL/6J) were fed by high fat diets supplemented or not by n-3 PUFA concentrate. There were 4 groups of animals: HF AL (high fed diet, ad libitum), HF+F AL (high fat diet supplemented by n-3 PUFA, ad libitum), HF CR (high fat diet, calorie restricted) and HF+F CR (high fed diet supplemented by n-3 PUFA, calorie restricted). After 5 weeks of treatment several experiments were done. Oral glucose tolerance test indicates that HF+F CR mice are more metabolically

flexible and insulin sensitive than all other groups. Isolated permeabilized adipocytes of HF+F CR mice have significantly higher oxidation of respiratory substrates than those from HF AL mice. This is in accordance with increased cytochrome b content and cytochrome c oxidase activity. The analysis of gene expression further showed upregulated mitochondrial biogenesis. Moreover, immunohistochemical evaluation of adipose tissue depots proved that HF+F CR mice have smaller adipocytes and less inflammation. In the current experiment the comparison of reactive oxygen species production in tissue of all groups will be done, because oxidative stress can play important role in the mechanism of n-3 PUFA and CR action. The time dependence of suggested changes will also be analysed in more detail.

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SEX-DEPENDENT EFFECT OF DIFFERENT DIETS ON GLUCOSE HOMEOSTASIS ARE COUPLED WITH ADIPOSE TISSUE PLASTICITY

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The goal of this study was to assessed the role of adipose tissue in less severe impairment of glucose homeostasis in females compared to males during the course of high-fat (HF) feeding in mice. Female and male mice of the C57BL/6N strain were fed either a chow or obesogenic HF diet for 15 or 35 weeks after weaning. Metabolic markers and hormones in plasma, glucose homeostasis, adipocyte size, inflammatory markers and cellularity of gonadal (gWAT) and subcutaneous (scWAT) adipose tissue depots were evaluated. HF-fed males were heavier than females until week~20, after which the body weight stabilized at similar level (55-58 g) in both sexes. Greater weight gain and fat accumulation in female were associated with larger adipocytes in gWAT and scWAT at week 35. While adipose tissue macrophage infiltration was in general less frequent in scWAT, it was reduced in both fat depots of female as compared to male mice, however the expression of inflammatory markers in gWAT was similar in both sexes at week 35. In females, later onset of the impairment of glucose homeostasis and better insulin sensitivity were associated with higher plasma levels of adiponectin (week 0, 15 and 35). Compared to males, female mice demonstrate increased capacity for adipocyte enlargement in response to a long-term HF-feeding, which is associated with reduced adipose tissue macrophage infiltration and with better insulin sensitivity. Our data suggest that adipose tissue expandability and adiponectin levels might play a role in the sex differences observed in obesity-associated metabolic disorders.

COMBINATION TREATMENTS WITH n-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS AND THIAZOLIDINE-DIONES EXERT BENEFICIAL EFFECTS IN PREVENTION OF OBESITY AND ASSOCIATED PATHOLOGIES IN MICE

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Fatty acids of marine origin, i.e. docosahexaenoic and eicosapentaenoic acid (DHA and EPA, respectively) act as hypolipidemics, but they do not improve glycemic control in diabetic patients. Thiazolidinediones (TZDs), like rosiglitazone and pioglitazone, i.e. specific activators of peroxisome proliferator-activated receptor-gamma improve whole-body insulin sensitivity. It was shown that a combination treatment using a DHA and EPA concentrate (DHA/EPA) and rosiglitazone provided, by complementary mechanisms, additive beneficial effects on dyslipidemia and impaired glucose tolerance (IGT) in obese mice(1). Aim of the project was to further study mechanism of action of TZDs, namely of roziglitazone and pioglitazone. Male C57BL/6 mice were fed high-fat diet. The effects of DHA/EPA (replacing 15 % dietary lipids), rosiglitazone (10 mg/kg diet), or pioglitazone (50 mg/kg), or combination of both DHA/EPA and rosiglitazone or pioglitazone on body weight, adiposity, metabolic markers and adiponectin in plasma, liver and muscle triacylglycerol accumulation analyzed. Intraperitoneal glucose tolerance test was used to characterize the changes of glucose homeostasis. Metabolic flexibility was tested between two limited condition – fasted and re-fed status. DHA/EPA and TZDs exerted additive effects in prevention of obesity, dyslipidemia, and IGT, while suppressing hepatic triacylglycerol accumulation and inducing adiponectin. The treatment also improved metabolic flexibility. In conclusion both type of TZD could be used in combination with DHA/EPA as complementary therapies to counteract dyslipidemia and insulin resistance. The combination treatment may reduce dose requirements and hence the incidence of adverse side-effects of the thiazolidinedione therapy.

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THE EFFECT OF n-3 POLYUNSATURATED FATTY ACIDS ONTO DIFFERENTIATION AND PROLIFERATION OF ADIPOCYTES IN KNOCKOUT MOUSE MODEL aP2-Cre-ER^{T2} PPARγ$^{L2/L2}$

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Peroxisome proliferator-activated receptor (PPAR) γ is a nuclear receptor and transcription factor which plays a crucial role in development of obesity. PPAR γ is mainly expressed in adipose tissue and regulates genes involved in the development of fat cells and their capacity to store lipids. The aim of this study was to demonstrate effects of long chain n-3 polyunsaturated fatty acids, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid, on the proliferation and differentiation capability of preadipocytes. In this study aP2-Cre-ER^{T2} PPARγ$^{L2/L2}$ knockout mouse were used with ablated PPAR γ in mature adipocytes by using tamoxifen-dependent Cre-ER^{T2} recombination system(1). 3-month old premutant mice were fed a corn oil based high fat diet (cHF; lipid content ~35 % wt/wt) for 8 weeks and then randomly assigned for 14 days or 42 days to various experimental group: (1) premutant PPARγ $^{ad+/+}$ mice (injected by corn oil) fed cHF; (2) mutant PPARγ $^{ad-/-}$ (injected by Tam) fed cHF ; (3) PPARγ $^{ad+/+}$mice fed cHF diet supplemented with n-3 LC-PUFA concentrate [cHF+F; product EPAX 1050 TG (46 % DHA, 14 % EPA)] replacing 15 % of dietary lipids; and (4) PPARγ $^{ad-/-}$ mice fed cHF+F. Compare with HF, diet enriched by EPA/DHA reduced body weight and size of mature adipocytes in abdominal adipose tissue. Levels of lipids and several hormones were measured in plasma at day 14 and day 42. Levels of TAG, NEFA and cholesterol were decreased only with n-3 PUFA contribution in both measured periods. HF+F group treated with tamoxifen amplified this action. For better understanding, what was happening in the adipose tissue after tamoxifen and n-3 PUFA treatment, we measured mRNA levels involved in adipogenesis, mitochondrial biogenesis and inflammation. Mitochondrial biogenesis was promoted by n-3 PUFA and this effect was intensified after tamoxifen treatment. Inflammation marker, such as tumor-necrosis factor α, was decreased after 42 day treatement on HF+F and groups treated by tamoxifen. Adipogenesis was not affected neither by n-3 PUFA nor tamoxifen.

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DOSE-DEPENDENT INVOLVEMENT OF THE ADIPONECTIN-AMPK AXIS IN INSULIN-SENSITIZING EFFECTS OF THIAZOLIDINEDIONES

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Thiazolidinediones (TZDs) such as rosiglitazone and pioglitazone are used for the treatment of insulin resistance (IR) in diabetic patients. These compounds are likely to improve glycaemic control mostly by repartitioning fat away from skeletal muscle, while augmenting insulin

action in liver, adipose tissue and skeletal muscle. Numerous clinical studies have demonstrated that TZDs, at doses eliciting maximum stimulation of insulin sensitivity, reduce accumulation of hepatic lipids (steatosis), which is frequently associated with systemic insulin resistance, and can be also used for the treatment of nonalcoholic steatohepatitis and alcoholic fatty liver disease. However, the therapy is associated with unwanted side effects such as oedema and weight gain, increased risk of heart failure, and bone loss. Novel treatment strategies are required allowing for the use of sub-optimal doses of TZDs in combination with other pharmacological or dietary interventions. The insulin-sensitizing effect of TZDs is thought to results from activation of PPARγ, associated with the induction of adiponectin and stimulation of AMP-activated protein kinase (AMPK) in the liver. Treatment with TZDs reduces obesity released adipocyte hypertrophy and low-grade tissue inflammation, while inducing secretion of insulin-sensitizing hormone adiponectin. The induction of adiponectin is probably responsible in large for the insulin-sensitizing effects of TZDs (adiponectin-AMPK axis). Our experiment of different doses of rosiglitazone shows that the only high dose of rosiglitazone significantly induces activity of α2 isoform of AMPK in the liver. And the low dose of rosiglitazone markedly induces expression of cell death-inducing DNA fragmentation factor α-like effector A (CIDEA) in the liver. CIDEA is expressed mainly in adipose tissue, inhibits AMPK activity, and plays a crucial role in lipid droplet formation. Moreover, hepatic content of triglycerides correlates with CIDEA expression level in this tissue. The level of adiponectin in plasma is influenced by the dose of rosiglitazone during the development of obesity and IR induced by high-fat feeding in wild-type mice. The stimulatory effect of rosiglitazone is specific for high molecular weight (HMW) form of adiponectin, which is associated with insulin sensitivity. Our data suggest that the mechanism underlying effects of sub-optimal doses of TZDs on insulin sensitivity may depend on a change of plasma lipid profile resulting from modulation of hepatic lipid metabolism by TZDs, independent of the adiponectin-AMPK axes. The next goal of this study is to characterize the dose-dependent mechanism of TZDs action using mice with genetically disrupted AMPK. Characterization of the mechanisms behind the dose-dependent effects TZDs will allow for designing novel combination treatment strategies, while reducing both the risks and the cost of the TZD therapy.

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MICRO-PATTERNED LAYERS OF FULLERENES (C_{60}) IN BONE TISSUE ENGINEERING

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Fullerenes have unique physicochemical properties that have been exploited for use in industry, medicine, nanoscience and biotechnology. In this study, fullerenes C_{60} were deposited on microscopic glass coverslips in the form of micro-patterned films through a metallic mask, and were tested for potential use in bone tissue engineering. Numerous studies have evaluated the therapeutic potential of fullerene derivates against oxidative stress-associated conditions, including cancer. On the other hand, there is a growing body of literature mentioning cytotoxic effects of these nanoparticles. Accumulation of fullerene aggregates has a negative effect on the growth, proliferation and viability of cells. We have therefore concentrated not only on the positive effects but also on these negative effects of fullerenes, which should be determined prior to potential biomedical applications. In this study, we evaluated the proliferation, viability and metabolic activity of human osteosarcoma cells (lines MG63 and U2OS), such as activity of mitochondrial dehydrogenases in living and metabolically active cells. We also monitored potential membrane and DNA damage and morphological changes of the cells. The DNA damage was analyzed by immunofluorescence staining of markers of DNA damage response, such as phosphorylation of histone H2AX and focal recruitment of p53-binding protein. After 7 days of cultivation, we observed no cytotoxic morphological changes, such as enlarged cells or cytosolic vacuole formation, which are signs of cellular senescence. In addition, there was no decreased viability of the cells and no increased induction of cell death. Moreover there was no increased level of membrane or DNA damage. These results suggest that C_{60} micro-patterned layers do not cause cytotoxic injury, and fullerenes can therefore be considered as a promising material in bone tissue engineering.

