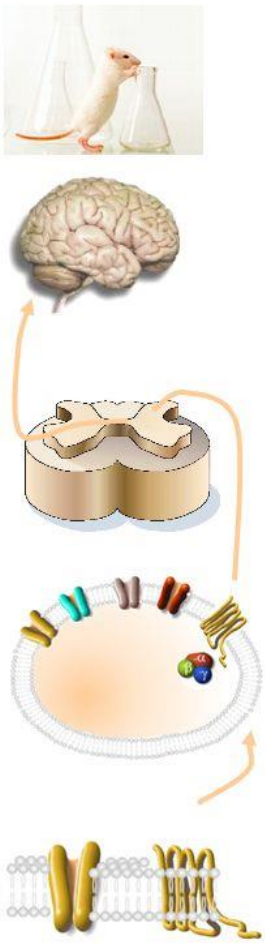
Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Physiology of the CAS, v. v. i.

(a) Background

Institute of Physiology CAS is Czech Republic's leading research institution in the field of ***normal and pathological physiology***. The Institute's mission is to deepen and broaden the fundamental knowledge on physiological and pathological processes implicated in specific ***metabolic***, ***cardiovascular*** and ***neuronal/brain*** functions (Fig. 1), thus paving the way to novel prevention, diagnostics and therapeutic procedures required to tackle serious medical conditions in humans.

Figure 1. ***Major research fields at the Institute and their complexity levels.***



NEUROPHYSIOLOGY	CARDIOVASCULAR PHYSIOLOGY	METABOLISM
System level		
- Circadian rhythms - Memory - Epilepsy - Alzheimer's disease - Pain	- Central and peripheral blood pressure control - Regulation of embryonic cardiac output - Pathophysiology of heart failure	- Neurohumoral control - Energy expenditure - Glucose homeostasis - Biomarkers - Nutritional interventions - Metabolic syndrome
Cellular level		
- Synaptic transmission - Neuromodulation - Nociception - Ionic channels: NMDA, TRP, nicotinic, purinergic - Metabotropic receptors: muscarinic, adrenergic - Secretion of pituitary hormones	- Calcium influx and calcium sensitization in contractility of resistance arteries - Calcium transients and ion channels - Gap junctional coupling - Isolated cardiac myocytes - Cell proliferation in cardiac growth and regeneration	- Intracellular signalling - Mitochondrial (dys)function - Membrane biophysics
Molecular level		
- Receptor structure-function - Gene and protein expression	- Adrenergic receptor number regulation - Mitochondrial membrane potential - RhoA/Rho kinase pathway in calcium sensitization	- Transport proteins - Reactive oxygen species - Structure of signalling proteins

Developmental aspects and studies at the genomic level are also important in our research.

Nowadays, 61 years after its foundation in 1954, the Institute consists of 23 scientific departments (***teams***) supported by six service departments, and a collaboration among them helps to accomplish its goals. A broad spectrum of methodological approaches is employed,

covering molecular, cellular and systemic levels (Fig. 1). A great majority of experiments is performed using animal and *in vitro* models, as reflected in Institute's results. Collaboration with clinical centres provides a challenging opportunity to extend such research endeavours ***from animals to humans***, i.e., to better characterise the basic mechanisms regarding their relevance for humans and to help preventing, diagnosing and treating diseases. Many of them represent a mounting problem in industrialized countries, such as those associated with obesity (diabetes and cardiovascular diseases), neurodegenerative diseases such as Alzheimer's, epilepsy, disorders of the control of circadian rhythms, pain etc.

(b) Metabolic research

One of the research fields pursued at the Institute since its early days is metabolism and its regulation. While some research teams concentrate on specific aspects of metabolism their research has implications for cardiovascular and neurophysiological research. The departments studying metabolism can be clustered into three groups, depending on the level of complexity. Specifically, this includes research into (*i*) the activity and regulation of ***selected protein complexes and transport proteins***, (*ii*) the function of ***mitochondria*** and the impact of mitochondrial dysfunction on health, and (*iii*) the mechanisms underlying ***obesity-associated metabolic disorders*** (metabolic syndrome), including their prevention and treatment such as nutritional interventions outlined below.

