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## IDENTIFICATION OF SPECIFIC PORE AMINO ACID RESIDUES INVOLVED IN THE ACTIVATION OF TRANSIENT RECEPTOR POTENTIAL ANKYRIN 1 (TRPA1) ION CHANNEL

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Sensory neurons express a wide range of ion channels and receptors, responsible for detection of potentially harmful stimuli. One of the recently cloned receptors expressed in primary afferent neurons is TRPA1, a member of a large family of TRP cation channels (1). This channel is activated by different sensory compounds such as allyl isothiocyanate (AITC), chemical pollutants, products of oxidative stress, temperature, osmolarity or depolarizing voltages. Very little is known about TRPA1 activation and modulation mechanisms and structural domains responsible for these processes. We used a site-directed mutagenesis approach to investigate the role of the putative inner pore region in the TRPA1 activation of hTRPA1. Based on assumed homology with known cation channel pores, we substituted selected residues predicted to line the S6 inner pore region by different amino acids and determined the sensitivity to activators for each mutant channel heterologously expressed in HEK293T cells. Whole-cell patch clamp technique was used to assess changes in the amplitude of AITC (200  $\mu$ M) and voltage (from -100 mV to 200 mV) induced currents. The TRPA1 mutants that exhibited significant changes in whole-cell currents were activated by 10  $\mu$ M AITC in cell-attached mode and analysed for changes in open probability and conductance. We identified several mutant channels that exhibited very small or undetectable macroscopic currents in response to AITC. Most of them were still activated at strongly depolarizing potentials indicating that changes in  $Ca^{2+}$  permeability induced by mutation may be responsible. We identified a conserved residue that is required for AITC-dependent gating, as alanine substitutions markedly altered the activation and deactivation process, and one residue at which alanine substitution greatly affected the open channel probability, without affecting the single channel conductance. These data suggests that the lower part of the inner S6 helix plays an important role in the gating of the TRPA1 channel and provides a possible general model for gating of some other related TRP channels.

(1) Story GM, et al Cell 112(6):S819-29,2003

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## CHARACTERIZATION OF THE CALMODULIN BINDING SITE ON THE C-TERMINUS OF THE RECEPTOR TRPM5

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Transient receptor potential (TRP) proteins are a diverse family of proteins with structural features typical of ion channels. They are divided into three main subfamilies: TRPC for 'canonical', TRPM for 'melastatin-like' and TRPV for 'vanilloid-receptor-like'. Eight vertebrate TRP channels consist the TRPM subfamily on the basis of structural similarity to the founding member (melastatin). TRPM5 is a non selective ion channel permeable for  $Ca^{2+}$  cations and as a member of the TRPM subfamily, plays an important role in taste receptors. The structure of TRPM5 consists of four subunits composed of a relatively short intracellular N terminus, six transmembrane-spanning domains, and an intracellular C-terminus. The aim of this study is to determine the binding site in C-terminus region of human TRPM5 that is able to interact with CaM with the use of in vitro binding assays. The whole length of C-terminus sequence of TRPM5 was subcloned into pET15b expression vector and used as a template for site directed mutagenesis. There were performed mutations of several amino acid residues that could potentially distort CaM binding. These residues were chosen on the basis of three-dimensional computer model. All His-tag fusion proteins were expressed in Rosetta<sup>TM</sup>(DE3) cells and purified using nickel-chelating sepharose. The homogeneity of the purified recombinant proteins were confirmed by SDS-PAGE electrophoresis. The ability of binding of the protein TRPM5 (amino acids 979-1141)

and its mutants to modified CaM will be tested by anisotropy measurement using dansyl-CaM or fluorescent probe Alexa Fluor488.

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## CATCHING THE MOVING TARGET: SPATIO-TEMPORAL COMPUTING ABILITY OF THE RAT INSIDE THE MOVING WORLD

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In their natural habitat, animals usually have to orient themselves in the world whose parts dynamically changed. Avoidance of a predator or approach the prey are possible variants of such behavior. In despite of many works described approach behavior in various kinds of animals and humans, suitable models of such behavior for the laboratory animals have been missing as yet. We studied navigation of rats toward a moving target. Thirteen rats were trained to reach a visible moving target in a circular water maze (diameter 185 cm). The target moved along the wall of the maze. Direction of the target movement (clockwise, counter-clockwise), speed (slower than the rat, faster than the rat) and starting position (northwest, northeast, south) changed between trials in a pseudorandom manner but they remained constant within the trials. The rats were not allowed to wait at the wall for the target. In this case the target was lifted up and it was returned to the surface of the water after it has passed the animal. Well trained rats used different target-approaching strategies according to the speed of the target. If the target moved slowly, the rats swam directly toward the target. If it moved fast, the rats reached the target either by swimming along a straight line to the place of collision or by keeping a constant angle between heading direction and the direction toward the target. The first strategy indicated that the rats can predict movement of the target. A computer simulation showed that the second strategy can be successful only if navigation is initiated at the right place with respect to the target. The experimental data showed that the rats swam to this place.

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## THE USE OF CONFOCAL AND TWO PHOTON EXCITATION MICROSCOPY TO STUDY TESTATE AMOEBAE

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Testate amoebae (Protozoa:Rhizopoda) are a group of unicellular animals (20 - 400  $\mu$ m), protected with the shell ( $SiO_2$ ,  $CaCO_3$ , proteins), living in fresh water habitats. They are used as model organisms in population ecology, ecotoxicology, paleoecology - thanks to their cosmopolitan disperse and species-specific ecological preferences together with low ecological valence to the changes of the environmental surroundings. Testate amoebae are usually examined using scanning electron microscopy (SEM) and environmental SEM (E-SEM). Scanning electron microscope allows for much better resolution than confocal laser scanning microscopy (CLSM) far sufficient for recognizing determinant features), but is limited to viewing surface of the specimen. Moreover, for species determination using morphology features, the shell often needs to be examined from all sides and the interior structures need to be visible. For this reason, we use CLSM and two-photon excitation (TPE) fluorescence microscopy to visualize the shell and inner structures of testate amoebae. We tried as many as 15 fluorescent dyes. We also performed checks for autofluorescence, acquired the z-stack through the whole animal and made 3D reconstructions of testate amoebae. We applied the

CLSM and TPE to visualize a cytoplasm inside the shell of a living organism, which is not possible by SEM. CLSM enables us to acquire images from depths up to 40  $\mu\text{m}$ , whereas TPE is able to penetrate to 60  $\mu\text{m}$ . We successfully used fluorescent dyes acid fuchsin to visualize the shell; BCECF, DIOC<sub>3</sub>(3), FITC, and propidium iodide to label inner structures. Mixotrophic species show autofluorescence of the chlorophyll a, membranes and the shell; heterotrophic species show autofluorescence of the shell only. These results contribute to morphological characteristics including taxonomical and ecophysiological research on testate amoebae. As far as we know, it is for the first time the CLSM, TPE and 3D reconstruction are used for studying inner organization of testate amoebae as well as for the first time acid fuchsin is used for their shell visualization.

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## TEMPERATURE DEPENDENCE OF NMDA RECEPTORS

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Ionotropic glutamate receptors (iGluRs) mediate most of the excitatory synaptic transmission in the CNS. N-methyl-D-aspartate (NMDA) receptors, a subclass of iGluRs, are characterized by Ca<sup>2+</sup> permeability and slow activation/deactivation kinetics, which makes them important for the processes of synaptic plasticity and excitotoxicity. These glutamate-gated ion channels are heterotetramers consisting of two NR1 and two NR2 (and/or NR3) subunits. In this study, temperature dependence of NR1/NR2B receptors expressed in human embryonic kidney (HEK) cells was studied using patch-clamp recordings. Whole-cell current responses to glutamate application were fitted to a six-state kinetic model of NMDA receptors activation and the rate constants describing each state transition were assessed at temperatures ranging from 21.9 to 46.5°C. Arrhenius plots of these rate constants showed that the most temperature-sensitive processes are the receptor desensitization ( $Q_{10} = 10.3$ ), resensitization ( $Q_{10} = 4.6$ ) and glutamate unbinding ( $Q_{10} = 3.6$ ). Time course of the current response of NR1/NR2B receptors induced by a brief (~2 ms) application of glutamate were fit by double exponential function ( $\tau_{\text{fast}}: Q_{10} = 3.7$ ,  $\tau_{\text{slow}}: Q_{10} = 2.7$ ). Similar values of  $Q_{10}$  were predicted by simulation of the responses based on the rate constants derived from the kinetic analysis. Surprisingly, deactivation time constants of NMDA receptors-mediated excitatory postsynaptic currents were characterized by low temperature sensitivity ( $\tau_{\text{fast}}: Q_{10} = 1.7$ ,  $\tau_{\text{slow}}: Q_{10} = 1.8$ ).

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## MITOCHONDRIAL COMPLEX I SUPEROXIDE PRODUCTION IS ATTENUATED BY UNCOUPLING

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Complex I (NADH:ubiquinone oxidoreductase) is the largest protein complex of mitochondrial respiratory chain. Excessive superoxide, generated by Complex I as a toxic by-product, can contribute to overall oxidative stress. Mitochondria-derived reactive oxygen species are involved in the pathogenesis of various clinical disorders including heart failure, hypoxia, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, and the physiological process of aging. Conditions for maximum superoxide production or its attenuation are not well understood. Unlike for Complex III, it has not been clear whether a Complex I-derived superoxide generation at forward electron transport is sensitive to membrane potential or protonmotive force. In order to investigate this, we used Amplex Red for H<sub>2</sub>O<sub>2</sub> monitoring, assessing the total mitochondrial superoxide production in isolated rat liver mitochondria. Mitochondria were allowed to respire either at state 4 or at state 3. Glutamate and malate were used as substrates for Complex I. Succinate was used as a substrate for Complex II. We have shown for the first time, that uncoupling diminishes rotenone-induced

H<sub>2</sub>O<sub>2</sub> production also in state 3, while similar attenuation was observed in state 4. Moreover, we have found that 5-(N-ethyl-N-isopropyl) amiloride is a real inhibitor of Complex I H<sup>+</sup> pumping (IC<sub>50</sub> of 27  $\mu\text{M}$ ) without affecting respiration. It also partially prevented suppression by FCCP of rotenone-induced H<sub>2</sub>O<sub>2</sub> production with Complex I substrates alone (glutamate and malate), but nearly completely with Complex I and II substrates. Sole 5-(N-ethyl-N-isopropyl) amiloride alone suppressed 20 % and 30 % of total H<sub>2</sub>O<sub>2</sub> production, respectively, under these conditions. Our data suggest that Complex I mitochondrial superoxide production can be attenuated by uncoupling, which means by acceleration of Complex I H<sup>+</sup> pumping due to the released respiratory control. However, when this acceleration is prevented by 5-(N-ethyl-N-isopropyl) amiloride inhibition, no attenuation of superoxide production takes place.