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THE INFLUENCE OF HYDROXYAPATITE PARTICLES IN COMPOSITE MATERIALS ON PROLIFERATION AND VIABILITY OF OSTEOBLAST-LIKE CELLS

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Currently, autologous bone grafts are the gold standard for bone substitutions in the reconstructive surgery. However, this approach is associated with additional surgical procedure followed by prolonged healing time and a risk of infection. That's why increased attention has been paid to the development of artificial material that could possibly replace the damaged bone tissue. Mature bone tissue is composed of two main parts: inorganic substance (60-70 %) and organic matrix (30-40 %). The organic part of the bone is represented mainly by cells, collagen fibers, glycoproteins and glycosaminoglykans. The inorganic part comprises minerals, especially hydroxyapatite (HA) and calcium phosphates. The hydroxyapatite particles are incorporated within the extracellular matrix, bound to the proteins such as osteocalcin, osteopontin or osteonectin, which make up approximately 3-5 % of the bone and provide active sites for biomineralization as well as for the cellular attachment. Arrangement of both organic and mineral components contributes to strength and resistance of the bone to the fracture. In the present study, we investigated the influence of the crystallinity, shape and the size of the hydroxyapatite a tricalcium phosphate (TCP) particles on growth and viability of bone cells. For this purpose we prepared materials composed of bioinert polysiloxane matrix and micro- or nanoparticles of HA or TCP. The proliferation, viability and differentiation of the human osteoblast-like MG 63 cells was studied *in vitro* in a conventional static cell culture system. Tissue culture polystyrene (PS) was used as a control. Cell numbers were evaluated in the interval of 1, 3 and 7 days by automated counting in a Beckman ViCell Analyser. The viability of MG 63 cells has been quantified using XTT test which is based on the conversion of the tetrazolium XTT dye by mitochondrial enzymes of viable cells. Cells were visualized by Alexa 488 and propidium iodide and visualized by confocal microscopy. Despite of some studies dealing with the positive effect of hydroxyapatite nanoparticles on proliferation of osteoblast-like cells (1), we have observed significantly higher cell densities and better cell viability on the composites with microparticles of hydroxyapatite or TCP. This could be caused by higher resorption of hydroxyapatite nanoparticles or different morphology and crystallinity of the composite material.

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ADHESION AND GROWTH OF HUMAN OSTEOBLAST-LIKE MG 63 CELLS IN CULTURES ON NANOFIBROUS PLGA MEMBRANES LOADED WITH NANODIAMONDS

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Nanofibrous scaffolds are promising cell carriers for the construction of advanced tissue replacements. Therefore, in this study, we constructed

composite nanofibrous membranes containing a copolymer of L-lactide and glycolide (PLGA, ratio 85:15) and diamond nanoparticles, prepared by the Radio Frequency Plasma Activated Chemical Vapour Deposition (RF PACVD) method (1). PLGA was dissolved in a mixture of methylene chloride and dimethyl formamide (ratio 2:3) at a concentration of 2.3 wt.%, and the nanodiamond (ND) powder was added at a concentration of 0.7 wt.% (after evaporation of the solvent, the final concentration was about 23 wt.%). The nanofibrous membranes were then created by an electrospinning technique (Nanospider^{TM} device, Elmarco Ltd., Liberec, Czech Republic). The nanocomposite membranes were sterilized by gamma irradiation, inserted into 24-well polystyrene plates, rinsed overnight with DMEM medium and seeded with human osteoblast-like MG 63 cells (17,000 cells/cm^2; 1.5 ml of the DMEM medium supplemented with 10 % foetal bovine serum). We found that the initial adhesion and subsequent growth of MG 63 cells were similar on both types of membranes. From day 1 to 7, the cell number on PLGA-ND membranes ranged from 10,800±950 cells/cm^2 to 170,300±6,150 cells/cm^2, and on pure PLGA membranes, it was from 11,300±1,200 cells/cm^2 to 168,000±4,500 cells/cm^2. The cell spreading areas were similar on both PLGA-ND and pure PLGA membranes (477±16 μm^2 and 442 ± 15 μm^2, respectively). Nevertheless, the cell population doubling time, calculated from day 1 to 7, was similar in all tested groups (43±1.3 hours, 43±0.6 hours and 42±0.7 hours in PLGA-ND, PLGA and polystyrene dishes, respectively). Thus, it can be concluded that composite PLGA-ND membranes provided good support for colonization with bone-derived cells, and that this material is promising for bone tissue engineering.

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POLYLACTIDE NANOFIBERS MODIFIED WITH HYDROXYAPATITE AS SCAFFOLDS FOR TISSUE ENGINEERING

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Recently, the use of various types of nanofibers has expanded in a range of tissue engineering applications, mainly for their ability to mimic the architecture of tissue at the nanoscale. In this study, we evaluated the adhesion and growth of osteoblast-like cells on poly (lactide acid) (PLA) nanofibers prepared by electrospinning and incorporated with hydroxyapatite (HA) nanoparticles. Nanofiber meshes enriched with 0 wt.%, 5 wt.% or 15 wt.% of HA on dry mass were prepared. On day 1, the lowest initial adhesion was found on samples with 15 wt.% HA, however, on day 7, no significant difference in numbers of total cells was found. The XTT test, i.e. measuring the activity of mitochondrial enzymes in viable cells, showed that the density of viable cells was slightly lower on scaffolds with 15 wt.% of HA. No significant difference in cell densities between samples with 5 wt.% of HA and samples without HA was found. Visualization of cells growing on materials revealed that the cells were distributed heterogeneously, especially on HA-containing samples. An enzyme-linked immunosorbent assay revealed that the concentration of osteocalcin was the highest in cells grown on samples with 15 wt.% of HA. Nanofiber PLA scaffolds certainly have potential in tissue engineering. While its incorporation with 5 wt.% of HA had no influence on cell adhesion and growth, 15 wt.% slightly decreased cell proliferation and at the same time the concentration of osteocalcin in the cells was increased on these samples, which suggests that HA promoted cell differentiation to a desired osteogenic phenotype.

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THE INFLUENCE OF DIFFERENT DENSITY OF TITANIUM CLUSTERS ON THE ADHESION AND GROWTH OF OSTEOBLAST-LIKE CELLS

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Materials that are currently widely used for surgical implants include 316L stainless steel, cobalt-chromium alloys, and titanium and its alloys. Amongst the metallic materials available for implant applications, titanium is considered to be the best for bone implants, and is extensively used in biomedical applications. Titanium-based materials have a relatively low modulus of elasticity, and thus approach the elasticity modulus of the natural bone more closely than other metallic materials. No less important is the surface modification of the implanted materials, because the physical and chemical properties of the material surface play a decisive role for the cell-material interaction. The optimal roughness for the adhesion and growth of bone cells should be approximately in tens of nanometers (measured by the R_a parameter). This beneficial effect of nanoscale surface roughness is explained by the adsorption of cell adhesion-mediating proteins, such as vitronectin, fibronectin, collagen and laminin, in an appropriate spatial conformation for binding the active sites on these molecules by the cell adhesion receptors. In addition, surfaces with nanoscale irregularities were found to adsorb preferentially vitronectin, which is recognized mainly by osteoblasts (1). The aim of this study was to test the influence of different densities of nanoscale irregularities on the material surface on the adhesion, growth and potential immune activation of MG 63 cells *in vitro*. Nanoclusters of Ti (approximately 50 nm) were deposited on microscopic glass slides in two different densities, i.e. low (~4 μg/cm^2) and high (~12 μg/cm^2), and then they were covered by 25 nm or 80 nm of TiO_2, which is known to support cell adhesion and growth by its wettability (2). Glass slides coated with a flat TiO_2 film without underlying Ti clusters was used as reference material. The number of cells on the tested surfaces was evaluated in three time intervals (i.e., on days 1, 3 and 7 after seeding). The concentration of proteins participating in cell adhesion (β_1-integrins, vinculin, talin) and cell immune activation (ICAM-1) was also measured by an enzyme-linked immunosorbent assay (ELISA) per mg of protein on day 7 after seeding. We found that the number of initially adhering cells (day 1) and the final cell population density (day 7) were highest on the reference sample with a flat TiO_2 film. The concentration of β_1-integrin adhesion receptors was also highest in cells on this sample. However, on day 7 after seeding, the cells on samples with a low density of clusters covered by a thicker layer of TiO_2 (80 nm) reached a higher population density than the cells on samples where low-density clusters were covered with a thinner TiO_2 film (25 nm). This was probably due to rounding and flattening of the prominences on the material surface by the thicker layer.

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THE ROLE OF BMH PROTEINS IN THE REGULATION OF YEAST NEUTRAL TREHALASE

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Trehalase (EC 3.2.1.28) is an intrinsic glycoprotein of the small intestine and renal brush-border membranes that hydrolyzes α,α-trehalose (1-α-D-glucopyranosyl α-D-glucopyranoside) to two glucose molecules in animals [1]. Three trehalases have been identified in Saccharomyces cerevisiae so far: neutral trehalase 1 (NTH1), neutral trehalase 2 (NTH2) and acidic trehalase 1 (ATH1). NTH1 is responsible for trehalose degradation, which is accumulated after stress [3]. The

activity of the NTH1 enzyme was just recently found to be mediated by BMH1 and BMH2 binding in yeast. Yeast BMH1 and BMH2 proteins (yeast 14-3-3 isoforms) form a complex with neutral trehalase after its phosphorylation by PKA. Either one of the two 14-3-3 yeast isoforms are required for complete activation of neutral trehalase (NTH1) [2]. However details concerning the mechanism of BMH-dependent activation of NTH1 remain still unknown. BMH proteins were expressed as described previously [4]. All mutants of NTH1 were expressed as 6xHis tag fusion proteins and were purified from Escherichia coli Rosetta cells using Chelating-Sepharose Fast Flow. The 6xHis tag was cleaved by incubation with thrombin. After the cleavage, NTH1 was purified using cation-exchange chromatography on HiTrap SP column and using gel filtration on Superdex 200 column afterwards. Purified NTH1 mutants were phosphorylated by cyclic AMP-dependent protein kinase (PKA). We showed that PKA phosphorylates NTH1 *in vitro* on three Ser residues: 20, 21 and 83. To find out which site or sites are essential for the 14-3-3 binding we produced NTH1 WT (both phosphorylated and non-phosphorylated), three NTH1 mutants containing single phosphorylation site, one double phosphorylated NTH1 mutant (at Ser20 and 21) and a mutant containing none of these studied phosphorylation sites as well. The interaction between BMH1 and BMH2 protein with enzyme NTH1 was monitored using native electrophoresis and sedimentation velocity measurements. The sedimentation equilibrium analysis was used to define the stoichiometry of NTH1/BMH complexes. Finally, we used enzyme kinetic measurements to monitor the BMH-dependent activation of NTH1.

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CHARACTERIZATION OF LIGANDS BINDING SITES ON THE TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL TRPM5

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Transient receptor potential (TRP) channels are diverse family of proteins with structural features typical of iont channels. They are involved in the perception of a wide range of physical and chemical stimuli, including temperature, pain, taste, light, osmolarity changes and pheromones. Recent studies have indicated that members of the TRP family of ion channels can function as calcium influx channels both in excitable and non-excitable tissues. The channel subunits have six transmembrane domains that most probably assemble into tetramers to form non-selective cationic channels, which allow for the influx of calcium ions into cells. The TRP family is subdivided in three main subfamilies: the TRPC (canonical) group, the TRPV (vanilloid) group and the TRPM (melastatin) group. Members of TRPM family are divided into 4 groups – TRPM1+3, TRPM2+8, TRPM4+5, TRPM6+7. TRPM4+5 are closely related cation channels that are ubiquitously expressed. TRPM5 plays an important role in taste and is found mainly in the intestine, taste buds, pancreas, stomach, lung, testis and brain. TRPM5 is a monovalent-specific, nonselective cation cation channel that carries Na^+, K^+, but not Ca^{2+} ions. It is directly activated by Ca^{2+}, but activation mechanism remains controversial. Channels TRPM4+5 play roles in Ca^{2+} modulation. TRPM4 channel is activated by Ca^{2+} is often via complex signaling cascades including Ca^{2+}-Calmodulin binding, but characterization of binding site has not been determined in case of TRPM5. On basis of a comparison of the similarity with TRPM4 and Calmodulin Target Database, there have been identified several Calmodulin binding sites. To detect Calmodulin binding site we used anisotropy measurements between Calmodulin labeled with fluorescent probe and series of different length of highly purified fusion proteins of C-terminus of TRPM5. Similarly was identified PIP_2 binding site in C-termini of TRPM5 using side directed mutagenesis. It could be deduced that both sites (CAM and PIP_2) were overlapped in C-terminus of TRPM5. This project can help us understand the regulation of TRPM5 ion channel.