The array of methods nowadays employed in metabolic research ranges from a cell biology level to advanced ***whole-body phenotyping*** in rodents, including behavioural testing, as well as the use of sophisticated ***animal models*** of human biology. For all of our studies, the animal models represent an essential tool. The Institute has always been recognized for its research on laboratory rats. In the last few years there has been a gradual shift of some groups to experimenting on mice. The situation in many other research centres worldwide is quite similar and reflects the increasing availability of various strains of transgenic mice. However, as the rats represent a superior models for studying metabolic phenotypes they are still frequently used at the Institute. Recent advances in the gene editing technologies (ZFN and TALEN nucleases) and new approaches to transgenesis help to overcome the historical limitations of the rat model. The Institute holds a major credit for the introduction of these powerful approaches and production of many new biomodels of human pathologies. In a collaborative project (Ivics et al. *Nat Protocol* **9**: 773, 2014) our researchers were directly involved in the development of a new approach to rapid high-efficiency germline transgenesis and sustained transgene expression in both mouse and rat, by using the Sleeping Beauty transposon system. The existence of such biomodels is essential to elucidate the molecular mechanisms and the genetic determinants of both rare and common diseases, such as the metabolic syndrome associated with aberrant lipid and glucose metabolism and hypertension. Namely the unique set of recombinant inbred strains, SHR congenic and SHR transgenic or knockout lines, and the availability of the appropriate genome sequences, enabled rapid screening for functional variants of candidate genes in the field of diabetes (Heinig et al. *Nature* **467**: 460, 2010), mitochondrial pathophysiology (Houstek et al. *Physiol Genomics* **46**: 671, 2014) and cardiovascular research (Langley et al. *Cardiovasc Res* **97**: 653, 2013). N.B.: SHR = spontaneously hypertensive rat

Characterisation of the activity and regulation of selected protein complexes and transport proteins represents the very first step towards understanding molecular aspects of cellular physiology. In research of protein structures, our primary focus lies in studying regulation of selected signalling proteins (14-3-3 in particular) whose functions are controlled through protein-protein interactions (Veisova et al. *Biochem J* **443**: 663, 2012). The expertise in protein structure characterisation also carries a big collaborative potential with cellular physiology groups as structural information about the studied proteins can be provided. This is very well exemplified by the collaboration of several research groups on the structural studies of TRPV1 and TRPA1 channels in neuronal cells (Boukalova et al. *J Biol Chem* **285**: 41455, 2010). Membrane transporters represent a wide and diverse group of proteins involved in the

transport of solutes across cytoplasmic or organellar, e.g., mitochondrial membranes. An important model employed in studies of this kind is the yeast cell. Our researchers participated in cloning and characterisation of about 15 new yeast transporters and discovered four new proteins involved in the regulation of intracellular cation homeostasis (Herrera et al. *Biochim Biophys Acta* **1838**: 127, 2014; Petrezselyova et al. *Biochim Biophys Acta* **1828**: 623, 2013). Such research on yeast can also have a therapeutic potential as membrane transporters can influence virulence and pathogenicity of *Candida* species (Bucek et al. *PLoS One* **9**: e93322, 2014). Similarly, mitochondrial carriers and in particular members of the uncoupling protein family (*UCPs*) were studied in the context of their regulation by the reactive oxygen species (*ROS*), a highly discussed and rather controversial topic in UCP physiology (Malingriaux et al. *PLoS One* **8**: e77786, 2013).

***Characterisation of mitochondrial (dys)function and its health impact*.** Mitochondria are key players in energy metabolism and harbour a number of metabolic pathways. Research into their functioning covered three specific areas:

(*i*) Inherited disorders of mitochondrial oxidative phosphorylation such as ATP synthase (see also Part g in the present document) and cytochrome c oxidase (Kovarova et al. *Biochim Biophys Acta* **1822**: 1114, 2012).