## 2-D ELECTROPHORETIC RESOLUTION OF PERCOLLR-PURIFIED PLASMA MEMBRANES ISOLATED FROM HEK293 CELLS EXPRESSING THYROTROPIN-RELEASING HORMONE RECEPTOR AND G11 $\alpha$ PROTEIN; COMPARISON OF CONTROL AND "DOWN-REGULATED" CELLS

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Our previous data indicated that specific down-regulation of trimeric Gq/G11 $\alpha$  proteins induced by prolonged stimulation with thyrotropin-releasing hormone (TRH) was not reflected in any detectable change in an overall composition of membrane proteins. This was evidenced by 2-D electrophoretic resolution followed by silver staining (1-4). Here we report, that application of an improved sample solubilization method and purification of plasma membranes in PercollR gradient results in a more sensitive detection of an overall protein composition of plasma membrane proteins. Sypro-Ruby stain indicated a clearly defined alternation of protein composition of plasma membranes isolated from "down-regulated cells", i.e. those exposed to TRH for a prolonged period of time. This occurred in parallel with specific decrease in the amount of Gq/G11 $\alpha$  proteins detected by an immunoblot assay and decrease in functional activity of these G proteins measured as agonist-stimulated high-affinity [35S]GTP $\gamma$ S binding.

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## NAVIGATIONAL COGNITIVE STRATEGIES IN HUMAN

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Spatial navigation is a complex cognitive ability, which helps us to find direction in our everyday living environment. Spatial memory provides processes of encoding, storage and consequently retrieval of information about environment in form of a „cognitive map”. Impairment of spatial memory (e.g. as a consequence of neurodegenerative disorders like Alzheimer disease) leads to disorientation as well as disruption of daily activities and thus to deterioration of subject's psychical state. To facilitate diagnostics of these navigational impairments it is necessary to clarify strategies of navigation used by healthy volunteers. My PhD study is therefore focused on creation of navigation tasks in real and virtual environments and their utilization in navigational ability research. Data obtained in

healthy volunteers will be used to form new tests for clinical diagnostic of navigational difficulties in patients with a high probability of developing of a form of dementia or another neurodegenerative disorder. In recent years navigational research focuses to the development of tasks using virtual reality (VR) environments. VR has a number of advantages compared to real environment. It enables flexible design of testing environments with variable complexity, size or structure, with detailed recording of subjects moving and behavior. The first step of our work (in collaboration with a team from Faculty of Mathematic and Physic at the Charles University) was therefore to create a virtual version of an already existing BVA (Blue Velvet Arena) apparatus for an early diagnosis of navigation disorders, which is placed in Hospital Motol, Prague. Seeing that VR has also some disadvantages (such as the absence of self movement) our goal was also to examine the influence of various immersion types (2D or 3D, field of view size-one or 3display screens) on navigation performance. This should lead to determining the methodology applicable for navigation assessment with following clinical use. This task was tested using both forms of BVA. Preliminary results suggest that VR navigation is comparable to real world orientation, but the field of view size or former virtual experiences have influence on angle and distance assessment accuracy. Currently the research of navigation strategies is in progress. For human navigation, mainly the visual information source is important. On usual terms we all use cues in the form of objects, color signs and so on for orientation. In case of their absence, information about environment geometry should be sufficient for navigation. The individual differences of using environmental geometry without cues to navigate seems to be however rather large. We suppose that by impoverishing of environment (by elimination of orientation signs) it would be possible to distinguish different types of navigation strategies, their usability in depleted environment and their combinations. Several healthy volunteers were tested in virtual maze without orientation cues to verify this assumption. Various tasks performed during navigation through this maze, drawing of map of the environment and consecutive interviews with these subjects help us to distinguish some navigation strategies. We have found pronounced individual differences in successful navigation performance in depleted environment. Our present findings are preliminary and we will examine and complete them by testing a bigger group of healthy subjects.

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#### BEHAVIOUR OF HUMAN ENDOTHELIAL CELLS AND FIBROBLASTS ON MICROPATTERNED SURFACES PREPARED BY PLASMA POLYMERISATION

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A hydrophobic surface of artificial vascular prostheses usually does not allow sufficient attachment and growth of endothelial cells. Adhesive polymeric microdomains give a possibility to improve cell attachment, control cell distribution over the biomaterial surface and influence cell adhesion, growth, and differentiation. In the present study we evaluated adhesion, growth and maturation of two cell types present in vessels, i.e. human saphenous vein endothelial cells (HSVEC) and human foreskin fibroblasts (HF) on micropatterned surfaces prepared by successive polymerisation of acrylic acid (AA) and 1,7-octadiene (OD). The densities of the cells were counted each day from micrographs during a 7-day of cultivation. The cells were stained for beta-actin and talin in both cell types, and for von Willebrand factor and CD31 in HSVEC. The concentrations of the same markers were measured by the enzyme-linked immunosorbent assay (ELISA). Both cell types grew preferentially on AA domains. On AA domains, the percentage of HSVEC and HF were in the range from 93.3 to 81.5 % and from 81.2 to

62.9 %, respectively, during 7-day cultivation which suggests that HSVEC are more sensitive to micropatterning. HSVEC grew aligned along the strip-like domains, but no alignment of HF was observed. Similarly, alignment of vascular smooth muscle cells was observed on micropatterned surface with 48 µm wide grooves (1). Both the size and shape of the domains has been found to influence spreading, morphology, proliferation, and development of  $\alpha$ -actin cytoskeleton in vascular smooth muscle cells (2). Cells growing on AA were more spread and better stained for beta-actin, and talin in both cell types, and for CD31 and von Willebrand factor in HSVEC. However, ELISA revealed that the concentrations of the same proteins were similar on both OD and AA. These results suggest, that plasma polymerisation is a method that allows the control of cell behaviour and can be potentially used in tissue engineering.

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#### RECOGNITION AND CHARACTERIZATION OF CALMODULIN-BINDING SEQUENCE IN THE C-TAIL OF TRPC6 CHANNEL

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Transient receptor potential channel TRPC6 is non-selective cation channel which modulates calcium level in eukaryotic cells in response to external signals. It consists of four subunits with six membrane-spanning domains and intracellular N- and C-termini. Calmodulin is the important mediator of calcium dependent regulation of TRPC6. The aim of this study is to map in detail C-terminal region of mouse TRPC6 that is capable of interacting with calmodulin. The specified sequence was subcloned into pET42b and used as a template for site directed mutagenesis. There were performed point mutations of several amino acid residues that could potentially disrupt calmodulin binding. These residues were chosen on the basis of three-dimensional computer model. Fusion proteins were expressed in *E. coli* and purified by affinity chromatography and gel filtration. The homogeneity was confirmed by SDS-PAGE and mass spectrometry. The ability of binding of recombinant proteins to calmodulin was tested by fluorescent anisotropy measurements using Calmodulin Alexa Fluor 488 dye. TRPC6 binds directly to calmodulin via its C-tail with a one to one stoichiometry in calcium-dependent manner. Our results show that amino acids R852, K856, I857A, K859, R860, K863 and R864 participate in calmodulin binding on C-terminus of TRPC6. The dissociation constants were determined. Several amino acid residues participating in binding of calmodulin in the C-tail of TRPC6 channel were determined using *in vitro* binding assays.

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#### ARE NANODIAMOND LAYERS SUITABLE FOR GROWTH OF ENDOTHELIAL AND OSEOBLAST-LIKE CELLS?

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We investigated the effects of two different nanodiamond films on adhesion and growth osteoblast-like MG 63 cells and endothelial CPAE cells. Nanocrystalline diamond films (NCD) were grown on (100) oriented silicon substrates (12 mm in diameter) by a microwave plasma-enhanced CVD method in the ellipsoidal cavity reactor [1]. Prior to the

deposition process, the silicon substrates were either polished to atomic flatness (*rms* about 1 nm, referred as nano Si) or mechanically lapped to the root mean square (*rms*) roughness up to 300 nm (micro Si). Thus, the resulting NCD layers were either nanostructured (*rms* = 8.2 nm, nano NCD) or displayed a hierarchically organized micro- and nanostructure (*rms* of 301.0 nm and 7.6 nm, respectively; micro-nano NCD) which resembled, at least to a certain degree, the architecture of natural tissues [2]. The deposited NCD films were treated in oxygen plasma to enhance the hydrophilic character of the diamond surface (water drop contact angle approx. 35°). The samples were seeded with human osteoblast-like MG 63 cells or endothelial CPAE (10 530 cells/cm<sup>2</sup> for both cell types). The number and viability of cells were detected on day 1, 3 and 5 after seeding with XTT kit a LIVE/DEAD viability/cytotoxicity kit. The viability of bone-derived cells, measured by a LIVE/DEAD viability/cytotoxicity kit, reached 98 % to 100 % on both types of NCD films. On day 3 after seeding, the MG 63 cells on the nanostructured NCD films (72,020 ± 6,540 cells/cm<sup>2</sup>) as well as on the hierarchically micro- and nanostructured films (60,200 ± 6,540 cells/cm<sup>2</sup>) reached significantly higher numbers than on the control polystyrene culture dish (40,750 ± 2,530 cells/cm<sup>2</sup>). Also the viability of CPAE on both types of NCD films was very high, ranging from 98 % to 100 %. In CPAE cells, the highest cell population density was obtained on nano NCD (44 035 ± 6853 cells/cm<sup>2</sup>). This value was significantly higher than that on micro-nano NCD, where the cell colonization might be hampered by the micro-sized surface irregularities [3].

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#### CHARACTERIZATION OF THE CALMODULIN BINDING DOMAIN ON THE C – TAIL OF VANILLOID RECEPTOR TRPV1

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Vanilloid receptor (TRPV1) is a member of the TRP ion channel superfamily. It is a thermosensitive channel that is predicted to be composed of six transmembrane segments, the pore region between the fifth and the sixth transmembrane domains and C- and N-intracellularly located termini. This nonselective cation channel allows monovalent and partially selectively divalent cations Ca<sup>2+</sup>, Mg<sup>2+</sup> to move into the cell. TRPV1 could be activated by a wide range of stimuli. The main point of our interest was the isolated C – terminus (CT) and its interaction with calmodulin. Despite TRPV1 - CT does not possess the classic calmodulin binding motif, it was described before that 35–amino acid long region (amino acids 767–801) in the C-terminus was able to bind calmodulin in a Ca<sup>2+</sup>-dependent (1) manner. We have constructed a molecular model of TRPV1 - CT (2) that predicts a single calmodulin binding domain. The main goal of our study was to provide the characterization of TRPV1-calmodulin interaction and to elucidate the participation of single aminoacid residues in the binding site for calmodulin on the purified part of the TRPV1 - CT by employing fluorescence spectroscopy. The part of rat C-tail of TRPV1 was subcloned into the expression vector pET32b which was used as a template for introduction point mutations. Expressed and purified fusion proteins were used for steady state anisotropy measurement, using Alexa Fluor 488 fluorescence probe and dissociation constants for all these constructions were estimated.

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#### GENETIC ORIGIN AND COMPLEMENTATION STUDIES OF ISOLATED ATP SYNTHASE DEFICIENCY

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ATP synthase provides 95 % of cellular ATP and its dysfunction results in deleterious mitochondrial disorders that typically affect pediatric population. Dysfunction of ATP synthase can be caused by mutations in mtDNA or in nuclear genes. Maternally transmitted disorders of ATP synthase are caused by heteroplasmic mutations of mtDNA encoded subunit ATP6 or rarely ATP8. In contrast, affected genes responsible for ATP synthase deficiency of nuclear origin are practically unknown, despite increasing number of cases diagnosed. Up to now, only in one patient mutation has been found in ATP synthase assembly factor ATP12. Investigation of other cases excluded mutations in any of 16 genes encoding enzyme subunits but recent gene expression analysis (1) and whole genome homozygosity mapping (2) uncovered mutation in putative mitochondrial protein of 30 kDa (TMEM70). The mutation was found in 25 homozygous patients and was absent in controls. The aim of our study was to test whether ATP synthase deficiency can be rescued by wild type TMEM70 gene. For this purpose TMEM70 cDNA was cloned into the pEF-DEST51 expression vector and fibroblasts from ATP synthase deficient patient and from controls were transfected with TMEM70 vector and empty vector using NHDF nucleofection kit (Amaxa). Cells were selected with blasticidin and ATP synthase content and composition was determined in isolated fibroblast mitochondria prepared by hypotonic shock method. Analysis by SDS-PAGE and WB using subunit-specific antibodies showed pronounced increase in the content of ATP synthase subunits belonging to both the F1 catalytic (alpha and beta subunits) and membraneous Fo part (subunits a, c, d) of the enzyme. BN-PAGE and WB analysis further showed that TMEM70 gene restored mitochondrial content of fully assembled ATP synthase complex of 620 kDa. Complementation effects were further confirmed by immunofluorescence studies of fibroblasts with anti ATP synthase antibodies. Parallel functional studies in our laboratory fully established that mutation in TMEM70 is responsible for isolated deficiency of ATP synthase.