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CAPILLARY ELECTROPHORESIS OF PROTEINS AND PEPTIDES USING CARBOXYMETHYL CHITOSAN-COATED CAPILLARY

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Capillary zone electrophoresis (CZE) is a reliable method for peptides/proteins separation. Its application is attractive due to the simplicity and automatization of methods, high separation efficiency, high resolution, lack of organic waste, minimal sample consumption and small volumes of mobile phases needed for analysis. However, the separation of peptides/proteins is complicated by their tendency to adsorb onto the surface of fused silica capillaries. This sticking of peptides/proteins is significant disturbing factor in their separations by CZE. Positively charged aminogroups of peptides/proteins tend to adsorb on negative charged silanol groups of the inner wall of fused silica capillary. It results in poor recovery, peak broadening and distortion. Therefore resolution and quantitative determination may also be seriously impaired. To overcome these problems several strategies have been proposed. In our experiment we used carboxymethyl chitosan (CMC) for permanent coating of fused silica capillary. This approach was successfully applied for CE separation of the test mixture of proteins and peptides (cytochrome c, chymotrypsinogen A, Gly-Phe, Ala-Pro-Gly and Pro-Gly-Gly) and of tryptic digest of albumin, transferin and their glycoforms. The important advantages of this coating are a simple preparation and its long life-time. Comparison of separation with an uncoated capillary and with CMC-coated capillary is also discussed.

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LEARNING, MEMORY AND COGNITIVE FLEXIBILITY IN NOGO-A-DEFICIENT TRANSGENIC RATS

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Nogo-A, a myelin associated inhibitory protein, is an important member of a class of axonal outgrowth inhibitors, expressed by neurons and glial cells, which are responsible for the lack of regenerative potential in adult CNS. Disruption of such inhibitory mechanisms may facilitate recovery after injuries of the CNS. Recently, a possible link between such proteins and expression of specific behaviors has come to the focus of researchers. A rat knockdown model exhibiting markedly decreased neuronal expression of Nogo-A has been developed on the genetic background of inbred albinotic Sprague-Dawley rats, which served as wildtype controls. As mice completely lacking Nogo-A exhibit schizophrenia-related endophenotypes, we hypothesized that neuron-selective knockdown of Nogo-A in the rat would result in neurodevelopmental abnormalities, reflecting in altered behavioral functions compared to intact Sprague-Dawley rats. Nogo-A-deficient rats were trained in a battery of place avoidance variants with increasing demands for segregation of spatial information and behavioral flexibility; and delayed-matching-to-place (DMP) version of the Morris water maze, both requiring intact hippocampus. Results showed that Nogo-A-deficient rats were impaired in the final stage of place avoidance battery (spatial reversal) but their visuospatial working memory and persistence of memory trace tested in the DMP was similar to controls. It is concluded that Nogo-A-deficient rats exhibit deficits in behavioral plasticity, but no overt disruption of hippocampal integrity. Further detailed studies focusing at behavioral manifestations of subtle hippocampal deficits would be required.

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INERTIAL INPUTS AND THEIR ROLE FOR SPATIAL COGNITION

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Effective spatial navigation requires remembering relevant places in the environment. This ability depends on the finding of relevant sources of the spatial information and their correct utilization. Generally, navigation depends on various types of sensory information. Visual, auditory or olfactory inputs are examples of "external sources" of spatial information. Proprioceptive, tactile or vestibular inputs are examples of "inner sources" of spatial information. By means of vestibular or proprioceptive inputs is possible to perceive so called inertial stimuli which are generated by active or passive movement of a subject. External sources of spatial information seem to be most important for navigation and inner sources of spatial information sometimes may be omitted. In present study we evaluated importance of perceiving inertial inputs generated by passive movement of animals for spatial cognition. We used behavioral method called "Active Place Avoidance Task" (AAPA). This task is used for testing cognitive abilities in rats. A rat which is placed on a rotating circular arena, should avoid an unmarked sector defined with respect to stable extra-arena cues. We tested the hypothesis that the inertial stimuli generated by the arena rotation may contribute to the performance in the task. These stimuli provide permanent information to the rat concerning changes in its position with respect to the external world with distal cues. Set of these cues represents reference frame in which the to-be-avoided sector is defined. We trained one group of rats on a stable arena while extra-arena cues rotated around the arena. This eliminated the inertial stimuli generated by the arena rotation while preserving other aspects of the task. Six out of seven rats from this group did not learn this modified task. The remaining rat learned it equally well as rats from a control group learned the standard active place avoidance task. After six days of training, we changed the tasks between the groups. The control rats solved the modified task as well as the standard task. We conclude that the inertial stimuli generated by the arena rotation are important for acquisition of the active place avoidance task but not for performance once the task has been mastered. We suggest that rats must perceive the distal extra-arena cues as stable in order to associate the position of the to-be-avoided sector with these cues.

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APPLICATIONS OF MULTIPLE REFERENCE FRAMES ENVIRONMENTS IN BEHAVIORAL RESEARCH

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This work has been motivated by the desire to enhance our knowledge about specific cognitive requirements of navigation in multiple reference frames environments and to understand the roles of the hippocampus and posterior parietal cortex in this behavior. The main conclusions of this study are: We have developed a novel behavioral test called the Enemy Avoidance Task. The initial set of experiments has shown that laboratory rats are able to plan their movement with respect to a to-be-avoided moving object. Behavioral performance in the task may be quantitatively evaluated. The aforementioned ability is crucially dependent on the functional integrity of the dorsal hippocampus. To the contrary, functional inactivation of the dorsal hippocampi by local infusion of tetrodotoxin did not cause any impairment in the ability of the animal to estimate its distance from a non-moving object. The finding suggests a specific role of the hippocampus in dynamic cognitive processes required for flexible navigation strategies such as continuous updating of information about the position of a moving stimulus. These results are at odds with the two major theories of hippocampal function (Cognitive map theory and Declarative memory theory) and therefore suggest that revision of the theories is necessary. Lesion to the posterior parietal cortex does not critically impair avoidance behavior in the Enemy Avoidance Task. We have analyzed the importance of inertial stimuli generated by arena rotation in the the AAPA task (avoidance of a room-frame bound sector on a rotating arena). Inertial stimuli are critically important in the acquisition phase of the task, but are not indispensable once the task is already learnt. Following an initial training period, visual stimuli are sufficient to solve the task. Lesion to the posterior parietal cortex does not cause major impairment in the place avoidance task on rotating arena in neither of the reference frames. However, indirect evidence from the "frame-preference test" shows that PPC lesion may produce changes in cognitive coordination, i.e. the ability to separate the frames. However, further research is needed to confirm the hypothesis.

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THE ROLE OF HIPPOCAMPUS IN OBJECT AND OBJECT-PLACE RECOGNITION

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The role of hippocampus in cognition is intensively studied. It is unclear whether hippocampus is necessary for recognizing position of a distant object and what it is its role in object recognition. To study these questions we have developed two novel operant-conditioning tasks: a) a task in which rats discriminate different objects presented on a computer screen (object recognition task) and b) a task in which rats discriminate different positions of the same object presented on the computer screen (object-place recognition task). Operant responses (lever presses) were reinforced when one particular object was displayed (object recognition task) or when the object was displayed in one particular position (object-place recognition task). After the rats reached an asymptotic performance, we have studied the role of hippocampus in these tasks by injecting a $GABA_A$-receptor agonist (muscimol 0.3 μg) into both hippocampi. The results showed that hippocampal inactivation impaired performance in both tasks without affecting motor activity. In the object-recognition task the rats were evaluating visual similarity between the rewarded object and a currently presented object. The hippocampal blockage affected specifically this process. The same rats were subsequently tested in a brightness discrimination task which is considered as hippomcapal independent. As expected the hippocampal inactivation had no effect on behavior in this control task. We conclude that a) the ability to recognize position of a distant object requires hippocampus and b) the perception of visual similarity of different objects depends on hippocampus.

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IMPAIRMENT OF SPATIAL COGNITION IN SCHIZOPHRENIA PATIENTS

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Spatial navigation is a complex cognitive ability involving cognitive functions, such as perception, attention, memory, and executive functions. Impairment of spatial cognition in schizophrenia (as a consequence of neurotransmitter systems dysfunction) leads to disorientation as well as disruption of daily activities. To test the spatial cognition abilities, we have developed a virtual version of the Blue Velvet Arena (BVA), apparatus situated in Prague Hospital Motol and used for an early diagnosis of navigation disorders. This apparatus is a human version of the well-known spatial navigation task developed for rats, called Morris water maze (MWM; Morris, 1984). The virtual

human version of the MWM examines the ability of subjects to remember the position of a hidden goal on the floor of an enclosed circular arena. In several succeeding trials they have to navigate to the hidden goal from different start locations using various visible orientation cues placed near the arena wall. To examine spatial navigational impairment in schizophrenia patients, first we assessed in healthy volunteers their ability to find the correct goal position and identify their cognitive strategies used to solve the task. We have developed a task requiring subjective estimation of the goal position after an unannounced cue(s) deletion. Our data indicates that some individual hierarchy appears between three visible orientation cues. This means that one of them as a dominant cue leads to more accurate navigation. Similar virtual BVA task is currently used to assess the spatial cognition impairment in schizophrenia patients. The task was adapted according to the working memory version of MWM, where the hidden goal is shifted after each 6 trials to a new position. The goal position appears and beeps after the subjects' entrance or after the specified time limit, so the subject obtains positive or negative feedback after each trial. Preliminary results from 10 patients show that the spatial memory is impaired in schizophrenia patients with respect to the level of cognition impairment measured by other neuropsychological tests. In comparison to the controls, these patients show significantly decreased efficiency of the distance traveled and increased latency in the first part of the test. Schizophrenia patients also show specific disturbed performance in the probe trials, during which the goal position does not appear after the entrance. The percentage of the time spent in the correct arena quadrant during the probe trials is significantly decreased among the patients. In addition the distance from the goal position averaged for one probe trial is in patients significantly increased. Our present findings are preliminary and we will examine and complete them by testing a bigger group of schizophrenia and control subjects.

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ELEVATED CORTICOSTERONE LEVELS: DELAYED EFFECTS ON HIPPOCAMPAL FORMATION VOLUME, CELL COUNTS AND NEURODEGENERATION MARKERS IN LABORATORY RAT

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We studied delayed effects of elevated plasma levels of corticosterone (CORT) on brain structure volume, neuronal quantity, and gross marks of neurodegeneration in the hippocampal formation of Long-Evans rats. Animals were exposed to increased CORT levels for three weeks via implanted subcutaneous pellets. The pellet contained 200 mg of corticosterone released over 21 days. Control animals received pellets containing cholesterol. Exogenous corticosterone treatment is pharmacologically important in animal stress model as well as in medicine. Volumetry, neuronal quantification and gross marks of degeneration were measured seven weeks after the termination of CORT treatment. We observed significant differences in volumes and especially in laterality of hippocampal subfields between control and CORT-treated animals. We found that the left hippocampus was substantially larger than the right hippocampus in the costicosterone-treated group, but not in the control group. In the control group, on the other hand, right hippocampal volume was markedly higher than all other measured volumes. Left hippocampal volume did not differ between the groups. The most interesting result of this study is the asymmetrical decresase of the volume of the right hippocampal formation, mostly in the subiculum and less in the dentate gyrus and the CA1-CA3 subfield. Surprisingly, our data show only little (CA1 and CA2 subfield) or no (dentate gyrus and CA3) changes in the neuronal quantity, but the cell counts were not evaluated for the subiculum, where the volume change was most notable. No marks of an ongoing neurodegenerative process were present, and numbers of degenerated neurons were not changed significantly. Therefore we conclude that the degenerative process occurred during the CORT treatment and ceased afterwards.

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BRAIN EVOKED POTENTIALS USING LOWCOST DIGITALIZATION CARDS

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Electrophysiological methods used in medical experience and research lead to detailed mapping of electrical activity of investigation part. Nowadays methods for recording and mathematical analysis electrophysiological signals increase, because increase requirement obtaining of more and complex information about patient. Electroencephalography (EEG) is the measurement of spontaneous electrical activity of the brain. Evoked potentials (EP) are changes of spontaneous electrical activity of the brain and represent responses to an external sensory stimulation. Shape of the evoked response varies in time. EEG and evoked responses processing is used for clinical diagnostic such as epileptic source localization. Nowadays in many experiments increase the need of synchronous measuring of electrophysiological signals together with video signals. Current systems are often specialized for either electrophysiology or video without possibility to both in one environment. Our aim is to develop universal system specialized to measure and analyze EEG signals and evoked responses synchronous with video signals. For our purposes we use multifunction cards M Series developed by a company called National Instruments (NI) and we developed software called VisionBrain. These multifunction cards were chosen because of their low cost. The other advantage is the fact that they together with quality amplifier are comparable with more expensive EEG systems currently used. These data acquisition cards have up to 80 analog inputs, 4 analog outputs and 48 digital I/O lines. The software VisionBrain enables to set up all inputs and outputs and programmable amplifiers NI-PGA in addition. Not less important task was to find an acceptable file format for storing measuring electrophysiological data and events altogether. For our purposes we designed our own binary file format called Brain Vision Data (*.bvd) and Brain Vision Events (*.bve). The synchronization between multifunction digitalization card and camera is ensured by two ways. If camera supports external triggering, synchronization is managed by using card's digital I/O. However, if camera doesn't support it, we can provide simplified synchronization through prepared LED diode. The software was tested with multifunction NI M Series PCI-6221 data acquisition card (16bit, 250 kS/s, 16 AI, 2 AO and 24 digital I/O lines) with homemade EEG four channel amplifiers for signal preconditioning. For video recording we have tested simultaneous image acquisition with high resolution webcam (Philips 900NC), industrial cameras UI-2230C and UI-2230M (Imaging Development Systems, Germany) and high sensitive cooled camera Retiga 2000R (Q-Imaging, Canada).