(*ii*) Production mechanisms of ROS (Mracek et al. *Biochim Biophys Acta* **1827**: 401, 2013), their detoxification (Gabrielova et al. *J Bioenerg Biomembr* **42**: 499, 2010) and *in vivo* regulation (Jaburek et al. *Int J Biochem Cell Biol* **45**: 816, 2013; Shabalina et al. *Biochim Biophys Acta* **1837**: 2017, 2014); the aim is to identify the role of ROS in the pathology of various diseases as well as their signalling role in healthy cells.

(*iii*) Metabolic requirements of cancer cells, accenting gene regulation of bioenergetically relevant pathways during carcinogenesis (Smolkova et al. *Int J Biochem Cell Biol* **43**: 950, 2010), potential effects of mitochondria-targeted anticancer drugs such as α-tocopheryl succinate (Rohlena et al. *Antioxid Redox Signal* **15**: 2923, 2011), and disruption of circadian regulation of gene transcription during tumorigenesis (Sotak et al. *Int J Cancer* **132**: 1032, 2013).

Mechanisms underlying obesity-associated metabolic disorders (metabolic syndrome) and possibilities for their prevention and treatment. To uncover the mechanisms by which *n*-3 fatty acids of marine origin (*omega-3*) exert their beneficial effects on health, we used mice with a genetically disrupted AMP-activated protein kinase (*AMPK*), and demonstrated that AMPK plays an important role in the preservation of hepatic insulin sensitivity to dietary omega-3, as well as in the reduction of hepatic steatosis in response to omega-3 (Jelenik et al. *Diabetes* **59**: 2737, 2010). We also demonstrated that the metabolic effects of omega-3 in dietary-obese mice were stronger when supplied as phospholipids (rather than as triacylglycerols) and that these effects were accompanied by a normalization of endocannabinoid system activity augmented in obesity (Rossmeisl et al. *PLoS One* **7**: e38834, 2012; *Biochim Biophys Acta* **1841**: 267, 2014). Results of several studies performed in mice indicated that the effects of omega-3 are augmented when combined with caloric restriction (Flachs et al. *Diabetologia* **54:** 2626, 2011) or with anti-diabetic drugs (Kus et al. *PLoS One* **6**: e27126, 2011; Kuda et al. *Diabetologia* **52**: 941, 2009) – ***these results have a direct medical impact*** (see Part g in the present document).

(c) Cardiovascular research

Institute's research into physiology and pathophysiology of the cardiovascular system may be divided into three main directions: (*i*) ischemic reperfusion and myocardial damage, (*ii*) hypertension, and (*iii*) vascular replacement.

Ischemic reperfusion and myocardial damage. We characterized the role of fatty acid translocase Cd36 in cardiac ischaemic tolerance (Neckar et al. *Physiol Genomics* **44**: 173, 2012), as well as the effect of its transgenic expression on (*i*) the incidence and severity of ischemic and reperfusion ventricular arrhythmias and (*ii*) reduced myocardial infarct size due to coronary artery occlusion (Klevstig et al. *Pflügers Arch* **465**: 1477, 2013). In a model of diabetic mice we analysed functional, morphological and molecular changes in diabetic

cardiomyopathy and demonstrated that hypoxia-inducible factor HIF-1α regulates an early cardiac response to diabetes, and that deregulation may increase the risk for diabetic cardiomyopathy (Bohuslavova et al. *BMC Endocrine Disorders* **14**: 11, 2014). The studies of improved ischemic tolerance of chronically hypoxic hearts defined the role of mitochondrial voltage-dependent calcium-activated potassium channels of high conductance (BK_{Ca}) in this process (Borchert et al. *Am J Physiol* **300**: H507, 2011) and characterized the salutary effects of reactive oxygen species on BK_{Ca} and cardioprotection. Activation of these channels can be a promising approach in mitigating myocardial injury associated with postischemic reperfusion (Borchert et al. *Exp Biol Med* **238**: 233, 2013). By studying the development and pathology of cardiac conduction system in wild-type and connexin40-deficient mice the role of connexin40 has been established (Sankova et al. *Cardiovasc Res* **95**: 469, 2012).