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

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Peroxisome proliferator-activated receptor (PPAR)  $\gamma$  is a nuclear receptor and transcription factor which plays a crucial role in development of obesity. PPAR  $\gamma$  is mainly expressed in adipose tissue and regulates various spectrum of 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  $\text{aP2-Cre-ER}^{\text{T2}}$   $\text{PPAR}\gamma^{\text{L2/L2}}$  knockout mouse were used with ablated PPAR  $\gamma$  in mature adipocytes by using tamoxifen-dependent Cre-ER<sup>T2</sup> recombination system. Adult transgenic male mice were fed high fat (35 % fat) diet (HF). Tamoxifen (TAM) (1 mg in 100  $\mu$ l of corn oil) was injected intraperitoneally once a day for 2 consecutive days. Mice were divided into two groups and injected with tamoxifen; one group was further fed high fat diet and the second group fed high fat diet enriched with EPA/DHA. After two weeks on the certain diet, mice were sacrificed. Subcutaneous adipose tissue was used mainly for

preparation of the primary culture of adipocytes. Abdominal adipose tissue was used for histological examination and gene expression analysis. In both tissues were measured DNA concentration and triacylglycerol content. Compare with HF, diet enriched with EPA/DHA reduces a size of mature adipocytes in abdominal adipose tissue (HF  $41.80 \pm 0.39 \mu\text{m}$ ; HF-TAM  $35.73 \pm 0.29 \mu\text{m}$ ; EPA/DHA  $37.31 \pm 0.34 \mu\text{m}$ ; EPA/DHA-TAM  $33.40 \pm 0.30 \mu\text{m}$ ). *In vitro* experiment demonstrated that EPA/DHA reduces percentage of differentiated adipocytes (HF  $14.58 \pm 2.38 \%$ ; HF-TAM  $19.44 \pm 3.41 \%$ ; EPA/DHA  $13.47 \pm 2.98 \%$ ; EPA/DHA-TAM  $8.34 \pm 1.59 \%$ ). EPA/DHA are important players in the proliferation and differentiation of adipocytes and it could be very strong instrument for the treatment of obesity.

#### POSSIBILITIES OF COMBINATION OF CONFOCAL MICROSCOPY AND SECOND HARMONIC GENERATION IMAGING FOR *IN VIVO* STUDY OF EXPERIMENTAL MELANOMA

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The second harmonic generation (SHG) imaging along with confocal laser scanning microscopy (CLSM) in reflectance mode can be considered as a simple, fast, and yet powerful tool for non-invasive *in vivo* studies. When used simultaneously, these microscopic methods can improve their diagnostic value. To investigate these possibilities, we performed observations on cancer tissue *in vivo*, in totally anesthetized animals, as well as *ex vivo* on freshly harvested tumor specimens. In this investigation, murine B16F10 melanoma cells were subcutaneously inoculated in syngenic mice. The experimental melanomas were studied after they developed up to 15–20 mm in diameter. Comparisons of microscopic structure were also done with experimental melanomas before and after microwave hyperthermia application. The microscopic images were acquired by three imaging modes using a Leica SP2 AOBs MP confocal laser scanning microscope: 1) 1-photon excitation (1P) imaging in reflectance mode; 2) SHG imaging, using 2-photon (2P) excitation, to detect collagen morphology and distribution; 3) 2P imaging of tissue autofluorescence. In many cases, both the analysis of separate channels and merging of images acquired from different channels proved to be useful for visualization of tissue changes after microwave hyperthermia treatment. We can conclude that confocal microscopy is suitable for *in vivo* imaging of skin and experimental melanoma, it can provide histopathological information by means of SHG imaging combined with reflectance imaging and it could assist diagnosis and therapeutic interventions.

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#### ENDOTHELIAL CELLS SEEDED ON PROTEIN MULTILAYER ASSEMBLIES UNDER SHEAR STRESS

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Surgical grafting with synthetic bypass encounters intimal hyperplasia and thrombogenicity. It was shown that endothelial cell seeding approximates the conduit to physiological vessel (1, 2). The purpose was to culture human endothelium on protein multilayers, expose them to shear stress and reveal adhesive and metabolic gene profile. Extracellular matrix (ECM) proteins laminin (LM) and fibronectin (FN) were attached to a thin layer of collagen I (Co) deposited on a glass slide providing CoLM and CoFN surfaces. Contact angle measurement and atomic force microscopy were performed. Human saphenous vein endothelial cells (HSVEC) were cultured on the assemblies for 48 h and their densities were assessed. The confluent monolayers were submitted to laminar shear stress of 12 dynes/cm<sup>2</sup> for 40, 80 and 120 minutes. RT qPCR of selected adhesion and metabolic genes was performed. Shear stress was excluded in the static control samples. The cell densities were 55±5 %, 77±4 % and 74±3 % of the seeding density on CoLM and 51±4 %, 73±4 % and 78±13 % on CoFN at 6, 24 and 48 h, respectively. After 40, 80 and 120-min-shear stress the densities amounted to 126±8 %, 124±8 % and 120±7 % of the static control on CoLM and 81±10 %, 80±39 % and 92±24 % on CoFN, respectively. The human endothelium formed a confluent monolayer on both ECM proteins surfaces with similar cell densities. The resistance to flow was better on CoLM than on CoFN but no significant cell detachment was observed on either surface after 120 min. The preliminary results do not indicate significant flow-related changes in the gene marker profile.

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#### IMPACT OF ECTOPIC SYNTHESIS OF MITOCHONDRIAL UNCOUPLING PROTEIN 1 IN WHITE ADIPOSE TISSUE ON THE WHOLE BODY METABOLISM IN MICE

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The development of obesity associated with insulin resistance is closely related to the accumulation of lipids in non-adipose tissues. Increased expression of uncoupling proteins (UCPs) in white adipose tissue is associated with conversion of lipid-storing white adipocytes into lipid-oxidizing cells, suggesting an efficient strategy for the treatment of obesity and metabolic syndrome. Transgenic mice with ectopic expression of mitochondrial uncoupling protein 1 (UCP1) in white adipose tissue (aP2-UCP1 mice) are partially resistant to diet-induced obesity and glucose intolerance (1, 2). We reported the main phenotype of aP2-UCP1 mice on the system level (resistance to genetic obesity or diet-induced obesity, improved glucose tolerance after long-term cHF feeding, hypotriglyceridemia) and change associated with white adipose tissue (reduced subcutaneous fat depots and gonadal fat, decreased lipogenesis and lipolysis, increased mitochondrial biogenesis) and with brown adipose tissue (brown fat atrophy, decreased norepinephrine-stimulated thermogenesis and sensitivity to acute cold stress (homozygotes)) (1, 2, 3, 4 and 5). The next main focus was the analysis of energy metabolism on the whole-body level (using indirect calorimetry system INCA Somedic, Sweden) and on the tissue level, glucose homeostasis and whole-body insulin sensitivity in the aP2-UCP1 mice. The expression of UCP1 in white fat of transgenic mice aP2-UCP1 mice was associated with improved glucose tolerance in the context of long term high-fat feeding. Respiratory uncoupling in this tissue prevented accumulation of lipids and down-regulation of Glut4 in skeletal muscle of mice fed high-fat diet. Down regulation of

adiponectin gene expression in the transgenic mice might be associated with increased infiltration of adipose tissue with macrophages. Transcript levels of genes involved in lipid metabolism suggested decreased lipogenesis and increased lipid oxidation in the skeletal muscle of transgenic mice. UCP1 over expression in white fat conferred protective effects against glucose intolerance and lipotoxicity induced by long-term high-fat feeding.

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#### THE EFFECT OF DIETARY EPA/DHA RATIO AND DOSAGE ON THE DEVELOPMENT OF OBESITY AND METABOLIC DISORDER IN MICE FED A HIGH-FAT DIET

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n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are abundant in marine fish oils, prevent development of obesity and insulin resistance in rodents fed high-fat diets (1). Epidemiological studies suggest similar beneficial effects in human populations. Furthermore, they act as potent hypolipidemics in both rodents and humans. In this study, we explored the effect of different dietary EPA/DHA ratio and the relative amount on the development of obesity and metabolic disorder in mice fed a high-fat diet. Six groups ( $n = 10$ ) of adult C57BL/6N male mice were fed with a standard low-fat, control high-fat (lipids ~ 35 % weight/weight) and various CHF-based experimental diets for 16 weeks. In the experimental diets, 5 or 15 % of dietary lipids was replaced with the EPA/DHA concentrate (EPAX AS, Norway), and the ratio of EPA to DHA was either 10:50 or 45:10. Body weight gain (BWG) and food consumption were monitored weekly. Blood was sampled after 2, 8 and 16 weeks of differential feeding under both fed and fasted conditions. At the end of the experiment, an intraperitoneal glucose tolerance test was performed after an overnight fast. Mice were killed by cervical dislocation under diethylether anaesthesia, EDTA-plasma was isolated from the truncal blood, while liver, muscle and adipose tissue depots were collected for future analyses. Glycemia was measured using calibrated glucometers. Plasma metabolites (triacylglycerols, cholesterol and non-esterified fatty acids (NEFA) were measured using enzymatic kits and spectrophotometry. There was no difference in the average food consumption during the whole experiment. Mice fed the diets with a higher EPA/DHA dose had a significantly decreased BWG (by 20 %), while diets with a lower EPA/DHA dose did not affect BWG compared to control mice fed a high-fat diet alone. Fasted blood glucose levels correlated positively with the BWG. Blood glucose in the fed state was not different among the groups of mice fed high-fat diets. All experimental diets significantly decreased the level of plasma triacylglycerols already after 2 weeks of feeding compared to control high-fat diet. Furthermore, the diet with a higher dose of EPA/DHA decreased plasma levels of triacylglycerols below those found in mice fed a low-fat diet (low-fat,  $98 \pm 10$  vs. 15% EPA/DHA,  $62 \pm 8$  mg/dL;  $p < 0.05$ ). The dietary ratio of EPA/DHA did not influence these differences. However, the hypolipidemic effect of the diet with a lower dose of EPA/DHA and lower EPA to DHA ratio diminished after a prolonged feeding (8 weeks). The levels of NEFA tended to decrease in all experimental groups compared to the control high-fat group. Our results suggest dose-dependent protective effects of dietary EPA/DHA in the development of obesity, hyperglycemia and hyperlipidemia in mice fed a high-fat diet, while a higher EPA to DHA ratio supports the maintenance of hypolipidemic effect during the long-term feeding.