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INVOLVEMENT OF NO IN THE NEUROVASCULAR COUPLING DURING CORTICAL EPILEPTIC ACTIVITY IN RATS

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Available *in vitro* data show the importance of nitric oxide (NO) of neuronal origin in the initiation of seizures. However, recent findings suggest a dual role for NO in seizure modulation, which seems to vary with the type of nitric oxide synthase (NOS) inhibitor and with the model of seizures used. In the present study we investigated the effect of 7-Nitroindazole, an *in vivo* inhibitor of the nNOS, on seizures

induced by transcallosal electrical stimulation in rats. Adult albino rats (250-350 g, n=14) were anaesthetized and epidural silver EEG electrodes were implanted over sensorimotor cortices. Regional cerebral blood flow during epileptic activity was measured by Laser Doppler flowmetry. Blood gas levels, neuronal activity, regional cerebral blood flow (rCBF) and blood pressure were measured so as behavioral tests which were also performed before and after an. i.p. bolus of 7-Nitroindazole (25 $mg.kg^{-1}$). We catheterized a common carotid artery to measure arterial blood pressure and arterial blood gasses. After postsurgical recovery animals were placed in a recording chamber. Seizures were elicited by biphasic constant current suprathreshold stimulus (8 Hz, 15 s) which was applied after 15 minutes recording of background effect of intraperitoneally administered 7-Nitroindasole (25 $mg.kg^{-1}$). Results from acute *in vivo* EEG measuring display controversies. The inhibition of nNOS by 7-Nitroindazole reduced blood pH. It also led to a slight increase of blood pressure and decreased baseline rCBF. Transcallosal electrical stimulation produced cortical epileptic afterdischarges which were paralleled by facial and fore limb clonic seizures. NOS inhibition also potentiated the severity of afterdischarges. In addition it impairs locomotor activity 30 minutes after administration, however 240 minutes after no effect was observed. Outcomes from EEG measurings show a proconvulsive action of 7-Nitroindazole in the rat model for myoclonic seizures. The administration of 7-Nitroindazole did not produce significant changes of blood pH. We conclude that a motor coordination deficit was found in 7-Nitroindazole treated animals. Furthermore we suggest that nNOS may play some role in the control of motor behaviour. The *in vivo* effect of NO is very complex and thus the anticonvulsive action is probably hidden by other systemic NO action. The elucidation of the mechanisms underlying epilepsy is crucial in order to gain a rational basis for developing novel therapies.

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POSITIVE ALLOSTERIC MODULATOR OF METABOTROPIC GLUTAMATE RECEPTORS SUBTYPE 4 (mGluR4)-PHCCC IN THREE DIFFERENT MODELS OF EPILEPTIC SEIZURES ELICITED IN IMMATURE RATS

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Metabotropic glutamate receptors (mGluRs) serve to modulate CNS excitability mainly by their presynaptic action. Activation of mGluRs Group III can reduce glutamate release and thus could be valuable in treatment of various neurological (epilepsies, Parkinson's disease) as well as psychiatric disorders (anxiety, depression). PHCCC, N-phenyl-7-(hydroxyimino) cyclopropa[b]chromen-1acarboxamide, is a positive allosteric modulator of metabotropic glutamate receptor subtype 4. Activation of this receptor seems to be beneficial in several types of Parkinson-like symptoms and produces anxiolytic-like effect in rodents. mGluR4 is involved in a generation mechanism of normal as well as pathological rhythms in both adult and developing brain. Because of insufficient evidence concerning influence of mGluR4 receptor subtype on epileptic seizures in immature brain, we studied effects of PHCCC administration in a three different models of seizures. A first experiment, pentylenetetrazol-induced convulsion was performed in 7-, 12-, 18- and 25-day-old rats. PTZ (100 mg/kg) was administered subcutaneously 30 min after pretreatment with PHCCC in doses of 1, 3, 10 and 20 mg/kg i.p. Rats were observed in isolation for 30 min and incidence, pattern and latencies of convulsions were registered. In the other two experiments animals went through surgical procedure: pups were anesthetized with ether and silver epidural stimulation and recording electrodes were implanted. Cortical afterdischarges (ADs) were elicited by low-frequency stimulation of sensorimotor cortex in rat pups 12, 18 and 25 days old. Stimulation series were repeated six times with 10-min intervals. Five min after the first AD, PHCCC was injected (3 or 10 mg/kg i.p. Total duration of ADs and severity of motor phenomena were evaluated. Pentyleneterazole (PTZ)-induced absence-like seizures experiment was performed only in 18- and 25-day-old rats. Four recording electrodes were used for EEG registration. Two 20-mg/kg doses of PTZ were administered with a 20-min interval. PHCCC was injected in a dose of 3 or 10 mg/kg 15 min after the first dose of PTZ. Latency, number and duration of the RMA were evaluated in 10 min intervals. Control animals in all three experiments received solvent, i.e. dimethylsulfoxide. The solvent did not significantly affect any model used. PHCCC did not significantly influence motor seizures induced by PTZ in any age group. In contrast, PHCCC administration caused significant ADs prolongation as well as increased incidence of RMA and their prolongation. These results suggest that mGluR4 activation during early stages of development have clear proconvulsant effect. It supports hypothesis that activated mGluR4 presynaptic receptors at corticothalamic connections reduce glutamate release in a manner which can drive to limitation of GABA-ergic inhibition in RTN and aggravates spike-and-wave EEG rhythm (1, 2)

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PERINATAL EPILEPTOGENESIS POST PHOTOTHROMIC ISCHEMIC STROKE INDUCED IN RATS AT P 7.

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The number of models available to study perinatal epileptogenesis after brain injury is currently limited. We aim to find the critical developmental period before initial jump in electroencephalogram development and use it to induce stroke, which may lead to epileptogenicity with epileptic manifestation in adulthood. To determine the age of rapid development of EEG activity, video-EEG and EMG were recorded at P 7, 9, 12, 15 in male Wister rats. Photothrombotic brain lesions were induced at P7 utilizing intravenous injection of Bengal Rose followed by 5 minutes laser illumination over sensomotor cortex. Possible motor deficits were assessed two months later. Animals underwent continuous video-EEG monitoring to asses epileptic activity, succeeded by transcardial perfusion and tissue staining. Results from spontaneous EEG-recording during development revealed biphasic rhytmogenesis; signal energy and entropy rose with age, highest in intervals P 7-P 9, P 12-P 15 respectively. P 7 was therefore selected as the ideal age to mimic these "critical developmental period". Photothrombotic lesions impaired sensorimotor performances and led to hyper-locomotion in adulthood. Video-EEG recorded at P 70 revealed partial hippocampal seizures with secondary generalization. Lesion diameters of 2.4-3.7 mm penetrated motor cortex reaching external capsule. Volume ratio analysis (ischemic/contralateral hemisphere) revealed significant volume reduction of ischemic hemisphere. In conclusion, P7 proved to be the ideal age corresponding to critical developmental period of cortical maturation represented by EEG signals. Stoke induced at this age lead to significant reduction in tissue volume, developmental epileptogenesis plus functional deficits and epileptic activity in adulthood.

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CAPSAICIN AND CAMPHOR: TWO DIFFERENT MECHANISMS OF ACTION ON TRPV1 CHANNEL

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The vanilloid transient receptor potential (TRPV1) ion channels are Ca^{2+} permeable cation channels specifically expressed in primary afferent nociceptors of the peripheral nervous system. TRPV1 can be activated and subsequently desensitized by various chemical and physical signals that interact allosterically with TRPV1 through separate molecular mechanisms: vanilloids (capsaicin), acids, hot temperature (>43 °C), voltage or camphor (1). From the cytoplasmic side, TRPV1 is critically modulated by membrane phosphoinositide 4,5-bisphosphate (PIP_2), the intracellular concentration of which varies as a result of calcium influx through the channel pore when it is open by capsaicin (2). Camphor, a naturally occurring monoterpene, activates the TRPV1 channel independently of the capsaicin binding site and strongly

desensitizes it by an, as yet, unclear mechanism. This highly amphipatic compound may exert its effects on TRPV1 through influencing the general membrane structural organization and surface electrostatics (3), consequently, through PIP_2-mediated effects. In this study, we used fluorescence resonance energy transfer (FRET), electrophysiology and Ca^{2+}-imaging in single live HEK293T cells in order to (i) characterize the general effects of camphor on the plasma membrane associated events, particularly on the changes in the distribution of membrane PIP_2, (ii) examine the effects of camphor on TRPV1 in the context of PIP_2-mediated regulation, (iii) explore the extent to which the two distinct forms of acute desensitization of TRPV1: camphor-induced (Ca^{2+}-independent) and capsaicin-induced (Ca^{2+}-dependent) are interrelated, and (iv) correlate these processes with changes in intracellular Ca^{2+} levels. We found that camphor markedly activated, immediately desensitized and blocked the TRPV1-mediated responses. Using combinations of camphor with either capsaicin, voltage or heat, we demonstrate that camphor activates TRPV1 through the preferential interaction with the capsaicin-occupied rather than with the closed-state form of the channel and, at higher concentrations (>5 mM), it is able to overcome TRPV1 desensitization. We suggest the possibility that camphor affects the membrane PIP_2 distribution (in contrast to capsaicin) so that this might be a sufficient priming signal for camphor-mediated modulation of TRPV1.

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THE C-TERMINAL BASIC RESIDUES CONTRIBUTE TO THE POLYMODAL ACTIVATION OF TRPA1

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The ankyrin transient receptor potential channel TRPA1 is a nonselective voltage-sensing cation channel. It is predominantly expressed in a subpopulation of nociceptor neurons. There it mainly acts as a polymodal sensor, activated by pungent compounds such as allyl isothiocyanate (AITC) (in mustard oil), cinnamaldehyde (in cinnamon) or acrolein (in smoke), as well as by low temperatures (<18 °C) (1). Like other TRP channels, TRPA1 assembles as a tetramer of subunits, each with six transmembrane helices. Both N and C termini of TRPA1 are predicted to be cytoplasmic and containing various protein-interaction and modulatory motifs. Almost 80 % of the N teminal region is formed by ubiquitous ligand-interaction motifs – ankyrin repeats. While several functionally important regions have been described along the N-terminus (EF hand, cysteines involved in covalent interactions with thiol-reactive electrophilic agonists), there is very little known about the functional importance of the C terminus. In an effort to characterise this part of TRPA1 channel, we focused on basic residues, which are likely candidates for interactions with common TRP channels regulatory molecules – negatively charged phosphoinositides (2,3). We have mutated 27 basic residues all along the C-terminal tail, and performed the whole-cell patch-clamp experiments to determine their role in human TRPA1 function. Several mutations which affected the AITC- and voltage-dependent gating were identified not only in the proximal part of the C-terminus (residues K969, R975, K988 and K989) but also in the fourth predicted helix centered around K1048 and K1052. There, single alanine mutations completely abolished AITC- and voltage-dependent activation. Two function-affecting mutations were also found even in the distal portion of the C-terminus (K1092A and R1099A). In addition, we characterized three charge-neutralizing 'gain-of-function' mutants (R975A, K988A and K989A) which exhibited higher sensitivity to depolarizing voltages, indicating that these residues may be directly involved in the voltage-dependent modulation of TRPA1. Together, our results identify basic residues in the C-terminus that are strongly involved in TRPA1 voltage and chemical sensitivity, and some of them may represent possible interaction sites for negatively charged molecules that are generally considered to modulate TRPA1 (4).