Hypertension. Here we focussed on the mechanisms underlying blood pressure regulation in hypertensive rats and on human-rat comparative genomics. Radiotelemetric blood pressure data obtained in recombinant inbred strains (developed and phenotyped at the Institute) demonstrated an extensive conservation of trans-regulated genes and their master regulators in both rat and human hypertension (Langley et al. *Cardiovasc Res* **97**: 653, 2013). This study represents an interesting new insight into the genetic causes of cardiovascular diseases. The experimental hypertension research, traditionally strong at the Institute, focused on three topics: (*i*) The role of chromogranin A (a protein catalysing the formation and cargo storage of regulated secretory granules in neuroendocrine cells and important for a range of catecholamine biosynthetic enzymes and catecholamines themselves) in the pathogenesis of hypertension (Friese et al. *Hum Mol Genet* **22**: 3624, 2013). (*ii*) The age-dependent participation of different pathogenetic mechanisms in salt hypertension in Dahl rats. We demonstrated an antihypertensive effect of high K^+ intake (manifesting itself in reduced sympathetic vasoconstriction) in immature animals but not in adults (Zicha et al. *Acta Physiol Oxf* **202**: 29, 2011). On the contrary, hypertension development and sympathetic outflow could be attenuated by chronic endothelin A receptor blockade or chronic antioxidant treatment only in adult but not in young Dahl rats (Vaneckova et al. *Acta Physiol (Oxf)* **208**: 340, 2013; Zicha et al. *Acta Physiol Oxf* **205**: 124, 2012). (*iii*) The role of calcium sensitization in the control of blood pressure studied in genetic (SHR) and salt hypertension (Dahl rats) using a new approach to determine calcium sensitization in conscious rats (Behuliak et al. *J Hypertens* **31**: 2025, 2013). We demonstrated that calcium sensitization is enhanced in salt hypertension but reduced in genetic hypertension. These findings open new research possibilities for the analysis of vascular contractile mechanisms in various forms of hypertension.

Vascular replacement. We tested chemically modified bypass grafts suitable for the adhesion and growth of cells, particularly endothelial cells. Artificial grafts currently used in clinical practice are usually made of polyethylene terephthalate (*PET*) and expanded polytetrafluoroethylene (*ePTFE*). These materials are relatively highly hydrophobic and thus less appropriate for the adhesion of endothelial cells, which is considered crucial for preventing thrombosis, inflammatory cell adhesion and vascular smooth muscle cell hyperplasia. In collaboration with the Institute of Macromolecular Chemistry, we thus modified these materials with fibrin layers prepared by *in vitro* simulation of a part of physiological haemocoagulation process. Fibrin was chosen because it could be isolated in sufficient quantity in the autologous form, i.e. from patient´s own blood, and the nanofibrous morphology of fibrin films resembling the morphology of native extracellular matrix can be controlled by the use of stimulators and inhibitors of fibrin formation from fibrinogen (Filova et al. *J Biomed Mater Res A* **102**: 698, 2014; Chlupac et al. *Tissue Eng Part A* **20**: 2253, 2014). We also obtained promising results with immobilized short, synthetic and non-immunogenic oligopeptides derived from fibronectin and other extracellular matrix molecules acting as ligands for the cell adhesion receptors, on the luminal surface of the prosthesis (Popelka et al. *Eur Polym J* **58**: 11, 2014).

(d) Neurophysiological research

Neurophysiological research carried out at the Institute covers a selected spectrum of problems ranging from the cellular mechanisms of neurotransmitter release and actions of

transmitters on their receptors to highly integrative functions of the central nervous system, such as memory and temporal regulation of physiological processes (circadian rhythms). Additionally, pathophysiological mechanisms are studied, namely those of neuropathic pain, cerebral ischemia or neuropsychiatric diseases such as epilepsy, Alzheimer's disease and schizophrenia.