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#### SIGNIFICANCE OF AMP-ACTIVATED PROTEIN KINASE FOR THE EFFECT OF LEPTIN

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Leptin is a hormone secreted by adipocytes in proportion to fat stores that plays a major role in regulating energy homeostasis by decreasing food intake and increasing energy expenditure. Leptin stimulates the oxidation of fatty acids and the uptake of glucose and prevents the accumulation of lipids in nonadipose tissue. Rodents with diet-induced obesity and most obese humans are resistant to the effects of leptin. Previous studies demonstrated that leptin modulates the activity of the AMP-activated protein kinase (AMPK) by stimulation of phosphorylation and activation of the alpha2 catalytic subunit of AMPK and that the AMPK is therefore a key regulator of the effect of leptin on food intake and fatty acid oxidation (1). The aim of the study was to investigate the significance of AMPK for the leptin effect in post-weaning mice using alpha2 AMPK KO mice. Male alpha2 AMPK KO mice and B6 mice were fed standard chow, housed one per cage and maintained on 30 °C. Fourteen days after weaning were injected with saline (control) or leptin intraperitoneally (3 mg/kg). Food intake of individually housed mice was assessed per 24 hours period. AMPK and ACC activity was measured in *gastrocnemius* muscles and plasma leptin levels were determined by RIA kits. The metabolic rate of mice was assessed by indirect calorimetry. We have demonstrated that leptin decreased respiratory exchange ratio (indicating increased fatty acid oxidation) in B6 mice, however there were no differences in alpha2 AMPK KO mice. We have observed the anorexigenic effect of leptin in B6 mice, but this effect was absent in alpha2 AMPK KO mice. However, alpha2 AMPK KO mice injected with leptin still tended to eat less than alpha2 KO mice injected with saline. Our results indicate that absence of alpha2 AMPK subunit leads to disruption of signaling pathway for leptin suggesting that alpha2 AMPK subunit is necessary for the effect of leptin.

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#### DIFFERENTIAL EFFECT OF TYROSINE TO ALANINE SUBSTITUTION IN THE FIRST TRANSMEMBRANE DOMAIN IN P2X7 AND OTHER P2X SUBUNITS

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Purinergic P2X receptors (P2XRs) are ion channels activated by extracellular ATP. In vertebrates, seven genes encode P2XR subunits (termed P2X<sub>1-7</sub>). All P2X subunits have two transmembrane domains (TM1 and TM2) connected with a large extracellular loop and ending with intracellular N- and C- termini. The P2X<sub>7</sub> receptor differs in several features from other P2X subtypes: (i) it is activated by ATP at concentrations greater than 100 μM, (ii) 2',3'-(benzoyl-4-benzoyl)-ATP (BzATP) has 30times higher potency than ATP, (iii) the receptor does not desensitize and (iv) its channel pore exhibits extremely large time-dependent dilatation during prolonged agonist application which can lead to cell damage. The reason for these differences between P2X subunits is not clear. Among the TM residues of all P2X subunits, only four are highly conserved, glycine and tyrosine of TM1 and glycine and aspartic acid of TM2. Earlier studies revealed that substitution of Tyr at P2X<sub>2</sub> generates a constitutively active channel with enhanced ATP sensitivity. Here we examined the effect of alanine substitution of this residue on receptor function using rat P2X<sub>2a</sub>, P2X<sub>2b</sub>, P2X<sub>3</sub>, P2X<sub>4</sub> and P2X<sub>7</sub> subunits. Mutants and wild-type (WT) receptors were expressed in HEK293 cells and agonist-induced currents were measured using whole-cell patch clamp recording. The deactivation time constant(s) after agonist washout and EC<sub>50</sub> values were examined. Membrane expression of all receptors was confirmed by immunocytochemistry. The P2X<sub>2a</sub>[Y43A], P2X<sub>2b</sub>[Y43A], P2X<sub>3</sub>[Y37A] and P2X<sub>4</sub>[Y42A] mutants exhibited 2-15 fold leftward shifts in the EC<sub>50</sub> values compared to WT receptors and significantly prolonged deactivation after washout of non-desensitizing concentrations of ATP. Except P2X<sub>3</sub>[Y37A] receptor,

these mutants showed also significantly enhanced sensitivity to  $\alpha\beta$ me-ATP. The P2X<sub>7</sub>[Y40A] mutant exhibited 2-fold leftward shift in EC<sub>50</sub> for BzATP compared to WT receptor and increased percentage of contribution of slow deactivation time constant to deactivation kinetics. However, no effect on fast and slow deactivation time constants and ATP EC<sub>50</sub> values was observed. These results indicate that the effect of substitution of conserved tyrosine in the first transmembrane domain is much smaller in P2X<sub>7</sub> subunit as compared to other P2X receptor subunits.

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#### STIMULUS-INDUCED LIGHT TRANSMITTANCE TRANSIENTS IN CA3 REGION IN-VITRO ARE GLUTAMATE DEPENDENT

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Change of the optical properties of the nervous tissue during its activation can be used as a marker of this activity. Our main interest is light transmittance (LT) that is associated with the dilution of intracellular scattering particles as water enters the cell during the activation. The intrinsic optical properties in acute hippocampal slices (400µm) were examined in CA3 region during the stimulation of Mossy fibers. Cooled 12-bit CCD-camera (RETIGA2000R) connected to the water immersion epifluorescence microscope (Olympus BX51WI) was used for image capturing. Synaptic activity was associated with increase in LT in CA3 region in stimulation intensity dependent manner. The change in maximal intensity increase of LT during 3T and 4T stimulation was  $150.6 \pm 16.3 \%$  and  $173.9 \pm 21.8 \%$  of 2T intensity. The changes in LT were almost completely abolished by TTX (1µM in ACSF), LT increase was led down to  $5.4 \pm 2.4 \%$  compared to the control measurement, indicating that the synaptic activation was responsible for the changes of LT. To enable glutamate release but prevent from binding glutamate to ionotropic glutamate receptors APV (50µM in ACSF) and CNQX (10µM in ACSF) were used. In this case LT was also decreased to  $57.9 \pm 4.9 \%$ . The signal was significantly blocked but the percentage of the change was distinctively higher than in the previous measurement with TTX. In conclusion, our data show significant contribution of the postsynaptic neuronal glutamate activation in the overall LT changes after the stimulation. We are at the moment elucidating the changes of LT concerning glial transport of glutamate via using TBOA, preliminary results showed the increase of LT  $159 \pm 19.62 \%$  we supported the idea of the glial contribution to the optical signal.

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#### IMPROVED ADHESION AND GROWTH OF HUMAN BONE-DERIVED CELLS ON NANOCRYSTALLINE DIAMOND FILMS

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Nanodiamond is considered as a promising material for applications in hard tissue surgery and bone tissue engineering. Therefore, we studied the adhesion, growth and osteogenic differentiation of human bone-derived cells in cultures on nanocrystalline diamond (NCD) films deposited on microscopic glass slides using a microwave plasma-enhanced chemical vapor deposition (MW PECVD) technique (substrate temperature 710 °C, atmosphere 0.8-1 % CH<sub>4</sub> in H<sub>2</sub>, pressure 20 Torr). After the growth period, the films were exposed to oxygen plasma and subsequently hydrogenated. Additionally, as NCD diamond can be doped with boron (aiming at active sensing applications *in vivo*), presence of charged states of the ionised B acceptors at the diamond film surface can have influence on the cell colonization. Therefore, in

order to study these properties, some of the NCD films were doped with boron (NCD-B) using trimethylboron (TMB) mixtures with H<sub>2</sub> in a concentration of 3000 – 30,000 ppm B:C. leading to  $\rho \sim 10^{-1} \text{ } \Omega \text{ cm}$  for the highest doped films. The neutron depth profiling showed that in the near surface region (< 800 nm) the boron content in the highest doped sample was about  $(1.9 \pm 0.3) \times 10^{21} \text{ B cm}^{-3}$  (i.e.,  $1.1 \pm 0.2 \text{ at. } \%$  of B). For cell culture experiments, only the low TMB-doped films (i.e., 3000 ppm) were used. The films were sterilized in ethanol and seeded with human osteoblast-like MG 63 cells (density about 17 000 cells/cm<sup>2</sup>, medium DMEM with 10 % of fetal bovine serum). Although both NCD and NCD-B films were relatively hydrophobic (wetting angle 85-90°), the cell number achieved on NCD on day 3 after seeding ( $56,280 \pm 1090 \text{ cells/cm}^2$ ) was significantly higher than that on NCD-B (by  $27 \pm 3 \%$ ), glass slides (by  $22 \pm 3 \%$ ) and polystyrene wells (by  $36 \pm 3 \%$ ). On day 7, the cell numbers on both NCD and NCD-B films ( $351,170 \pm 16,530$  and  $310,020 \pm 10,410 \text{ cells/cm}^2$ , respectively) became significantly higher than the values on glass slides and polystyrene dishes ( $218,800 \pm 12,340$  and  $223,400 \pm 9,290 \text{ cells/cm}^2$ , respectively). Immunofluorescence staining showed that the cells on both NCD films assembled fine streak- or dot-like focal adhesion plaques containing  $\alpha_v$  integrins or talin, and a mesh-like beta-actin cytoskeleton. These beneficial effects of NCD films on cell adhesion and growth could be attributed to the substrate nanostructure. As revealed by atomic force microscopy, the root mean square roughness (rms) was 18.2 nm and 10.0 nm in NCD and NCD-B, respectively. In addition, the cells on NCD-B were brightly stained for osteocalcin, a calcium-binding extracellular matrix glycoprotein and important marker of osteogenic differentiation. Thus, both tested nanodiamond films gave good support for adhesion and growth of bone-derived cells and could be used for coating of bone-anchoring parts of bone and dental implants.

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#### GAP PERMEASES IN CANDIDA ALBICANS

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*Candida* cells can proliferate in many different niches within the host and must be able to sense the environment in order to express only those genes that help to utilize optimally all nutrient sources in the area. Sensing and uptake of amino acids, which are present in mammalian hosts in high concentration and which constitute major source of nitrogen, would have a central role in the growth of *C. albicans*. Also the requirement of amino-acid transporters for hyphal morphogenesis induction and virulence was shown. In *Saccharomyces cerevisiae*, one general amino acid permease (Gap1) exists. It is not only required for transport, but also for amino-acid sensing and activation of signal transduction pathways that induce many intracellular changes. In *C. albicans* genome, a whole family (6 members) of Gap1 orthologues exists, but none of them have been characterized. We would like to elucidate the role of individual CaGap permeases in the virulence and pathogenicity of this species, together with the characterization of their transport properties. For this, we employ 1) deletion of one or both GAP alleles in the *C. albicans* SN87 (leu<sup>-</sup>, his<sup>-</sup>) strain, using fusion PCR strategy (Noble S. M. and Johnson A. D., 2004, Eukaryotic Cell, 298-309) and heterologous markers *C. maltosa* LEU2 and *C. dubliniensis* HIS1, followed by a phenotype tests of all *C. albicans* gapΔ mutants (growth, morphology) on the media with different sources of nitrogen and carbon, and 2) heterologous expression of CaGAP genes in a *S. cerevisiae* mutants lacking its own amino-acid transporters HS100-3C (*can1*, *lyp1*, *gap1*) or m4584 (*gap1*, *gnp1*, *tat1*, *tat2*, *bap1*, *bap2*, *agp1*) in order to reveal the substrate specificity and kinetic parameters of individual CaGap permeases.