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INHIBITORY EFFECT OF NEWLY SYNTHESIZED NEUROSTEROIDS AND THEIR ACCESS TO THE NMDA RECEPTOR

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N-methyl-D-aspartate (NMDA) receptors play important roles in development, synaptic plasticity, learning, and memory, however, abnormal activation of NMDA receptors is thought to mediate neuronal degeneration (1). NMDA receptor activity can be influenced by exogenous and endogenous ligands, including neurosteroids – endogenous steroids that are synthesized and act in the central nervous system. Allosteric modulators influence activity of NMDA receptor-channels positively or negatively with biological consequences. 20-oxo-5β-pregnan-3α-yl sulfate (pregnanolone sulfate, PAS) is an endogenous neurosteroid that inhibits NMDA receptors and is neuroprotective. To delineate the mechanism of NMDA receptor inhibition by PAS patch-clamp and imaging recordings from HEK293 cells expressing NR1/NR2B receptors and cultured rat hippocampal neurons were used. We have prepared eighteen pregnanolone derivatives substituted at C3 with negatively and positively charged chemical groups in different distances from C3 as well as with uncharged residues and in addition two fluorescence tagged steroids. The results of our electrophysiological experiments show that kinetics of the steroid inhibition is slow and not typical for drug-receptor interaction in the aqueous solution. In addition, the recovery from inhibition was accelerated by β- and γ-cyclodextrin. Values of IC_{50} assessed for novel synthetic C3 analogs of PAS differ by more than 30-fold. The values of IC_{50} were positively correlated with the steroid lipophilicity. The onset of inhibition induced by C3 analogs of PAS can range from agonist-dependent to agonist-independent. The onset and offset of cell staining by fluorescent analogs of PAS is slower than that determined of the onset and offset of these steroids induced inhibition of current responses mediated by NMDA receptors. We conclude that steroid accumulation in plasma membrane is route, from which it accesses a site on the NMDA receptor.

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A FUNCTIONAL STUDY OF ION CHANNELS INVOLVED IN NOCICEPTIVE SIGNALLING

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The detection of noxious external stimuli by peripheral sensory neurons and their conduction up to the higher levels of the central nervous system are crucial for maintaining the integrity of an organism. These processes depend on the composition of specific ion channels along the nociceptive nerve fibers. Until this time, many of these multi-molecular complexes have been identified and characterized (1). However, many of their specialized physiological roles, particularly the molecular

mechanisms underlying the encoding and modulation of nociceptive signals remain to be investigated. The aim of our work is to contribute to the understanding of some of these processes, especially of these related to the activity of ion channels which are mainly expressed in primary afferent sensory neurons and are major contributors of nociceptive signalling. Among the nociceptor-specific ion channels, several members of the TRP *(Transient Receptor Potential)* protein family (e.g. TRPV1, TRPM8, TRPA1) are considered as detectors of potentially harmful stimuli. They are polymodal ion channels activated by a plethora of pungent or irritant chemical substances and also by changes in the environmental temperature. The other major subclasses of ion channels contributing to the conduction of noxious stimuli are tetrodotoxin-sensitive (Nav1.3, Nav1.7), tetrodotoxin-resistant voltage-gated sodium channels (Nav1.8, Nav1.9) and voltage-gated potassium channels (e.g. K_{DR}, K_A). We used fluorescence, electrophysiological and molecular biological methods to study the activation mechanisms and structure-function relationship of native and heterologously expressed TRPV1, TRPM8 and TRPA1 channels. We also characterize the role of specific voltage-gated ion channels in the conduction and modulation of innocuous and noxious temperature stimuli in cultured dorsal root ganglion neurons. Our results bring new data on the function of ion channels involved in nociceptive signalling, standardize approaches and outline aims for upcoming studies.

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A MODEL OF AUDITORY PROCESSING INSPIRED BY BINAURAL ADAPTATION

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Auditory brainstem calculates horizontal direction (azimuth) of sound from the ITD (interaural time delay). Two theories exist about how brainstem circuit calculates the azimuth from the ITD. Jeffress proposed in 1948 that azimuth is computed by an array of delay lines (1). The anatomical existence of such array in mammals is still under debate. Our group in 2005 explored the possibility of the stochastic algorithm (2). We use the simplest yes-or-no neuronal model. The computation of output quantities is based on the stochastic processing. For simplicity of the model, we use the ergodic assumption that averaging over the population of neurons and averaging over several time periods yield same outputs. We study various parameters of the model and their influence on the output of the model. Finally we measure performance of the neuronal circuit using the concept of ideal observer.

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EXPLORING MUSCLE SYNERGIES OF DROSOPHILA FLIGHT APPARATUS USING INDEPENDENT COMPONENT ANALYSIS OF WINGBEAT KINEMATICS

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Flies are considered to be the mini-masters of flight, surpassing all other flying organisms in flight maneuverability. Their remarkable flight abilities are attributable to their highly sophisticated flight apparatus. In Diptera the flight apparatus is physiologically and functionally differentiated into two major groups, the power muscles and steering muscles. Steering muscles modulate the wing kinematics in order to fine tune the wing oscillations and hence generate various maneuvers. There are neuro-physiological indications that instead of acting as individual muscles they might act as independently controlled groups of muscles. In our work we aim to identify such muscle synergies from observed changes in wing kinematics. For real time analysis of the wing kinematics, wingbeat of tethered flies (*Drosophila melanogaster*) were recorded with a high speed computer vision system. Wing positions were sampled at a rate of 6250 Hz with a precision of 1°. Wingbeat parameters like the amplitude, ventral flip angles and dorsal flip angles were extracted, and analyzed using independent component analysis (ICA). ICA is a statistical method for obtaining maximally mutually independent linear combinations of the multivariate signals analyzed. ICA of left and right wingbeat amplitudes reveals that their difference is controlled almost independently from their weighted sum. At a more fundamental level, from ICA of the left and right dorsal and ventral flip angles we consistently obtain the difference of ventral flip angles as one of the independent components**.** These results imply relative independence of the control of laterally asymmetric changes of the ventral flip angles. In order to understand neuro-physiological implications of the ICA results, we have proposed a linear decomposition of the controlling factors of the four wing flip angles into six laterally symmetric and anti-symmetric units.

DIFFERENT EFFECTS OF ALLOSTERIC MODULATORS ON AGONIST BINDING AND ACTIVATION OF THE M_3 MUSCARINIC RECEPTOR

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Allosteric modulators of muscarinic receptors demonstrate a noteworthy high degree of subtype selectivity in influencing affinity of orthosteric ligands that is unique for both interacting pair classical/allosteric ligand and muscarinic receptor subtype (1). We unexpectedly observed a discrepancy between allosteric modulation of alcuronium of the affinity of acetylcholine analog carbachol (Kd) measured under equilibrium and allosteric modulation of its potency measured in functional assay as carbachol-stimulated GTP-γ^{35}S binding to membranes expressing M_3 muscarinic receptor subtype. Despite a strong negative binding cooperativity alcuronium had no effect on potency of carbachol to activate interacting G-proteins. Another modulator brucine that increases affinity of carbachol at M_3 receptor demonstrated a large decrease in potency of carbachol to stimulate GTP-γ^{35}S binding. To further probe this divergence of binding and functional effects we determined influence of brucine and alcuronium on activation of preferential Gq/11 and non-preferential Gi/o G-proteins using scintillation proximity assay. Likewise carbachol stimulation of GTP-γ^{35}S binding to all G-proteins, brucine decreased the potency of carbachol in activating both Gq/11 and Gi/o G-proteins, and alcuronium had no influence on the potency of activation of either G-protein. In following experiments we tested whether changes in kinetics of agonist-induced receptor activation could explain these seemingly incompatible observations. To this end we employed nicotinic antagonist rapacuronium that during clinical testing demonstrated serious bronchospasm ascribed to its putative positive allosteric interaction with endogenous acetylcholine at M_3 receptors (2). In equilibrium binding experiments we found negative cooperativity of rapacuronium with acetylcholine binding at all muscarinic receptor subtypes (3). However, rapacuronium at low concentrations increased both potency and efficacy of acetylcholine in receptor activation at the M_3 receptors while at high concentration caused anticipated reduction of potency. Kinetic experiments in the presence of low concentration of rapacuronium exhibited a large transient increase in the rate of acetylcholine-induced GTP-γ^{35}S binding. Results conclusively demonstrate deviations of allosteric modulation of functional outcomes from that anticipated on the ground of equilibrium binding studies. They point to their differential action on receptor activation and binding that are mainly manifested under non-equilibrium conditions that are prevalent *in vivo*. They emphasize the importance of fast functional tests for determining the effects of allosteric modulators on signal transduction.

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AMYLOID β_{1-42} AFFECTS MUSCARINIC RECEPTORS EXPRESSED IN CHO CELLS AND IN THE MICE MODEL OF ALZHEIMER'S DISEASE

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It has been suggested that impaired cholinergic neurotransmission is related to geriatric memory dysfunction and Alzheimer´s disease (1). In studies on the mouse model of Alzheimer disease (double transgenic mouse APPswe/PS1dE9) we investigated influence of *in vivo* amyloid-β fragments accumulation on muscarinic receptor density and function in cerebral cortex membranes during aging. In young (2-month-old) animals we did not find differences between transgenic and non-transgenic littermate animals (2). However, in both groups we found age-dependent reduction in receptor density and decline in receptor activation that were significantly more pronounced in transgenic animals (2,3). Weakening of receptor activation was apparent already in young adult (5-6-month-old) transgenic animals and appeared before amyloid deposits formation. In this age group, we did not detect impairment of presynaptic M2 and M4 receptor subtype function when studied separately in *ex vivo* functional tests. In subsequent experiments we tried to detect a presumed damaging influence of chronically applied low concentration of $A\beta_{1-42}$ (100 nM for 4 days) on muscarinic receptor subtypes individually expressed in Chinese hamster ovary (CHO) cells. A series of displacement experiments using agonist carbachol and [^{3}H]N-methylscopolamine as a tracer revealed decreased affinity of agonist high-affinity receptor binding site in M1 subtype and an increase in relative number of these high-affinity sites in M1 and M3 receptors. These results agree with published data reporting selective impairment of M1 receptors (which is the predominant subtype in both human and rodent brains) in studies using autopsied brains of Alzheimer´s disease patients. On the other hand, in contrast to experiments with APPswe/Ps1dE9 mice, using this simple model of CHO muscarinic receptors expressing cells, we were unable to detect any changes in GTPγS-binding parametres in any receptor subtype.

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MUSCARINIC ACETYLCHOLINE RECEPTORS SUBTYPES DIFFER IN INTRACELLULAR CALCIUM MOBILIZATION INDUCED BY XANOMELINE

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Muscarinic acetylcholine receptors belong to a large family of G-protein coupled receptors. Five subtypes of muscarinic receptors (M_1-M_5) can be divided to two groups depending on the type of G-protein they interact with. Odd subtypes (M_1, M_3, M_5) couple preferentially with $G_{q/11}$ class of G-proteins whereas even subtypes (M_2, M_4) transmit the signal across the plasma membrane by $G_{i/o}$ G-proteins. Muscarinic neurotransmission have important physiological roles in both central nervous system and periphery. A decline of cerebral cortex muscarinic transmision, the characteristic for dementia in elderly and Alzheimer´s disease, is mainly due to reduction of M_1 receptor-mediated transmission. Selective M_1 receptor agonists are thus sought as promising drugs. Muscarinic agonists exhibit very little selectivity because of conserved nature of orthosteric binding site. Xanomeline is a functionally selective M_1/M_4 muscarinic receptor agonist. It binds reversibly to the orthosteric site of all subtypes with similar high affinity. In addition, it also binds to an allosteric site of all subtypes in a wash-resistant (WR) manner with similar low affinity (1, 2). Our aim is to unravel mechanisms establishing selectivity of xanomeline. To this end we employed Chinese hamster ovary cells expressing individual subtypes of muscarinic receptors and fast microfluorometry of intracellular calcium to follow xanomeline-induced receptor activation. In case of M_2 and M_4 receptors we used cells transiently transfected with $G_{q/16}$ G-protein α-subunit that does not discriminate between receptor subtypes and mediates calcium mobilization. We found that in contrast to other subtypes stimulation of M_1 or M_4 receptors for 1 min elevated levels of intracellular calcium that persisted for more than one hour in the absence of xanomeline. On the other hand, even 10-min application of xanomeline did not bring steady elevation of intracellular calcium at M_5 receptors. WR binding of xanomeline to this subtype antagonized activation by full agonist carbachol. At M_1 receptors, orthosteric antagonist N-methylscopolamine blocked receptor activation by xanomeline but did not prevent formation of xanomeline WR binding. These data are compatible with the view of xanomeline acting as an ectopic agonist. Differences among receptor subtypes in kinetics of activation by xanomeline and its long-term effects on intracellular calcium levels may constitute the basis for xanomeline functional selectivity.