Research into the functioning of ion channels and G-protein coupled receptors in both peripheral and central nervous systems included an investigation of the molecular mechanisms underlying the effects of so-called allosteric modulators, i.e., agents affecting the activity of a receptor at a site other than the receptor's active (orthosteric) site. The medical (pharmaceutical) potential of such research is obvious. In the NMDA (N-Methyl-D-aspartic acid) subgroup of ***ionotropic glutamate receptors***, we aimed to identify clinically relevant antagonists, namely pregnanolone sulphate and its newly synthetized analogues, capable of preferentially blocking excitotoxic receptor activation, without interfering with its functions required for normal synaptic transmission and neural plasticity (Borovska et al. *Br J Pharmacol* **166**: 1069, 2012). The research considerably extended our knowledge on the function, structure, trafficking, molecular genetics, and pharmacology of ligand-gated ion channels including glutamate and acetylcholine channels (Kaniakova et al. *J Biol Chem* **287**: 26423, 2012). Thermally gated ***transient receptor potential (TRP) ion channels*** were of particular interest from the pain and nociception perspective. These channels are expressed in polymodal primary sensory neurons and on presynaptic endings of primary afferents in the spinal cord dorsal horn, and we attempted to elucidate the mechanisms underlying their (*i*) profound involvement in the development of acute and chronic pain states (Boukalova et al. *J Biol Chem* **285**: 41455, 2010; *Biochim Biophys Acta* **1833**: 520, 2013; Marsakova et al. *Anesthesiology* **116**: 903, 2012) and (*ii*) potentiation under pathological conditions when their activity may be affected by a number of cytokines and endogenous agonists (Spicarova et al. *J Neuroinflammation* **8**: 177, 2011). The outcome of these studies may facilitate the development of new compounds to treat cognitive disorders, chronical pain states as well as improve learning and memory. In a broader domestic collaboration, we patented a novel neuroprotective steroidal derivative (Rambousek et al. *Neuropharmacology* **61**: 61, 2011).
Attempts to elucidate the physiological role and the structure-function relationship of ***purinergic ATP-gated P2X ion channels*** revealed that P2X2, P2X7 and P2X4 mRNAs are most abundant in the hypothalamic suprachiasmatic nuclei neurons known to release ATP. This nucleoside triphosphate was shown to activate presynaptic P2X2 receptors and to modulate inhibitory synaptic transmission (Bhattacharya et al. *J Neurosci* **33**: 8035, 2013).

Research into the molecular mechanisms underlying the action of ivermectin, a positive allosteric regulator of several ligand-gated ion channels including P2X4 purinoreceptors contributed to the understanding of allosteric pharmacology of muscarinic receptors, molecular mechanisms of their activation and coupling with G-proteins. The main topics tackled in the field of biochemical ***physiology and pharmacology of cholinergic neurons*** included: (*i*) synthesis, storage, and release of acetylcholine and its presynaptic autoregulation; (*ii*) molecular pharmacology of muscarinic receptors including allosteric modulation of receptor activation, interaction of muscarinic receptors with G-proteins and modelling of muscarinic receptor signal transduction (Jakubik et al. *Mol Pharmacol* **86**: 180, 2014); and (*iii*) cholinergic mechanisms in the pathogenesis of Alzheimer´s disease, namely the effect of beta-amyloid protein on acetylcholine metabolism and muscarinic transmission (Janickova et al. *Neuropharmacology* **67**: 272, 2013; Machova et al. *Neurobiol Dis* **38**: 27, 2010).