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## POWERFUL SOFTWARE FOR IMAGE ACQUISITION AND ITS PROCESSING

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To record image data is common task and also a lot of acquisition systems specialized in imaging exists. But nowadays, the need of not only image, but synchronous image and electrophysiological recording is rapidly growing up in broad field of sciences. However, the acquisition systems that are available nowadays are mostly tools specialized in either imaging or electrophysiology without the possibility to control both components in one environment. The aim of this study was to develop software component for image recording and analysis, which is part of software enabling synchronous image and electrophysiological data acquisition and analysis and also enabling triggering external devices and powerful Matlab interoperability. The video acquisition component, together with parts of presented software, was programmed using Microsoft .NET Framework 2.0 library and C# programming language and thus it is suitable for any MS Windows based computer. This component is one of the three main components of the software. The others are graphical user interface and signal acquisition component. The user interface controls both acquisition components. The component can cooperate with several camera devices, such as standard web cams, DV cams and selected high sensitive cameras for microscopy use (UI, Q-Imaging, etc.), and capture image data. Manufacturer's camera drivers are used to control sensitive microscopy cameras and Microsoft application programming interface DirectShow to control standard USB and DV cameras. Acquired data can be saved into two different types of file, commonly used AVI file and our own file type called BVG (Brain Vision Graphics) providing more flexibility. Synchronization between this component and signal acquisition component is performed either by cameras' input and output trigger signals in the case camera enables external triggering, or by flickering of LED diode in predefined sequence if external triggering is not feasible. Trigger mode of cameras also enables to grab images in non-equidistant intervals. The component as a part of the whole acquisition software have been successfully tested in image acquisition mode with high resolution webcam (Philips 900NC), industrial cameras UI-2230C and UI-2230M (Imaging Development Systems, Germany) and high sensitive cooled camera Retiga 4000R and Retiga 2000R (Q-Imaging, Canada).

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## ROLE OF Na<sup>+</sup>/H<sup>+</sup> ANTIPORTERS IN SALT TOLERANCE OF CANDIDA SPECIES

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Na<sup>+</sup>/H<sup>+</sup> transport systems exist in all pro- and eukaryotic organisms. Beside the exchange of sodium cations against protons they can have various functions inside the cells. The Na<sup>+</sup>/H<sup>+</sup> antiporter from the eukaryotic yeast *S. cerevisiae*, Nha1p, is one of the best-studied ones. It transports alkali metal cations and protons and is involved in regulation of K<sup>+</sup> homeostasis, cell volume, intracellular pH, the maintenance of membrane potential and in cell response to osmotic shock. Some dozen human pathogenic species exist in the fungi kingdom, among them, the most important ones belong to the genus *Candida*. *C. albicans* is the most prevalent yeast pathogen in humans followed by *C. glabrata* and others like *C. dubliniensis* and *C. parapsilosis*. All these species are able to react against changes in their environment like shift of pH value, nutrients availability and osmolarity. Some of them can exist in different morphological states; yeast, pseudohyphal and hyphal form. In previous studies the *C. albicans* Na<sup>+</sup>/H<sup>+</sup> antiporter Cnh1p was heterologously expressed in salt sensitive *S. cerevisiae* BW31a cells. It showed a broad substrate specificity for alkali metal cations (Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, Rb<sup>+</sup>) and deletion of *CNH1* gene in *C. albicans* leads to higher sensitivity to K<sup>+</sup> and Rb<sup>+</sup>. In order to characterize the function of Cnh1p

homologs from three other *Candida* species (diploid *C. dubliniensis*, haploid *C. glabrata* and diploid *C. parapsilosis*) two approaches were used: 1) The three *Candida* antiporters were heterologously expressed in *S. cerevisiae* salt-sensitive BW31a cells which lack their own Na<sup>+</sup> antiporters. Expression of *Candida* Cnh1 antiporters in BW31a with a C-terminal GFP tag localized the proteins in the plasma membrane of cells. In all cases, the expression increased the cells' tolerance to Na<sup>+</sup>, K<sup>+</sup>, and Rb<sup>+</sup>. An enhanced Li<sup>+</sup> tolerance could be observed for cells expressing *CaCnh1p* and *CpCnh1p* but not for those expressing *CdCnh1p* and *CgCnh1p*. To measure the transport activity of antiporters the efflux rates of Na<sup>+</sup> and K<sup>+</sup> were estimated. *CaCnh1p* and *CpCnh1p* have the highest transport activities, they export about two times more Na<sup>+</sup> and K<sup>+</sup> than *CdCnh1p* and *CgCnh1p*. 2) To investigate the antiporters' role in *Candida* species, deletion of *CNH1* genes via homologous recombination was started. In haploid *C. glabrata*, a *cnh1Δ* mutant was constructed. Compared to wild type, the mutant showed a lower tolerance to high K<sup>+</sup> and Rb<sup>+</sup> concentrations and a higher tolerance to Li<sup>+</sup>. Reintegration of *CgCNH1* in the mutant restored wt phenotype. First results suggest the involvement of Cnh1 proteins in the salt tolerance and potassium homeostasis of *Candida* species, further studies will elucidate their role in cell physiology and pathogenicity.

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## ADDITIVE EFFECTS OF n-3 POLYUNSATURATED FATTY ACIDS AND THIAZOLIDINEDIONE IN THE AMELIORATION OF ADIPOSE TISSUE INFLAMMATION, DYSLIPIDEMIA, AND INSULIN RESISTANCE IN MICE FED HIGH-FAT DIET

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Polyunsaturated fatty acids of n-3 series, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), act as hypolipidemic, antiinflammatory, and cardioprotective agents, and they prevent development of obesity and insulin resistance in rodents fed high-fat (HF) diets. However, they do not improve glycemic control in diabetic patients. Thiazolidinediones including rosiglitazone are increasingly used for therapy of type 2 diabetics, in spite of unwanted side-effects. We tested a hypothesis that a combination treatment by EPA/DHA and rosiglitazone would provide beneficial additive effects in the reduction of obesity, low-grade adipose tissue inflammation, insulin resistance, and associated disorders in mice fed HF diet. Adult male C57BL/6J mice were fed for up to 20 weeks either corn oil-based HF diet or HF diet containing (i) 15 % of its lipids replaced by EPA/DHA concentrate (14 % EPA, 46 % DHA; EPAX 1050 TG, EPAX AS, Norway); (ii) rosiglitazone (10 mg/kg diet); and (iii) EPA/DHA and rosiglitazone at doses as above (EPA/DHA-rosiglitazone). Weight gain and obesity-associated disorders were ameliorated, in an additive manner, by EPA/DHA and rosiglitazone. The systemic effects of the treatment correlated with the significant reduction of low-grade inflammation of adipose tissue, namely accumulation of macrophages in crown-like structures embracing non-viable adipocytes, which was nearly abolished by EPA/DHA-rosiglitazone. Additive impact of the combination treatment in the prevention of hypertrophy of adipocytes was also found. Beneficial additive effects on plasma lipids, muscle ceramide level, glucose tolerance and plasma levels of adiponectin were observed. A combined use of n-3 PUFA and thiazolidinediones generated additive effects in the treatment of obesity-associated disorders. The combination treatment may be used in diabetic patients to reduce dose requirements and hence the incidence of adverse side-effects of the thiazolidinedione therapy.

## INDUCTION OF MUSCLE THERMOGENESIS BY HIGH-FAT DIET IN MICE: ASSOCIATION WITH OBESITY-RESISTANCE

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High-fat (HF) diet is known to be a strong obesogenic stimulus. It can be counterbalanced by stimulation of energy expenditure and lipid oxidation in response to the meal. Aim of this study was to reveal whether muscle nonshivering thermogenesis could be stimulated by HF diet. The other aim was to support the hypothesis of leptin and AMP-activated protein kinase (AMPK) involvement in this mechanism. Two mouse strains which differ in propensity to obesity were used, obesity-resistant A/J and obesity-prone C57BL/6J (B/6J) mouse strain. In the different propensity seems to play a major role one of the hormones produced by adipose tissue – leptin. The A/J mice known are known to increase its production in response to HF diet. For experiments male mice born and maintained at 30 °C to eliminate effect of cold stress were used. Four-week-old mice were randomly weaned onto a low-fat (LF) or HF diet. At the age of 6 weeks experiments were performed and tissues for *in-vitro* experiments and biochemical analysis were collected. In the A/J LF mice during cold exposure (4 °C) strong hypothermia was detected while the A/J HF, B/6J LF and B/6J HF mice were cold-tolerant. Cold-sensitivity of the A/J LF mice was associated with relatively low whole-body energy expenditure. This defect was normalized by HF diet. In A/J HF mice whole body energy expenditure in 30 °C was increased compared to LF. Further in oxidative soleus muscle of A/J mice HF diet caused increase of phosphorylation and total content of AMPK and by the activator of AMPK-AICAR stimulated fatty acid oxidation in parallel with significantly increased levels of leptin. These results documenting a shift from carbohydrate to fatty acid oxidation was also supported by gene expression data. Our results suggest a role of muscle nonshivering thermogenesis and lipid oxidation in the obesity-resistant phenotype of A/J mice and indicate that HF diet could induce thermogenesis in oxidative muscle, possibly by the leptin-AMPK axis.

## SOFTWARE TOOL FOR ELECTROPHYSIOLOGICAL DATA ACQUISITION AND ITS ANALYSIS

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Recording physiological signals is common part of sophisticated experimental systems. In many cases it is necessary to combine recording of required signals with subsequent mathematical analysis. Another useful component for experiments is a synchronous detection with video signals. Therefore, the aim of present study was to develop software for continuous recording and analysis of physiological signals and making them work together with the system oriented to a video signal. This software was developed for Microsoft .NET technology and is built on .NET framework 2.0 by C# programming language. The system consists of two main interface components. A programmable interface for typical device settings and graphical user interface for display signals. Both interfaces could be at least supplemented by component for storing data into file. Not less important task was to find an acceptable file format for storing measuring data and events altogether. For our purposes we developed our own binary file format called Brain Vision Data (BVD). This file is divided into several sections, the main header containing information about an experiment and necessary device settings, data stored block-by-block and finally follows section with events and marks. The advantage of BVD file is in the ability which allows users to save many types of signals including predefined events. The software works with multifunction cards of M series produced by National Instruments, which communicates and transfers data through the PCI bus (PCI 62xx). These data acquisition

cards have up to 80 analog inputs and 4 analog outputs with 16 or 18 bits A/D convertors and 48 digital I/O lines. The software also enables to set up programmable amplifier called NI-PGA and use 6 DMA channels for exporting data. Both data acquisition card and developed software are a part of a complex system where it is possible to record continuously physiological signals synchronous together with video signals. Communication between data acquisition card and camera is realized either through trigger signal from camera or TCP port using .NET remotable objects, which allow separating both systems into two independent units. Mathematical analysis is realized using Matlab and .NET interoperability.