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GENE EXPRESSION OF CHOLINERGIC MARKERS IS INTACT IN CEREBRAL CORTEX OF YOUNG ADULT APPswe/PS1dE9 MICE

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Decline in cognitive functions that is gradual in the course of natural aging but progressive during Alzheimer disease (AD) is in general accompanied by a disruption of central cholinergic system (1). Major pathological hallmarks of the advanced stage of the disease found at autopsy are presence of amyloid plaques and neurofibrillary tangles. The increase in concentration of soluble β-amyloid fragments precedes both clinical symptoms and characteristic pathology. Now it is generally accepted that the soluble β-amyloid fragments in not fully understood way initiate and drive progression of AD (2). Double transgenic mouse model APPswe/PS1dE9 of AD opens up possibilities for studying effects of *in vivo* β-amyloid accumulation on brain function. These mice overproduce human β-amyloid and at the age of about 7 months start to form amyloid deposits. We have previously found significant reduction of presynaptic (vesicular acetylcholine transporter and choline acetyltranferase) as well as postsynaptic (muscarinic receptors) cholinergic marker proteins and weakening of their function (3, 4). Using qPCR we investigated whether the decrease in cholinergic proteins concentration was due to lower gene expression. For this purpose we used brain cortices dissected from 7-month-old littermate control and transgenic mice that already display transgene-related deficits in protein expression. However, we did not find any significant changes in vesicular acetylcholine transporter, choline acetyltransferase, and individual subtypes of muscarinic receptors gene expression. These findings indicate that the early changes in cholinergic muscarinic transmission are not due to expression of respective genes but rather due to events on posttranscriptional level. Relatively small decreases in muscarinic receptor protein concentration compared to a large functional deterioration of muscarinic transmission (decrease in both potency and efficacy of stimulation of GTP-γ^{35}S binding by agonist) prompts for studies on molecular mechanisms of signal transduction by muscarinic receptors. Such knowledge is the necessary background for development of drugs that could improve failing muscarinic transmission. To this end we have now started preparation of cell lines stably transfected with M1-M5 receptors marked with fluorescent protein. This will help us to study influences of different muscarinic orthosteric and alosteric ligands on receptor localization in membrane, turnover, and conformation changes in real time determined using advanced optical techniques.

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CIRCADIAN CLOCK IN THE RAT GUT

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In most organisms, circadian system governs timing of many biological processes with a period of about 24 h. In the gastrointestinal tract, many physiological functions such as gut motility, activities of intestinal enzymes and cell proliferation exhibit diurnal rhythms. The basic molecular core clock mechanism responsible for generating circadian rhythms is composed of interlinked feedback loops between the clock genes and their protein products. The aim of the study was to determine daily rhythms in expression of clock genes within the rat duodenum, jejunum, ileum and colon and to ascertain whether clocks within the individual parts of the gut are mutually synchronized. The next aim was to ascertain an effect of restricted feeding regime (RF) on phase of the circadian clock in the colon. Moreover, development of circadian clock gene expression in the colon during ontogenesis was studied. For characterization of clock gene expression profiles in different parts of the intestine, adult rats were maintained under light-dark regime with 12 h of light and 12 h of darkness (LD 12:12) and fed *ad libitum* or they had access to food restricted for only 6 h during the daytime. On the day of experiment, the rats were sampled every 4 h throughout the circadian cycle. To detect circadian phases of the individual intestinal clocks, the rats were sampled every 1 h during the light phase of the LD cycle. For developmental study, pregnant rats and newborn pups with their mothers were kept under regime LD 12:12 and fed *ad libitum*. The fetuses and pups were sampled at embryonic day 20 and postnatal days 2, 10, 20, 30. In all experiments, daily expression profiles of clock genes *Per1*, *Per2*, *Rev-erbα* and *Bmal1* were examined by quantitative RT-PCR. In animals entrained to LD12:12 and fed *ad libitum*, the clock genes were expressed rhythmically in all studied parts of the gut. The rhythms exhibited differences in their phases, such that the rhythm in duodenum was phase-advanced to that in the colon. In the colon, the RF regime phase-shifted the gene expression profiles. Circadian rhythmicity of clock gene expression was detected only since P20. Our data demonstrate that individual parts of the gastrointestinal tract have their own circadian clocks, which are synchronized with a phase-delay along the cranio-caudal axis and these clocks may be entrained by RF. Moreover, rhythms in expression of clock genes develop gradually during ontogenesis and are fully developed only around weaning.

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ROLE OF MATERNAL MELATONIN IN ENTRAINMENT OF THE CIRCADIAN CLOCK IN THE RAT SUPRACHIASMATIC NUCLEI DURING PRENATAL DEVELOPMENT

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Most organisms exposed to daily changes in external light and darkness evolved internal circadian system, which drives daily rhythms in many bodily functions, ranging from gene transcription to behavior. In mammals, central oscillator is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. During ontogenesis, rhythmic activity of the SCN begins already during prenatal development. During this period, maternal SCN imposes rhythmical signals to the fetal SCN. The nature of these rhythmical maternal cues might be diverse and exact mechanism of the maternal entrainment of the fetal SCN clock remains unknown. The aim of this study was to investigate whether maternal melatonin may play a role in communication between the maternal and fetal SCN. Pregnant intact or pinealectomized rats were exposed to constant light throughout their pregnancy to abolish rhythm in melatonin synthesis. Half of the pregnant rats received regular daily injection of melatonin (1 mg/kg) during five days before delivery, i.e., from embryonic day 17 till 21. The other half of rats served as controls and was treated with vehicle under the same conditions. In 1-day-old pups, daily profiles of *Avp* and *c-fos* gene expression the SCN were examined by *in situ* hybridization. The data demonstrated that the phase of the gene expression rhythms in the SCN of pups born to melatonin treated mothers differed significantly to those born to vehicle treated mothers. The acrophases of these rhythms in the SCN of pups whose mothers received melatonin were significantly phase-advanced as compared to the control group. The results provide evidence that maternal melatonin may efficiently entrain the fetal SCN clock.

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PHOTOPERIODIC MODULATION OF THE CIRCADIAN CLOCK IN THE SUPRACHIASMATIC NUCLEUS AND LIVER

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Most physiological processes in mammals follow daily oscillations. These circadian rhythms are driven by central oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus and subsidiary peripheral oscillators in nearly every bodily cell. Recent findings have shown that changes in duration of day length, i.e., photoperiod, affect both central and peripheral clocks. The aim of this study was to elucidate the dynamics of adjustment of the central SCN clock and of the peripheral circadian clock in the liver to a change of the photoperiod. The dynamics of the change from a long photoperiod, with 18 h of light, to a short photoperiod, with 6 h of light, was studied. Moreover, a role of a feeding regime in photoperiodic entrainment of the peripheral clock in the liver was examined. The C57Bl6 mice were maintained under a long and short photoperiod, respectively, for at least 4 weeks. Thereafter, the photoperiod was changed symmetrically to a short or long photoperiod, respectively. In a separate experiment, the mice remained under the short photoperiod, but the feeding regime was changed to simulate the long photoperiod. On the day of sampling, the mice were released into darkness and sacrificed in 2 h intervals throughout the circadian cycle. The expression profiles of clock genes Per1, Per2 and Rev-erbα were determined in the rostral, middle and caudal parts of SCN by in situ hybridization, and expression of Per2 and Rev-erbα in the liver by real-time RT-PCR. In the experiments focusing on the change from the long to the short photoperiod, expression profiles were assessed 3, 5 and 13 days after the photoperiod change. To ascertain the role of the feeding regime in photoperiodic modulation of the hepatic clock, the expression profiles of Per2 and Rev-erbα were assayed 5 days after the change from the short to the long photoperiod and 5 days after the change of feeding regime simulating the long photoperiod. These results showed that while the clock gene expression rhythms in the individual parts of SCN were desynchronized under the long photoperiod, they attained synchrony after transition to the short photoperiod. The adjustment was achieved mostly via advancing the expression decline, but with different rates depending on the particular clock gene. In the peripheral liver clock, Per2 rhythm was adjusted to the transition by advancing the expression decline, Rev-erbα by advancing the expression rise. The findings thus suggest that the mechanisms of adjustment to the change of the photoperiod in the central SCN clock and in the peripheral clock are different. Moreover, the results indicate that in the liver, expression of Rev-erbα and Per2 might be controlled via different mechanisms. While Rev-erbα expression is controlled mostly by rhythmic cues related to feeding, Per2 expression is likely also partially driven by the SCN clock.

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EFFECT OF MOLSIDOMINE AND TEMPOL ON ISCHEMIC TOLERANCE IN CHRONICALLY HYPOXIC RAT HEARTS

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Chronic hypoxia (CH) is a cardioprotective phenomenon increasing myocardial tolerance to acute ischemia/reperfusion injury. Adaptation to CH is associated with increased formation of reactive oxygen species (ROS) which may play a role in both the induction of protective cardiac phenotype and the pathogenesis of pulmonary hypertension (PH). The aim was to find out whether a nitric oxide donor (molsidomine) and a superoxide dismutase mimetic (tempol) attenuate i) the development of PH and ii) the increased ischemic tolerance of CH hearts. Adult male Wistar rats were adapted to continuous normobaric CH (FIO_2 = 0.1, 4 weeks). Age-matched controls were maintained under normoxic conditions. Subgroups of the animals were treated with molsidomine or tempol, given either acutely before ischemia or chronically during hypoxic adaptation. Right ventricular systolic pressure (RVSP) was significantly increased in CH rats (46.5±3.1 mm Hg) compared with the normoxic group (27.3±1.1 mm Hg). Chronic treatment with tempol attenuated the increase in RVSP (33.9±1.6 mm Hg), while no effect was observed in chronic molsidomine-treated group (41.4±2.2 mm Hg). Adaptation to CH significantly decreased infarct size (induced by 20-min ischemia and 3-h reperfusion) in CH rats (43.4±2.9 % of the area at risk) compared with normoxic controls (57.5±2.7 %). Chronic tempol treatment abolished the protection induced by CH (55.3±2.6 %). Acute tempol treatment had any effect on infarct size in neither group. Chronic molsidomine treatment decreased infarct size only in the normoxic group (50.7±2.3 %), while acute treatment markedly protected both normoxic (32.3±5.7 %) and CH (26.0±2.3 %) groups. No significant difference in the incidence of the ischemic and reperfusion arrhythmias among the groups was observed. Our results confirm that ROS play a role in the development of PH and cardioprotection induced by CH.

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ENHANCED TOLERANCE TO ISCHEMIA IN THE DIABETIC HEART IS NOT SUPPRESSED BY β1-ADRENERGIC INHIBITION WITH METOPROLOL

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Despite higher vulnerability to ischemia/reperfusion injury (I/R) in diabetics, experimental studies revealed paradoxically enhanced ischemic tolerance in the diabetic myocardium (1,2) that has been proposed to share some molecular pathways with other forms of endogenous cardioprotection, such as ischemic preconditioning (IPC) and/or adaptation to chronic intermittent hypoxia (CIH) (1,3). In the non-diabetic (ND) heart, protective mechanisms in some models of IPC and adaptation to CIH have been found to involve β1-adrenergic receptor (β1-AR) activation (4,5). The present study was designed to invesitigate the role of β1-AR in the mechanisms of ischemic tolerance in the diabetic heart using a selective β1-AR blocker metoprolol (M, 50 mg/kg/day) given for 10 days prior to acute I/R challenge. 24 h after M withdrawal, Langendorff-perfused hearts of M-treated and untreated diabetic rats (STZ 65 mg/kg, i.p., 1 week, blood glucose >20 mmol/l), as well as of age-matched ND control animals were subjected to 30-min occlusion of the LAD coronary artery for the measurement of ischemia-induced ventricular arrhythmias and 2-h reperfusion for the determination of the infarct size (IS). Results: Lower susceptibility to I/R in the diabetic hearts was documented by a 66 % reduction of IS (expressed in % of area at risk) from 48±4 % in ND controls to 16±2 % associated with a decreased total number of premature ventricular complexes (PVC) from 397±94 in controls to 198±38 ($P<0.05$) and shorter duration of ventricular tachycardia. In the ND hearts, chronic treatment with M modified neither the size of infarction (43±2 %; $P>0.05$) nor arrhythmogenesis. In the diabetic hearts, M failed to reverse both, antiinfarct and antiarrhythmic protection (IS 23±3 %; PVC 168±47; $P>0.05$ vs. respective controls). Conclusions: The results suggest that the activity of β1-AR is not involved in the development of the improved cardiac ischemic tolerance during the acute phase of STZ-induced diabetes mellitus in rats.