Research into the ***developmental aspects of epilepsy*** demonstrated that intense epileptic activity leads to both acute and long-lasting morphological and functional alterations, often of progressive nature in rats younger than two weeks (Kubova & Mares *Neuroscience* **235**: 232, 2013). Mechanisms responsible for the damage in the immature brain included oxidative stress and mitochondrial dysfunction (Folbergrová et al. *Exp Neurol* **233**: 421, 2012). In immature rats, we confirmed the expected anticonvulsant and neuroprotective effect of agonists of groups II and III (as well as group I) of metabotropic glutamate receptor (Folbergrová et al. *Neuropharmacology* **54**: 665, 2008, Lojkova-Janeckova et al. *Epilepsia* **50**: 665, 2009). In adult animals, repeated spontaneous brief temporal lobe seizures promoted

increased hippocampal neurogenesis in the absence of status epilepticus and severe cell loss, thus providing a rationale to develop pharmacological strategies aimed at modulating neurogenesis as a treatment to reverse learning and memory deficits in temporal lobe epilepsy (Jiruska et al. *Neurobiol Dis* **54:** 492, 2013).

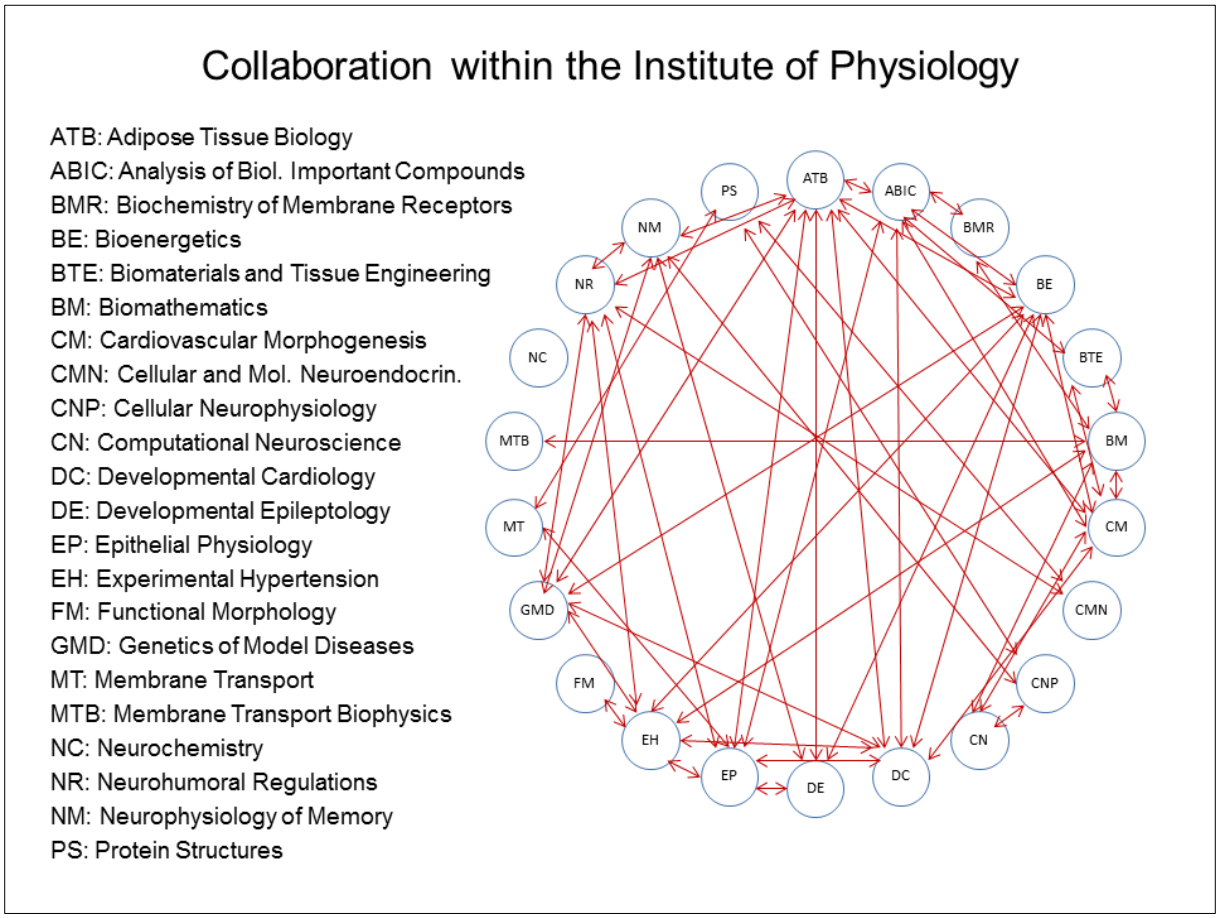
To shed light on ***the mechanisms of temporal organization of physiological processes*** on daily and seasonal basis, we studied (in physiological and pathological conditions) the circadian system composed of the central clock located in the suprachiasmatic nuclei of the hypothalamus, and peripheral clocks found in nearly all bodily cells. We found that maternal feeding regime may entrain clocks of their fetuses (Novakova et al. *J Biol Rhythms* **25**: 350, 2010; Sumova et al. *Prog Brain Res* **199**: 83, 2012), and studied aberrations of the circadian system in animal models of colorectal cancer, hypertension and metabolic diseases (Sladek et al. *PLoS One* **7**: e46951, 2012; Sotak et al. *Int J Cancer* **132**: 1032, 2013). In human studies, we revealed malfunctioning of the circadian system in patients with a rare genetic disease, Smith-Magenis Syndrome (SMS) by demonstrating that in the SMS patients, the disrupted regulation of sleep and hormone levels are likely due to the inability of their central clock to efficiently drive daily rhythms (Novakova et al. *J Clin Endocrinol Metab* **97**: E312, 2012). These findings help to understand the ontogeny of the circadian system, and are relevant to new chronotherapeutic approaches to a range of diseases. Moreover, we provided the first evidence that in humans (tested in real-life conditions), the individual chronotype (i.e., preference for bedtime) affects phasing of their peripheral molecular clocks. Thus, the extreme chronotypes may have their clocks out of phase with the social time, which may explain a higher incidence of some diseases in them (Novakova et al. *Chronobiol Int* **30**: 607, 2013).