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## INFLUENCE OF LC-PUFA INTAKE DURING PERINATAL PERIOD ON METABOLISM OF SKELETAL MUSCLE AND POSSIBLE PROGRAMMING OF METABOLISM

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Long-chain polyunsaturated fatty acids (LC-PUFA) are essential for normal development. During pregnancy, fatty acids are transported from maternal circulation across the placenta and play an important role in the regulation of many cell functions including regulation of gene expression. Thus intake of LC-PUFA during perinatal period could influence the development of metabolism and possibly cause persistent changes into adulthood. This study was designed to elucidate acute and lasting effects of intake of dietary fish oil during pregnancy and lactation on offspring metabolism and to specify the role of n-3 polyunsaturated fatty acids (PUFA) in the postnatal induction of uncoupling protein 3 (UCP3) and other genes involved in energy metabolism in muscle. Adult C57BL/6N female mice were divided into two groups that were fed non-purified low fat diet (6 % fat, wt/wt) that differed in lipid composition. One diet contained 6 % fat based on soybean oil (Chow6) whereas in the other was 5 % of total lipids content replaced by n-3 PUFA concentrate (Chow6-F2). Mice remained on the diets during pregnancy and lactation. After weaning After weaning pups were randomly divided and put on high fat (HF, 35 % fat, wt/wt) or remained on chow diet. Milk consumption and lipid profile of milk were assessed. Gene expressions in oxidative (*soleus*) and mixed (*gastrocnemius*) muscles using qRT-PCR and plasma lipid markers were quantified. We have demonstrated that n-3 LC-PUFA have no effect on milk production by dams and milk consumption by pups. Milk from Chow6-F2 group contains significantly higher amount of EPA and DHA. Expression of UCP3 in male gastrocnemius muscle was higher in group fed HF diet, whereas in soleus muscle there were no obvious changes. The situation in females was completely opposite. Sexual dimorphism in the activation of UCP3 and other genes involved in energy metabolism by high fat diet was discovered. Interaction between muscle type and gender was demonstrated.

## CHANGES IN CALSEQUESTRIN EXPRESSION IN HYPOTHYROID RAT FAST AND SLOW SKELETAL AND CARDIAC MUSCLES

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Calsequestrin (CSQ) is the main Ca<sup>2+</sup>-binding protein of the sarcoplasmic reticulum. It has two forms: skeletal calsequestrin (CSQ1) present at high level in fast-twitch and at medium level in slow-twitch muscles and cardiac calsequestrin (CSQ2) present in cardiac and slow-twitch muscles. CSQ controls the ryanodine receptor channel to which it may be anchored by triadin and junctin (1). Many hormones exert a strong systematic influence on skeletal muscles during development as well as in the adult stage. For the thyroid hormones, it has been shown in rats that both hypothyroid (HY) and hyperthyroid states were paralleled by modifications in the content of myosin heavy chain isoforms and their mRNA transcripts (2-4). We have investigated whether the decreased level of the thyroid hormones might alter calsequestrin

expression in the slow soleus (SOL) muscle, the fast extensor digitorum longus (EDL) muscle and the heart of adult female inbred Lewis strain rats. HY rats were treated with 0.05 % solution of methimazole (2-mercapto-1- methylimidazole, Sigma) in drinking water. CSQ was determined by SDS-PAGE followed by western blot analysis. Gene expression was assessed using reverse transcription and subsequent real time polymerase chain reaction (RT-PCR). Our results have confirmed that the mRNA transcript levels for CSQ1 are the highest in the EDL, medium in the SOL and the lowest in the heart. The HY status decreased the CSQ1 expression in the EDL and SOL. The CSQ1 levels on western blots were higher in the EDL than in the SOL and not detected in the heart. Its levels in HY EDL and SOL samples were slightly decreased, similarly as its RNA transcript. The mRNA transcript levels for CSQ2 were the highest in the heart, medium in the SOL and the lowest in the EDL muscle. The HY status slightly increased the CSQ2 mRNA expression in the EDL compared to the EU status. We can thus conclude that thyroid hormones may alter not only the muscle myosin heavy chain isoforms, but also the calcium homeostasis.

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#### THE EFFECT OF CHOLESTEROL DEPLETION ON THE THYROTROPIN RELEASING HORMONE RECEPTOR DISTRIBUTION AND MOBILITY IN INTACT CELLS

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Membrane integrity plays an important role in GPCR signaling. Here we investigated effect of disruption of plasma membrane integrity by cholesterol depletion on the thyrotropin releasing hormone receptor (TRH-R) distribution and membrane mobility in HEK293 cells which stably express high levels of TRH-R fused to the "enhanced" form of green fluorescent protein (TRH-R-eGFP). The confocal laser scanning microscopy (CLSM 488nm/100x1.4 Plan APO) was used to visualize the TRH-R fused to eGFP. The changes in TRH-R-eGFP membrane mobility were analyzed by FRAP (fluorescence recovery after photobleaching). In order to deplete cholesterol from the plasma membrane, the intact cells were treated with 10 mM  $\beta$ -cyclodextrin ( $\beta$ -CD). Distribution of TRH-R was mostly homogenous and only cell processes and areas where cells attach to each other showed higher intensity of fluorescence than the rest of plasma membrane. This picture remained the same in control as well as  $\beta$ -CD treated cells. The  $\beta$ -CD-exposure of HEK293 cells under carefully controlled conditions (temperature, mechanical stress induced by small flow of cultivation media) was also without effect on an overall cell morphology. However, as revealed by FRAP, cholesterol depletion induced clearly significant change of receptor mobility. Cholesterol depleted cells exhibited almost two times faster recovery of TRH-R-eGFP fluorescence signal in the photobleached area than control cells. In neither case the recovery of fluorescence intensity was complete, i.e. the intensity of fluorescence has not returned to levels prior to photo-bleaching. The "recovered" signal in cholesterol-depleted cells was significantly higher than in control cells. Our results indicate that although the distribution of TRH-R in intact cell membrane is mostly homogenous it is in part located in large structures detected in cell processes and at the cell-cell interfaces; cholesterol-depleting agent  $\beta$ -CD increases mobility of TRH-R in cell membrane of intact cells.

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#### VASCULAR SMOOTH MUSCLE CELLS IN CULTURE ON MODIFIED HIGH DENSITY POLYETHYLENE FOR TISSUE ENGINEERING

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Synthetic polymers are widely used in medicine and various biotechnologies, such as construction of body implants, carriers for cells for transplantation or *in vitro* cultivation. The attractiveness of polymers for cell colonization can be affected by physical and chemical modification of the polymer surface. In this study, high density polyethylene (HDPE), often used as model material for the development of tissue replacements, was modified by Ar plasma discharge on Balzers SCD 050 device (exposure time 50 seconds, discharge power 1.7 W), and then grafted with various biomolecules, such as glycine (Gly), bovine serum albumin (BSA) or polyethylene glykol (PEG), and/or sputtered with carbon (C). Thus, we created the following material modifications: pristine HDPE, plasma-modified HDPE, HDPE+Gly, HDPE+C, HDPE+BSA, HDPE+PEG, HDPE+BSA+C and tissue culture polystyrene. The materials were seeded with rat aortic smooth muscle cells (SMC; passage 8-9, 17 000 cells/cm<sup>2</sup>, medium DMEM with 10 % of fetal bovine serum). On day 1 after seeding, the highest number of initially adhered cells was found on plasma-modified HDPE with PEG. On day 2, the cell number on all modified HDPE samples became significantly higher than that on non-modified HDPE. On day 5, the highest cell number was observed again on HDPE + PEG. On all modified samples, the cells were better spread and formed well-developed filaments containing alpha-actin, a marker of SMC differentiation and contractility, although its concentration per mg of protein was unchanged.

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#### EFFECT OF CHRONIC CAPTOPRIL TREATMENT ON ADRENERGIC VASOCONSTRICTION IN SPONTANEOUSLY HYPERTENSIVE RATS

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The administration of captopril, an angiotensin-converting-enzyme inhibitor, is an effective antihypertensive treatment of spontaneously hypertensive rats (SHR), which are characterized by sympathetic nervous system (SNS) hyperactivity and increased vascular reactivity to norepinephrine (NE) based upon enhanced Ca<sup>2+</sup> influx through voltage-dependent calcium channels (VDCC). Captopril treatment prevents the development of hypertension in SHR by reducing sympathetic tone. Therefore, we aimed to study the effect of chronic captopril treatment on blood pressure (BP) responsiveness to NE and to assess the VDCC-dependent component of NE vasoconstriction in conscious SHR. Rats were subjected to chronic captopril treatment from the age of 4 weeks. At the age of 10 weeks, pressor responses to increasing non-cumulative doses of NE (0.01-40  $\mu$ g/kg, i.v.) were measured before and after VDCC blockade (nifedipine, 0.4 mg/kg, i.v.). The maximal BP rises after each of norepinephrine doses were used to construct dose-response curves. We noticed a steeper slope of the curve and decreased ED50 values in SHR compared to WKY, but there were no differences in maximal pressor responses to NE. Captopril pre-treatment changed neither the threshold nor the maximal pressure response to NE, but it decreased curve slope and increased ED50. Nifedipine considerably shifted dose-response curves to the right in both untreated and captopril-treated SHR so that they became almost identical. Blood pressure reduction by antihypertensive treatment with captopril in developing SHR is mediated by the reduction of nifedipine-sensitive component of sympathetic vasoconstriction. This effect seems to be strengthened by concomitant prevention of pressure-dependent structural changes of resistance vessels and/or improvement of endothelial function.

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### CHRONIC INTERMITTENT HYPOXIA STIMULATES 11HSD2 IN RAT HEART MYOCARDIUM

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The ability of the cells to respond to corticosteroid hormones depends on co-expression of their receptors and 11 $\beta$ -hydroxysteroid dehydrogenases (11HSD) that convert glucocorticoids to their biologically inactive 11-oxo derivatives or vice versa. Although both 11HSD type 1 (11HSD1) and type 2 (11HSD2) are able to oxidize biologically active glucocorticoids (cortisol, corticosterone) to their inactive 11-keto forms (cortisone and 11-dehydrocorticosterone), 11HSD1 reduces predominantly 11-keto form to active glucocorticoids and thus these enzymes play a crucial role in corticosteroid physiology by regulating glucocorticoid and mineralocorticoid receptor (GR, MR) activation. Increasing evidence suggests that gluco- and mineralocorticoids modulate cardiovascular homeostasis including hypertrophy and fibrosis. In addition, transgenic mice overexpressing 11HSD2 in cardiomyocytes spontaneously develop cardiac hypertrophy, fibrosis, and heart failure. To examine whether similar changes of cardiac metabolism of glucocorticoids are associated with physiologically more relevant cardiac hypertrophy and fibrosis we studied hearts of rats exposed to chronic intermittent hypoxia (7000 m, 8 h/day, 5 weeks) that show right ventricle (RV) hypertrophy, fibrosis and inflammation. The aim of this study was therefore to determine whether adaptation to hypoxia induces changes in expression of 11HSD2, GR, MR, and osteopontin (an inflammatory marker) in RV. The abundance of mRNAs was measured by semiquantitative RT-PCR using TaqMan probes and the enzyme activity by radiometric assay using [<sup>3</sup>H]corticosterone. Hypoxia increased the oxidation of corticosterone to 11-dehydrocorticosterone and the level of 11HSD2 mRNA in both the myocardium and isolated cardiomyocytes. In contrast, the expression of GR and MR was not changed either in RV myocardium or in isolated cardiomyocytes. Osteopontin was increased in hypoxic rats more than ten times. These results show that adaptation of cardiac tissue to hypoxia, hypertrophy and fibrosis stimulates inactivation of biologically active glucocorticoids to their inactive derivatives without any changes in expression of corticosteroid receptors. The described changes emphasize the physiological and pathophysiological role of corticosteroids in cardiac homeostasis.