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AMP-ACTIVATED PROTEIN KINASE α2-SUBUNIT DEFICIENCY DOES NOT INFLUENCE THE INFARCT SIZE-LIMITING EFFECT OF CHRONIC HYPOXIA

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AMP-activated protein kinase (AMPK) is an important sensor and regulator of whole body energy status that plays a pivotal role in regulating cellular metabolism under stress conditions. During myocardial ischemia, a rapid activation of AMPK occurs, resulting in activating key steps in both glucose and fatty acid metabolism. AMPK has also been implicated in ischemic preconditioning. To find out whether α2-subunit of AMPK is involved in cardioprotection afforded by chronic hypoxia, we compared infarct size in transgenic AMPK $\alpha2^{-/-}$ and wild-type (WT) male and female mice adapted to either continuous normobaric hypoxia (CNH; FIO_2 = 0.1; 3 weeks) or more severe intermittent hypobaric hypoxia (IHH; PO_2 = 8.6 kPa; 8 h/day, 5 weeks). Isolated Langendorff-perfused hearts were subjected to 45-min global no-flow ischemia and 60-min reperfusion. Infarct size, determined by TTC staining and normalized to the size of the left ventricle, was similar in both normoxic strains and sexes (WT males 40.1±2.3 %, females 39.6±1.2 %; AMPK $\alpha2^{-/-}$ males 43.1±2.2 %, females 42.5±1.3 %). Adaptation to IHH significantly decreased infarct size in both WT (males 32.7±2.0 %, females 31.4±1.1 %) and transgenic animals (males 33.1±2.0 %, females 31.2±1.3 %), while the protective effect of CNH was less pronounced; no significant strain- and sex-dependent difference was detected. The increase in coronary flow induced by IHH was significantly more pronounced in transgenic AMPK $\alpha2^{-/-}$ mice than in WT. We also measured myocardial (left ventricle) levels of triglycerides in normoxic and IHH males and females: the levels were similar in both normoxic strains and sexes (WT males 20.0±1.1 mg/g, females 17.9±0.9 mg/g; AMPK $\alpha2^{-/-}$ males 18.4±1.4 mg/g, females 16.5±0.7 mg/g). WT animals adapted to IHH exhibited high triglycerides levels than AMPK $\alpha2^{-/-}$ group. The results suggest that the α2-subunit of AMPK does not play a major role in the infarct size-limiting mechanism of chronic hypoxia, but can be involved in myocardial remodeling and metabolic adaptation.

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MOLECULAR ANALYSIS OF NORMAL AND HYPOPLASTIC CHICK EMBRYONIC VENTRICLES

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The term hypoplastic left heart syndrome (HLHS) describes a spectrum of cardiac abnormalities characterized by marked hypoplasia of the left ventricle. Hypoplasia of the left hear structures is noted, with enlargement and hypertrophy of the right heart. In a chick model of HLHS, we employed the Affymetrix gene chip technology to gain a better insight into molecular characteristics of embryonic and early fetal myocardial differentiation. RNA isolated from normal left (LV) and right (RV) chick ventricles on ED 6, 8, and 10 was checked for quality using Agilent capillary electrophoresis, and after cDNA synthesis hybridized on the chick whole genome chip. In addition, RNA from

ED8 LV and RV of hearts with developed left ventricular hypoplasia after left atrial ligation was treated in the same fashion. Comparisons were made of LV vs. RV at different stages of development, as well as between the control and HLHS group. Interestingly, the number of genes upregulated in the LV was much lower than those higher in the RV. The HLHS group showed delayed molecular differentiation, similar to fetal gene activation in adult heart failure models. This suggests that differentiation of the working myocardium lies more in a restriction of gene expression program, with specific upregulation of select few genes, such as MYH11, MYR8, GBJ6:gap junction protein, beta 6, and others from contractile proteins/myocyte differentiation group of genes, FGF13, NF1 from proliferation/growth factors genes, extracellular matrix genes, metabolism associate gene, and transcription factors. With this data we want to explain roles and involvement of selected genes expressed in the ventricular myocardium at different stages of development as well as their connection with congenital heart disease.

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MODULATION OF NOCICEPTIVE SYNAPTIC TRANSMISSION BY N-ACYL PHOSPHATIDYLETHANOLAMINE (NAPE) IN THE SPINAL CORD DORSAL HORN

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Modulation of synaptic transmission in the spinal cord dorsal horn plays a key role in nociceptive signalling. Recent studies have indicated a great importance of presynaptic TRPV1 receptors (transient receptor potential vanilloid) in this process. TRPV1 receptors in the CNS may be activated by endogenous agonists. One of them, anandamide, is best known also as a potent cannabinoid receptors agonist. Anandamide is synthesized from N-acyl phosphatidylethanolamine (NAPE) via NAPE-phospholipase D (NAPE-PLD). This precursor might play a pivotal role in attenuation or amplification of nociceptive signalling at the spinal cord level. The aim of this study was to determine the effect of NAPE application on synaptic transmission by recording miniature excitatory postsynaptic currents (mEPSCs) from the superficial dorsal horn neurons in acute spinal cord slices prepared from rats 19-23 days old. The recording solution contained 10 μM bicuculline, 5 μM strychnine and 0.5 μM tetrodotoxin (TTX). The basal frequency of the mEPSCs was 2.23 Hz in the 23 recorded neurons. Application of 20 μM NAPE lowered the frequency to 47 % of the control level. Higher concentration of NAPE (200 μM) evoked even more robust inhibition (16 % of the control) in another set of experiments. There was a partial recovery of the mEPSCs frequency after a 5 min washout period, reaching 73 % and 27 % of the preapplication level for the 20 μM and 200 μM NAPE concentrations respectively. At the end of the experiment, each neuron was tested also for presence of capsaicin (0.5 μM) evoked response. Our results showed that NAPE application had strong inhibitory effect on the mEPSCs frequency. This was probably mediated by presynaptic CB1 receptors activation on the nociceptive primary afferents in the spinal cord. We did not find evidence of TRPV1 receptors activation under the conditions of our experiments. Further investigation of NAPE modulatory function may reveal new potential targets for pain therapy.

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TICKING GUT. CIRCADIAN RHYTHMS WITHIN COLONIC CRYPTS

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Organisms living in rhythmically changing environment possess timing machine based on molecular clocks in both the central nervous system and peripheral tissues including liver and alimentary tract where the clock genes exhibit rhythmic expression. The circadian clock regulates hundreds of functions including proliferation and intestinal digestion and absorption. We have shown recently in intestinal epithelium the rhythmic expression of Na(+)/H(+) antiporter *Nhe3* and the cell cycle regulator *Wee1*, which are suggested to be clock-controlled genes. Moreover, we have demonstrated rhythmic expression of another ion transporter *Dra* (Downregulated in adenoma) and γ subunit of the sodium channel *ENaC*. As these studies showed rhythms in scrapped mucosa from a large area of colonic epithelia, the goal of the study was therefore to elucidate whether there are differences in the distribution and phase shift of circadian clock and rhythms along the base-to-surface axis of colonic crypts. Adult male Wistar rats were kept in light-dark regime LD 12:12. Sample collection was performed every 4 hours during 24 hours, colonic tissue was snap-frozen and histological sections were prepared using cryostat. Laser capture microdissection was employed for specific tissue collection of crypt base and mouth, respectively. Total RNA was isolated and examined by quantitative real-time RT-PCR. We demonstrated the functional intestinal circadian clock (rhythmic expression of clock genes mRNA) in both crypt base and mouth with exactly the same phase of rhythm along the crypt axis. Furthermore we found rhythmic expression of the cell cycle regulator *Wee1* mRNA in both crypt base and mouth and rhythmic expression of *γENaC* and *Nhe3* mRNA in apical part of crypts. In conclusion, we determined phase-synchronized expression of clock genes and clock-controlled genes in basal vs. apical part of colonic crypts. The data demonstrate for the first time the functional synchronization of clocks in particular crypts.

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11β-HSD1, H6PDH AND GLUCOCORTICOID RECEPTOR PROFILE DURING ONTOGENY IN LYMPHOID TISSUES

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Glucocorticoids are known to modulate immunological processes by influence of development and effector functions of immune system. The ability of the cells to respond to glucocorticoid hormones depends on their plasma concentration, the responsiveness of the target cells, the number of their receptors (GR) and the local metabolism of glucocorticoids predominated by 11β-hydroxysteroid dehydrogenase type 1 and 2 (11β-HSD1, 11β-HSD2). 11β-HSD1 reduces predominantly 11-keto forms (cortisone, 11-dehydrocorticosterone) to active glucocorticoids (cortisol, corticosterone) whereas 11β-HSD2 operates strictly as an oxidase that converts biologically active glucocorticoids to their inactive 11-keto forms. It is suggested that reaction catalysed by 11β-HSD1 utilizes NADPH which is regenerated by hexose-6-phosphate dehydrogenase (H6PDH) in the lumen of endoplasmic reticulum. Evidence suggests that glucocorticoids play essential roles in development of immune system and differentiation of some immune cells, therefore our study was aimed to detect developmental pattern of local glucocorticoid metabolism in immune organs. We studied the mRNA expression of 11β-HSD1, H6PDH and GR in thymus, spleen, mesenteric lymph nodes and for comparison also in liver of Wistar male rats during suckling, weaning, prepuberty, adulthood and aging. The abundance of mRNA was measured by qRT-PCR using TaqMan probes. PCR reaction was performed as a duplex measurement of the gene of interest and the housekeeping gene (GAPDH). The activity of H6PDH was measured fluorometrically. In the majority of investigated tissues (thymus, liver, spleen) the expression of 11β-HSD1 mRNA and GR mRNA increased significantly from suckling till adulthood. In contrary to these tissues, the lymphatic nodes showed the highest expression in suckling period and decreased during weaning. The pattern of expression for H6PDH mRNA was similar to 11β-HSD1 mRNA only in spleen and lymphatic nodes but not in liver and thymus. When compared with adult animals hepatic and splenic 11β-HSD1 expression was decreased in the 24-month old rats but similar ageing process was not found in lymphatic nodes. Expression of GR decreased only in liver and expression of H6PDH was not changed in any of the investigated tissue. These results show that developmental patterns are different in the investigated organs and that the capacity of the local metabolism of glucocorticoids might be on different level during ontogeny. The data also highlight a tissue-specific dysregulation of glucocorticoid metabolism during ageing.

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ALKALI-METAL-CATION HOMEOSTASIS AND RESISTANCE TO AZOLES IN *CANDIDA* YEAST SPECIES

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The family of *Candida species*, normally a harmless human commensal of the gastrointestinal and genitourinary tract, can become a human pathogen under certain circumstances. Mainly in immunocompromised patients, *Candidas* cause a wide range of infections and are the most prevalent pathogenic yeasts. Azoles form the most important group of drugs with antifungal activity. They destabilize plasma membrane of *Candida* cells. Among others, plasma membrane integrity is crucial for the maintenance of cation homeostasis. *Candida* cells sustain high internal concentration of potassium cations that are involved in osmotic regulation, pH regulation, enzymatic function and other processes. In contrast, they keep the internal concentration of sodium cations low in view of their intracellular toxicity. In general, *Candida species* differ in their resistance to azole-antimycotics and their halotolerance. The combination of subinhibitory concentrations of fluconazole and NaCl has been shown to inhibit the growth of both fluconazole-sensitive and fluconazole-resistant *C. albicans* strains (1). In our study, we have tested the influence of salts and azoles on the growth of four *Candida* species (*C. albicans, C. dubliniensis, C. parapsilosis and C. glabrata*). We have found that all tested *Candida* species can generally tolerate high concentrations of fluconazole or salts separately, but the combination of both compounds inhibits their growth effectively. The level of inhibition of single *Candida* species differs strongly. On the one hand, the most sensitive species is *C. dubliniensis*, and on the other hand, *C. glabrata* is able to survive salt- and toxic-stresses the most effectively. Measurement of intracellular content of sodium and potassium cations and estimation of relative membrane potential together with the use of *Candida* mutants lacking plasma-membrane transporters of alkali metal cations should explain relationship between resistance to fluconazole and alkali-metal-cation homeostasis.