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### EFFECT OF CORTICOSTERONE (CS) AND CORTICOTROPINE-RELEASING HORMONE (CRH) ON BEHAVIOUR OF RATS IN VARIOUS BEHAVIOUR TASKS

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Higher levels of corticoids were reported in some diseases, as Cushing disease, depression, posttraumatic stress disorder and dementia of alzheimeric type. It has been shown long-time increased plasma levels of corticoids caused neurodegeneration in central nervous system, namely hippocampus. For this reason it is important to study how corticosterone affect cognitive functions. Are this observed changes caused by corticosterone only? Effects of long-time increased exogenic corticotropine-releasing hormone (CRH) and/or corticosterone (CS) on behaviour of 37 male adult laboratory rats (*Rattus norvegicus*, Long-Evans) were studied. Six various behaviour tasks were used: two tests in Morris Water Maze (MWM), Active Allothetic Place Avoidance (AAPA), Step Through (ST), Step Down (SD), Conditioned Taste Aversion (CTA). Substances were administrated continuously: CRH by osmotic pump into cerebral lateral ventricle, during 4 weeks; CS by subcutaneous pellet for 3 weeks. The animals were divided in 4 groups. The first group -  $\blacktriangle$ CS - high levels of CS 9.52 mg/animal/day, n = 10. The second group -  $\blacktriangle$ CRH leading to  $\blacktriangle$ CS - CRH 1.5  $\mu$ g/animal/day, n = 11. The third group -  $\blacktriangle$ CRH + adrenalectomy  $\blacktriangledown$ CS - CRH 1.5  $\mu$ g/animal/day, adrenalectomy + substitution CS 1.4 mg/animal/day by

subcut. minipellet, n = 6. The fourth group - controls - without treatment, n = 10. The experiments were realized either during administration of substances, or at the time after administration. CRH and CS yielded different results. In all three experimental groups the change of behaviour was observed. The group -  $\blacktriangle$ CS - 3-5 weeks after administration in CTA and MWM showed deterioration of long-term memory. In addition, early in the period of administration we observed the change of behaviour in ST, which can be interpreted as deteriorating in short-term and in long-term memory. In the group -  $\blacktriangle$ CRH leading to  $\blacktriangle$ CS - was found significant persistent deficit in cognitive coordination in the task AAPA, which had been found already after short time of CRH administration. The third group -  $\blacktriangle$ CRH + adrenalectomy  $\blacktriangledown$ CS - exhibited in ST, AAPA especially elevated anxiety which probably caused slower learning but not an overt specific memory deficit versus controls in all tasks used. The results suggest that exogenous administration of CRH and CS differentially affect the various behaviors of rats tested with the present battery of memory tasks.

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### CHANGES IN MITOCHONDRIAL NETWORK MORPHOLOGY OF MAMMALIAN CELLS OBSERVED BY 4PI MICROSCOPY

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Mitochondria are essential organelles for life of eukaryotic cells. They supply the cell with energy from oxidative phosphorylation and have prominent function in various physiological processes. Mitochondria acquire specialized morphology and reorganize it under OXPHOS variations and external stimuli. Overall morphology changes dramatically, often in response to the metabolite needs of the cell. A growing body of evidence indicates that mitochondrial fusion and fission have important roles in establishing, maintaining and remodeling mitochondria. In the last decade, many studies have identified and characterized proteins responsible for organelle fusion and division, including the dynamin-related GTPases - mitofusins MFN1,2 (Fzo1p in yeast) and OPA1 (Mgm1p in yeast) for fusion and Drp1 (Dnm1p) and Fis1 for division and have demonstrated that a balance of these two antagonistic activities controls mitochondrial morphology(1). The observed various mitochondrial shapes show characteristic features at the 100 - 500 nm size scale in three dimensions which cannot be investigated properly by traditional confocal microscopy due to the diffraction limit. So we have used 4Pi microscopy (2), which provides ~250 nm lateral and ~100-150 nm axial resolution to investigate morphological alterations of mitochondrial networks in a biomedically relevant study of insulinoma INS-1E cells and hepatocellular carcinoma HEP-G2 cells under different cultivation conditions. (Both cell lines stably expressing matrix targeted redox-sensitive GFP). We studied 3D shapes of intact and disintegrated mitochondrial reticulum observed at rotenone-inhibited respiration or at uncoupling by FCCP. Cells were cultured for 2-4 days to 70 % cell confluence on 4Pi coverslips coated with poly-L-lysine. The cells were treated with 1  $\mu$ M FCCP, 20  $\mu$ M rotenone or both together, respectively, for 20 min at 37°C. Then the coverslips were fixed with 0.3 % glutaraldehyde. The coverslips with fixed cells were mounted and sealed onto the 4Pi metal holders containing coverslips with mirrors over the 87.5 % glycerol/PBS mounting medium. Data were collected with Leica TCS 4Pi microscope equipped with pairs of 100 $\times$ /1.35 NA glycerol immersion objectives. GFP fluorescence was recorded using two-photon excitation at 906 nm wavelength for INS-1E cells and 910 nm for HEP-G2 cells. The most astonishing observation was that OXPHOS mitochondria in fact represent a single continuous reticulum (single mitochondrion). The diameter of mitochondrial tubules was determined from the original measured data stacks by two methods, referred further as "PST" and "ruler" method, respectively. With rotenone inhibiting respiration to ~10 %, disintegration into several reticula and numerous ~300 nm spheres or short tubules was observed. De-energization by uncoupling additionally led to formation of rings and bulky cisternae of 1.4 $\pm$ 0.4  $\mu$ m diameter. Rotenone and uncoupler acted synergically in INS-1E cells and increased fusion forming bowl-like shapes. In HEP-G2 cells fission partially ceased with FCCP plus rotenone. In conclusion, using 4Pi

microscopy for visualisation of mitochondria enables us to uncover true shape and sizes of mitochondrial tubules and the influence of mitochondrial activity, *i.e.* energy production on mitochondrial morphology in mammalian cells.

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#### BIOENERGETIC PARAMETERS OF GLUCOSE-STIMULATED INSULINOMA INS-1E CELLS

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Insulinoma INS-1E cell line developed at the University of Geneva seems to be a promising model of  $\beta$ -cells usable for glucose-sensing function studies. Intending to investigate basic bioenergetic properties - respiration and *in situ* mitochondrial membrane potential ( $\Delta\psi_m$ ) - in relation to glucose-stimulated insulin secretion (GSIS), we studied glucose dependent phenomena in INS-1E cells. Glucose-depleted INS-1E cells increased immediately their respiration and  $\Delta\psi_m$  upon glucose addition. Instant insulin secretion increased with glucose, saturating at 20 mM. Similar relationships were detected for respiration burst and *in situ*  $\Delta\psi_m$  jump. The endogenous state 3/ state 4 respiratory ratio hyperbolically increased with glucose as well, approaching the maximum oxidative phosphorylation rate at maximum GSIS. Attempting to assess the basis of the "toxic" effect of fatty acids on insulin secretion, GSIS was studied in the presence of linoleic acid. Addition of 10  $\mu$ M linoleic acid diminished respiration increase,  $\Delta\psi_m$  jump, magnitude of insulin release, and reduced state 3/state 4 dependencies on glucose. Halfmaximum for respiration change upon glucose addition was shifted from  $\sim 7$  mM to  $>10$  mM in the absence and presence of 10  $\mu$ M linoleic acid, respectively. This correlated with a shift for halfmaximum of  $\Delta\psi_m$  increase upon GSIS. Moreover, we have revealed a high amount of mitochondrial uncoupling protein-2 (UCP2) transcript in INS-1E cells. We hypothesize that UCP2 participates in the measured effects of fatty acids.

#### SPATIAL BEHAVIOR OF THE RAT IN SPATIALLY NON-STABLE ENVIRONMENTS

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For navigational purposes animals use salient and stable cues, as they provide easily accessible and reliable information about the topography of space. These cues might directly point to a place (cued navigation) or the position of the place is remembered within a chart made by multiple cues (map navigation). It has been demonstrated that in order to lay out an optimal path through an environment animals preferentially record and integrate position of stable landmarks and ignore moving or otherwise unstable landmarks. However, in nature, it often occurs that animals must organize their spatial behavior also with respect to moving objects - other animals (predator, prey, sexual partner...), or with respect to moving, compact parts of the environment (eg. "floating islands"). Both situations have been introduced in laboratory and several behavioral tasks have been described. Place avoidance (PA) task require rats to avoid a directly imperceptible sector on a circular metallic arena to preserve themselves from being a mildly foot-shocked. Since the arena can be slowly and continuously rotated, rats have two independent sources of navigational cues at their disposal - arena based (*i.e.* in reference frame of the arena) and room based (*i.e.* in reference frame of the room). Then, several modifications of the task can be made to attenuate one and accentuate the use of the other reference frame. When unveiling brain structures and circuits underlying such kind of navigation, it has been found that segregation of the spatial information into two reference frames is strongly hippocampus-dependent but parietal cortex-independent. However, parietal cortex still plays some role during reference frame selection. Other behavioral tasks focused on navigation with respect to a single moving cue. For example in Robot Avoidance Task rat searches for pellets on a dry circular arena, and at the same time avoids approaching of small programmable robot below

distance of 25 cm, otherwise it gets a mild electric footshock. Unexpectedly, performance in this task was found to be affected by inactivation of dorsal hippocampus; lesion of parietal cortex had no effect. These behavioral tasks represent a new approach within rodent spatial cognition research. They introduce navigation based on moving single cues or subset of cues, a bit unjustly neglected part of rodent natural spatial behavior.

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#### LENTIVIRAL TRANSFECTION OF MITOCHONDRIAL ADDRESSED GFP GENE AND GENES FOR OTHER FLUORESCENT PROTEINS

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Lentiviral vectors represent efficient tools for delivery and stable integration of transgenes both *in vitro* and *in vivo*. They can transduce a wide range of targets and can be used for generating transgenic animals from several species. Finally, this gene delivery system can mediate the knockdown of endogenous genes by RNA interference via production of small hairpin RNAs. The first basic approach of our research will be preparation of GFP gene with mitochondrial addressing sequence (MAS), its lentiviral transfection to isolated islets of Langerhans and  $\beta$ -cells *in vivo* in rats (control Wistar rats vs. Goto-Kakizaki model of type 2 diabetes), HeLa, INS1E and HepG2 cells. Later we would like to use other genes (for example CIDEa and UCP2) with MAS for the transfection. As the second aim of our study we will construct several lentiviral vectors for RNA interference to silence various types of genes involved in mitochondrial physiology.

#### THE CONTRIBUTION OF RAT DORSAL HIPPOCAMPUS AND PARIETAL CORTEX TO NAVIGATION IN NON-STATIONARY ENVIRONMENT

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Vast majority of rodent spatial tasks are done in more or less fixed environments so that the cues for navigation are mutually coherent. However, in their day-to-day lives, humans and animals encounter dissociations of multiple subsets of navigational cues produced by motion of parts of the environment and are required to maintain orientation towards each subset (frame of reference) independently. Therefore, we have conducted two navigation experiments with rats involving spatial dissociations of different kinds. Then we explored the contribution of parietal cortex and dorsal hippocampus, brain structures known to play a key role in spatial information processing. Both experiments were conducted on dry circular arena ( $d=82$  cm) in 20 min daily sessions and employed adult males of Long-Evans rats. In the first behavioral task, the animals were required to keep a safety distance of at least 25 cm from a single moving object (small mobile robot) to avoid a mild footshock. The robot was moving forward ( $v=15$  cm/s) until it hit the wall, waited for 15 seconds, then turned in a pseudorandom angle and continued. First group of animals ( $n=8$ ) with implanted hippocampal cannulas were trained 6 days to reach asymptotic level of performance. On the 7th day they received bilateral injection of 1  $\mu$ l of tetrodotoxin solution (5 ng/ $\mu$ l) in their dorsal hippocampi, after which significant deterioration of performance was observed ( $p<0.001$ ) while leaving locomotion unaffected. Other group of parietal-lesioned animals ( $n=8$ ) showed insignificant ( $p=0.1$ ) amelioration in the learning curve compared to controls. In the second experiment, animals were trained to avoid a sector of the arena of about 60 degrees. The sector was unlabeled, so that the animals could learn to identify it both according to local cues on the arena and distant cues in the experimental room. On the 7th day of training the arena started to rotate slowly around its central axis (approx. 1 rpm) and shocks were switched off. Data were then analysed off-line to show which subset of cues the animals prefer, *i.e.* whether they will continue to avoid in the arena frame of reference

or in the room frame of reference. While parietal-lesioned rats showed no clear preference for any of the frames, sham-operated controls chose almost consistently the room frame. All experiments were in accord with the directive of the European Communities Council No. 86/609/EEC.

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### THE ADHESION AND GROWTH OF HUMAN OSTEOBLAST-LIKE MG 63 CELLS ON TITANIUM DIOXIDE LAYERS OF DIFFERENT NANOROUGHNESS

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Tissue engineering can be defined as a set of tools that uses living cells to aid tissue formation or regeneration. It has been shown that the cell behaviour strongly depends on physical and chemical properties of material surface [1-3]. In the present study we compared the influence of different surface nanoscale topographies of titanium dioxide films on the adhesion, proliferation and maturation of human osteoblast-like MG 63 cells. These films were prepared by means of DC reactive magnetron sputtering of Ti target on microscopic glass slides of different  $R_a$  roughness (0 nm, 40 nm, 100 nm and 170 nm) and seeded with the cells in a density of 7900 cells/cm<sup>2</sup>. On day one after seeding, the spreading area and cell densities on all TiO<sub>2</sub> films, microscopic glass slides, and tissue culture polystyrene were similar. However, on day seven, the cell number on TiO<sub>2</sub> on glass with roughness of 100 nm (367,000 ± 34,000 cells/cm<sup>2</sup>) was significantly higher than that on polystyrene (261,000 ± 13,000 cells/cm<sup>2</sup>) and on uncoated glass (285,000 ± 44,000 cells/cm<sup>2</sup>). The improved cell colonization might be due to a beneficial action of an advantageous material nanostructure (irregularities of 100 nm), which might enable adsorption of cell adhesion-mediating extracellular matrix molecules in the most appropriate spectrum and spatial conformation compared to the other nanostructured TiO<sub>2</sub> layers (irregularities 40 and 170 nm) [3]. In addition, these cells displayed fine dot-like focal adhesion plaques containing  $\alpha_v$ - and  $\beta_1$ -integrins and talin, developed streak-like focal adhesion plaques containing vinculin, fine mesh-like beta-actin cytoskeleton, and also were stained brightly for osteocalcin, a marker of osteogenic cell maturation. It can be concluded that the TiO<sub>2</sub> films, especially those with the surface nanoroughness of 100 nm, supported adhesion, growth and maturation of human bone-derived cells. Thus, they could be used for coating bone implants in order to improve their integration with the surrounding bone tissue. However, these findings require further investigation.

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### EXPRESSION OF P2X RECEPTORS IN SUPRACHIASMATIC AND SUPRAOPTIC NUCLEI OF HYPOTHALAMUS

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In the central nervous system, purinergic P2X receptors have been implicated in the effect of extracellular ATP as an autocrine or paracrine intercellular transmitter. Hypothalamus is a prominent region of ATP receptor expression. Previous studies have shown the presence of several P2X receptor subtypes in supraoptic nucleus (SON),

particularly, P2X<sub>2</sub> receptor immunoreactivity has been described at both pre- and postsynaptic sites. In this study we compared the distribution of P2X receptor subunits in suprachiasmatic nuclei (SCN) that control circadian changes in a variety of physiological and behavioral functions, including metabolism, body temperature, hormone secretion, feeding and sleep and activity cycles, and SON. We used in-situ hybridization and immunohistochemistry combined with confocal microscopy to examine expression of P2X receptor mRNAs and proteins in the SCN and SON. Calcium imaging was used to test the effect of extracellular ATP on single cell function in Fura2AM-loaded acutely isolated hypothalamic slices. Experiments were performed on brain slices from adult or 21-day-old rats. In-situ hybridization showed the presence of P2X<sub>2</sub>, P2X<sub>3</sub>, and P2X<sub>4</sub> receptor transcripts both in the SON and the SCN. Immunohistochemistry revealed the presence of P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>4</sub>, and P2X<sub>5</sub> receptor proteins in SON but not in SCN. In contrast to the SON, there was also a lower percentage of extracellular ATP-induced calcium responses in the SCN. These results demonstrate variability in the distribution of P2X receptors within hypothalamus and differences in responsiveness to extracellular ATP which is considered to be a dominant messenger for neuron-glia and glia-glia communication in the brain. Further studies are required to elucidate whether low expression of P2X receptors in the SCN is related to its specific function as endogenous clock pacemaker and/or is due to low number of glia cells in this region.

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### ANALYSIS OF SPATIAL POINT PATTERNS IN ULTRASTRUCTURAL IMMUNOGOLD LABELLING OF CELL COMPARTMENTS BASED ON 3-D IMAGE DATA

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In biological investigations requiring statistical analysis of immunogold clustering, inhibition, colocalization or mutual inhibition patterns the concept of random point field theory is used. In [1], for the 2-D case, the clustering or colocalization of gold particles was detected using various characteristics of the distribution of distances between them. The generalization, to the 3-D case of electron tomography data, was found by using the results of [2]. In general, it is necessary to use simultaneously several quantities of different types to analyze and characterize a given point structure. In particular, this means that basing conclusions upon mean values or distance functions, or second-order quantities only, is not sufficient. None of these quantities alone can, in general, determine uniquely the distribution of a point structure, unless it arises from a Poisson point field. Therefore, a combined use of intensities, a distance distribution and a second-order quantity is necessary. If empirically given point patterns are studied, we have to distinguish between two levels of investigation: a primary stage of explorative data analysis and, usually at a later stage, attempts at the development/fitting of a suitably parametrized mathematical model that is consistent with the observed point patterns. Here we restricted ourselves to the exploratory level. In all our investigation we supposed the 3-D point pattern was homogeneous and isotropic. The most basic information is an estimate of the intensity  $\lambda$  of the point process, i.e. the mean number of points per unit area. Next an unbiased estimator for the square of  $\lambda$  was used [2]. As an estimator for the second-order product density the following formula was used [2]:

$$\hat{\rho}^{(2)}(\mathbf{r}) = \frac{1}{4\pi r^2} \sum_{\mathbf{x}_i, \mathbf{x}_j \in B, i \neq j} \frac{k_h(\mathbf{r} - \|\mathbf{x}_i - \mathbf{x}_j\|)}{\text{vol}(B_i \cap B_j)}$$

where Epanechnikov kernel with a suitable bandwidth  $h$  is used and the summation is over all distinct points of the given realization of the point process in a rectangular observation window  $B$ . It enabled us to estimate the pair correlation function by dividing it by the estimator of the square of intensity  $\lambda$ , which is amenable to the known statistical interpretation: In the case of complete spatial randomness, i.e., the homogeneous Poisson process it equals one; values below one indicate repulsion, whereas values above one indicate clustering; a hard-core effect leads to an initial segment with zero values. Similarly, estimators for other second-order characteristics in 3-D, i.e., the Ripley's K-function, L-

function, nearest-neighbor distance distribution function, spherical contact distribution function and the Baddeley's J-function were computed. The computations were made in Wolfram Mathematica®.

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#### ALLOTHETIC AND IDIOTHETIC NAVIGATION IN TRIANGULAR AND CIRCULAR POOLS

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The purpose of the present study is to develop a task based on navigation to a single intramaze location that can be implemented with the same success from any point at the circumference of the circular pool and from three angles at the equilateral triangular pool either by allothesis (in light) or by idiothesis (in darkness). Convenient test location is centrally located hidden platform.

We trained adult male Long-Evans rats in logically following sequences with 1-min probe trials (in absence of the escape platform) in triangular pool and circular pool in light and in darkness to find how the different start position of the rat affects the performance of the rat by allothesis and by idiothesis. We used equilateral triangular pool fitting inside the circular pool, the size of the circular pool (190 cm in diameter) corresponds to the circumscribed circle of the triangular pool. The rats trained in equilateral triangular pool facing the corner in light reached the asymptotic value 4s of escape latency and trained the same way in darkness reached the asymptotic value 9s of escape latency. They successively developed the strategy to swim to several corners, divide the last corner and swim straight to the center. On the last trial in darkness the swim is almost direct from the corner to the platform. Different start positions of the rats in triangle in light did not affect the performance. The escape latencies of performances of the rats trained during one day first in the triangular pool in light conditions afterwards in darkness and finally in circular pool in darkness are significantly different. At the end of the experiment the rats were tested in the circular pool facing the wall from the 3 different start positions corresponding to the corners of the equilateral triangular pool in light and dark conditions. During learning in darkness in the circular pool the rats first swam cross the whole water maze, they touched several times the walls of the pool and they learned to swim perpendicularly to the circumference (1). All experiments complied with the Czech law and with the directive of the European Communities Council No. 86/609/EEC on protection of laboratory animals.

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#### SUBUNIT COMPOSITION AND FUNCTIONAL PROPERTIES OF GABA<sub>A</sub> RECEPTORS IN ANTERIOR PITUITARY CELLS

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The release of hormones from anterior pituitary gland is controlled at the level of a gland by various neuropeptides as well as by several classical neurotransmitters such as dopamine, noradrenaline and  $\gamma$ -amino butyric acid (GABA). Precious studies have shown that anterior pituitary cells express GABA<sub>A</sub> receptor-channels, but their structure, distribution within the secretory cell types, and nature of actions have not been clarified. In the present study we addressed these questions using cultured anterior pituitary cells from postpubertal female rats and

immortalized  $\alpha$ T3-1 and GH<sub>3</sub> cells, RT-PCR analysis, immunocytochemistry, calcium imaging and electrophysiology. Our results show that mRNAs for all GABA<sub>A</sub> receptor subunits are expressed in pituitary cells and that  $\alpha$ 1/ $\beta$ 1 subunit proteins are present in all secretory cells. In gramicidin-perforated cells, GABA induced dose-dependent increases in current amplitude that were inhibited by bicuculline and picrotoxin and facilitated by diazepam and zolpidem in a concentration-dependent manner. In intact cells, GABA and the GABA<sub>A</sub> receptor agonist muscimol caused a rapid and transient increase in intracellular calcium, whereas the GABA<sub>B</sub> receptor agonist baclofen was ineffective, suggesting that chloride-mediated depolarization activates voltage-gated calcium channels. Consistent with this finding, RT-PCR analysis indicated high expression of NKCC1, but not KCC2 cation/chloride transporter mRNAs in pituitary cells. Furthermore, the GABA<sub>A</sub> channel reversal potential for chloride was positive to the baseline membrane potential in most cells and the activation of ion channels by GABA resulted in modulation of the pattern of spontaneous electrical activity. These results indicate that secretory pituitary cells express functional GABA<sub>A</sub> receptor-channels that are depolarizing.

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