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GLYCEROL TRANSPORTERS IN THE OSMOTOLERANT YEAST ZYGOSACCHAROMYCES ROUXII

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The microorganisms must adapt to environmental changes, including changes of the water activity. Most of the yeast species use glycerol for the osmotic stability. Upon an increase of external osmotic pressure, cells accumulate glycerol, and its intracellular stock is released upon hypoosmotic conditions. Though glycerol, as a small uncharged molecule, is able to diffuse across the cell membranes, this process is not quick enough to ensure the needs of osmoregulation and specific glycerol transporters must be involved. The model yeast *Saccharomyces cerevisiae* disposes two systems for glycerol uptake and efflux. One of them, encoded by the *FPS1* gene, is an aquaporine-like channel that is opened to facilitate a quick release of glycerol upon hypoosmotic conditions and kept closed when cells need to accumulate glycerol (1). The second system, Stl1, is an active transporter mediating the uptake of glycerol in symport with protons (2). However, the expression and activity of this transporter is quite low and this is supposed to be one of the key differences between *S. cerevisiae* and so called osmotolerant yeast species. Osmotolerant yeasts are supposed to possess very efficient systems for the uptake of glycerol that help them to accumulate enough intracellular glycerol with relatively low level of energy-demanding glycerol synthesis. The osmotolerant yeast *Zygosaccharomyces rouxii* belongs among the food-spoilage yeast able to survive in the presence of extremely high concentrations of sugars and salts. Glycerol uptake measurements showed a very rapid and robust influx of external glycerol upon osmotic shock in this species. Searching the recently sequenced genome of *Z. rouxii* we have found two putative orthologues of the *S. cerevisiae STL1* (*ZrSTL1* and *ZrSTL2*). For the characterisation of transport properties and physiological roles of their products two approaches have been used. First of them is the deletion of both genes in *Z. rouxii* and physiological characterisation of obtained mutants. The second approach consists in their cloning and expression in the osmosensitive *S.cerevisiae* strains or in a mutant strain lacking its own *STL1*. Our results indicate that both genes are crucial for typical osmotolerant properties, because mutants lacking *ZrSTL1* and/or *ZrSTL2* lost the ability to grow upon high osmotic pressure. After having compared the growth condition of *Z.rouxii Δstl1* and *Δstl2* mutants we can say that Stl1p is probably more important for growth in the presence of high salt concentrations. This observation is supported by the growth of *S. cerevisiae* osmosensitive *Δhog1* mutants expressing *ZrSTL1* or *ZrSTL2* upon osmotic stress. Thanks to the vectors with GFP-STL1/2 fusion we could localize both genes in *S. cerevisiae* cells. The majority of fluorescence has been detected at the plasma membrane and this is consistent with the proposed function of Stl1/2p as plasma-membrane transport proteins.

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YEAST NA^+/H^+ ANTIPORTER IS REGULATED BY 14-3-3 PROTEINS

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Yeast *Saccharomyces cerevisiae* is important and well known model for studies of alkali-metal-cations homeostasis in eukaryotic cells. High-affinity active transporters Trk1 and Trk2 are responsible for uptake of required potassium; the efflux of surplus potassium or toxic sodium is ensured by Ena ATPases, Nha1 Na^+/H^+-antiporter and Tok1 potassium channel. Several regulators of K^+ (Na^+) transporters are already known, but overall picture of the maintenance of alkali-metal-cation homeostasis is still unclear. The 14-3-3 protein family is among the candidate regulators. The *BMH1* and *BMH2* genes encode members of ubiquitous 14-3-3 protein family in yeast *S. cerevisiae* which are highly important for various processes; e.g. signaling pathways and regulation of subcellular localization of many proteins. The role of major Bmh1p and minor Bmh2p in potassium and sodium homeostasis was studied using genetic approach, thus several series of mutant strains lacking various combinations of alkali-metal-cation uptake (*trk1 trk2*) and/or efflux (*ena1-5 nha1 tok1*) systems simultaneously with the *bmh1* or *bmh2* deletions were constructed. The phenotype changes brought about by multiple deletions were estimated in various conditions including salt stress or higher concentrations of drugs, and the changes of membrane potential were measured. Furthermore, the relevance of phenotype changes was confirmed by complementation using plasmids harboring *BMH1* or *BMH2* gene. Interestingly, the phenotype of the *bmh1* deletion could be complemented by *BMH2* overexpression indicating similar function of both genes. Though *bmh1* deletion increased sensitivity of all strains to cationic drugs (Hygromycin B or Spermine), no change in a membrane potential was observed. The drug sensitivity was not connected to alkali-metal cation homeostasis. In spite of the previously shown indirect interaction between Trk1 and Bmh1 proteins, deletion of *bmh1* evoked significant growth inhibition in media with high salts (NaCl, KCl, LiCl) only in strains, which contained functional Nha1 antiporter. Interaction of Nha1p and 14-3-3 proteins was confirmed on protein-protein level by the BiFC method.

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mtRNA SPECIES SHOW DIFFERENT PATTERN OF DISTRIBUTION IN MITOCHONDRIAL TUBULES

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Mitochondrial DNA (mtDNA) quality determines human lifespan and its mutations are responsible for many diseases. Details of mitochondrial genetics are still poorly understood, including

transcription and replication of mtDNA. The aim of this study was visualization of spatial distribution of various mtRNA species in tubules of mitochondrial reticulum. In order to visualize mtRNA in co-localization with loci of its transcription, mitochondrial nucleoids, we used fluorescent in situ hybridization with molecular beacon system (1). Molecular beacons were designed to anneal on ND5 mRNA (transcribed from H-strand) or ND6 mRNA (from L-strand) and on two non-coding RNAs (LSPminor and LSPmajor) transcribed from mtDNA regulatory element (D-loop). In case of non-coding RNAs we proposed lower fluorescence signal and its localization around mitochondrial nucleoids. Experiments were done using HepG2 cells and images were obtained by confocal microscopy. Our results show differentiated distribution of particular RNA species. As expected, the highest intensity of fluorescence is around putative mitochondrial nucleoids and is fading away along mitochondrial tubules. This system will be applied on high resolution microscopy (STED microscopy) and ATTO fluorescent dyes will be employed. Connection of high resolution, specific nucleases treatment and smart design of molecular beacons has potential to provide details about mtDNA replication, transcription and mtRNA lifespan. Monitoring of mitochondrial heteroplasmy will be further discussed.

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METABOLISM OF GLUTAMINE IN CANCER CELLS AND ITS THERAPEUTIC IMPLICATION

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Altered energy metabolism is a hallmark of cancer progression. The hypothesis of metabolic remodelling of cancer cells means that metabolic pathways which normally serve as main source of ATP and NADH are utilized to produce metabolic precursors necessary for proliferation of the cells. In Krebs cycle, citrate is exported from mitochondria in order to support lipid synthesis, resulting in so-called truncated Krebs cycle. Later on the Krebs cycle is complemented by glutaminolysis, an anaplerotic pathway where glutamine enters the Krebs cycle due to the transformation to 2-oxoglutarate. Recently, glutamine itself has been shown to be a precursor of citrate by "reductive carboxylation" pathway working counter-Krebs cycle direction. The key reaction of reverse pathway is transformation of 2-oxoglutarate to isocitrate catalyzed by isocitrate dehydrogenase 2, NADPH-dependent enzyme of mitochondrial matrix. Pathway further follows by the reverse aconitase reaction. Therefore, glutamine may be a direct precursor of lipids. We hypothesize that reductive carboxylation pathway might be important for maintaining cell growht even under the hypoxic and aglycemic conditions that normally accompany tumor progression. The aim of this project is to study glutaminolysis in cancer cells and to quantify both normal and reverse glutaminolytic/ Krebs cycle flux in breast carcinoma cell line HTB-126 under the cancer-specific conditions, especially hypoxia and aglycemia. To this end, we employ GC-MS analysis to quantify the incorporation of ^{13}C labelled glutamine into the key metabolites such as malate, citrate, palmitate, and CO_2 to characterize specific metabolic pathway involved and to reveal conditions under which glutamine becomes a lipogenic precursor. This study initiates a brand new progress of cancer research which could bring an original and effective therapeutic modalities based on of the inhibition of specific enzymes important for cancer cell survival.

VISUALIZATION OF MITOCHONDRIAL NUCLEOIDS IN HEPG2 CELLS

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Mitochondrial nucleoids are specific nucleoprotein complexes which contain several copies of mitochondrial DNA (mtDNA) – in humans about 6 small, circular and double-stranded molecules. Utilization and maintenance of this DNA is completely dependent on "nucleoid" accessor proteins encoded by the nucleus and imported into mitochondria, such as mitochondrial transcription factor A (TFAM), mitochondrial single-strand DNA-binding protein (mtSSB) or polymerase γ (Polγ). In humans, mt-nucleoids are about 68 nm in diameter whereas the diameter of mitochondrial tubules is about 200-400 nm. Recently, super resolution fluorescence microscopy techniques have emerged. These new techniques (4Pi, STED, Biplane FPALM microscopy) are able to break the diffraction limit described by Abbe (about 250 nm) and can provide a new insight into mitochondrial network and mt-nucleoids organization (1). In order to visualize mt-nucleoids in cells, we have prepared constructs coding for conjugates of mtDNA coating protein TFAM with EGFP for conventional confocal microscopy or EOS fluorescent protein for Biplane FPALM. Using confocal microscopy we have found co-localizations of each TFAM-conjugated fluorophores with TMRE or mitochondria-addressed DSRed fluorescent protein contouring mitochondrial reticulum network. In addition, SYTO16 mtDNA staining and immunocytology with anti-TFAM or anti-SSB antibodies were performed. We compared distribution of mt-nucleoids in cells with different metabolic states (glycolytic x OXPHOS). In further experiments rotenone, which inhibits mitochondrial Complex I and thus induces superoxide production, was employed. This lead to increased fission of mitochondrial reticulum network and also redistribution of nucleoids inside the fragmented network. We demonstrated that rotenone treatment leads to decrease in number and increase in size of mt-nucleoids in HEPG2 cells.

UP-REGULATION OF ADENYLYLCYCLASE I AND II INDUCED BY LONG-TERM EXPOSURE OF RATS TO MORPHINE FADES AWAY 20 DAYS AFTER MORPHINE WITHDRAWAL

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The amount of adenylyl cyclase I and II was determined in plasma membranes (PM) isolated from cerebral cortex of morphine-treated rats. The specific content of Na, K-ATPase was measured in all types of membranes as negative standard. The content of trimeric G protein α and β subunits was also determined. ACI (ACII) was increased 8x (2,5x) in PM isolated from morphine-treated rats when compared with membranes isolated from control animals; the amount of Na,K-ATPase was unchanged. Surprisingly, the increase of ACI and II was not detected in PM isolated from morphine-treated rats 20 days after morphine withdrawal when compared with control animals. Thus, the marked increase in specific content of ACI and ACII noticed in PM obtained from morphine-treated animals faded away 20 of days after the last dose of the drug. The specific content of trimeric G protein α and β subunits was unchanged.

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EARLY POSTNATAL DEVELOPMENT OF RAT BRAIN IS ACCOMPANIED BY GENERATION OF LIPOFUSCIN-LIKE PIGMENTS

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Fluorescent end-products of lipid peroxidation, lipofuscin-like pigments (LFP), are generated during five postnatal days in rat brain. Maximum LFP concentration is achieved on the second postnatal day, PD2. Starting from postnatal day 10 (PD10), LFP concentration returns to prenatal values. A new rise in LFP concentration is observed at 3 months of age, in 90-days old rats. LFP were characterized by fluorescence spectroscopy and HPLC and it was possible to discern several tens of fluorescent compounds of unknown structure that are generated and metabolized during early development. We suggest that LFP are formed after respiratory burst of microglia phagocytosing apoptotic cells.