In ***cognitive neuroscience***, we focused on spatial navigation in dynamic environments and behavioural flexibility, and discovered an intriguing phenomenon linked to the need to continuously update information about the position of a small programmable robot the experimental rats were forced to avoid. Functional inactivation of hippocampus disrupted the avoidance behaviour (Telensky et al. *Proc Natl Acad Sci USA* **108**: 5414, 2011). In addition, we have shown that hippocampus is crucial for recognition of objects on a computer screen (the ”virtual reality in rats“ project, Levcik et al. *Hippocampus* **23**: 153, 2013), and dorsal hippocampus for recognizing positions of objects located in an inaccessible part of the experimental environment (Klement et al. *Behav Brain Res* **207**: 480, 2010).

(e) Collaborations within the Institute

Needless to say, research cooperation within the Institute ***streamlines its scientific endeavours*** and contributes to a ***more efficient utilization*** of available resources. A wealth of mutually beneficial collaborations among Institute’s teams has been established, as summarized in Fig. 2. This includes ***joint research projects*** (both bilateral and framework ones, such as the national Centres of Excellence described below (see Section f), and those funded by EU) as well as ***joint publications***.

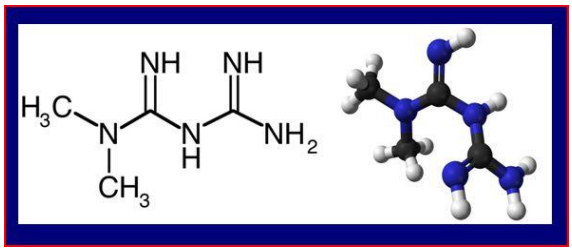
Figure 2. ***Collaboration among the Institute's teams (marked by arrows).***



Selected collaborative efforts within the Institute:

- Departments of Epithelial Physiology and Neurohumoral Regulations have been studying **the development and entrainment of the colonic circadian clock** (e.g., Polidarova et al. *Chronobiol Int* **28**:204, 2011; Sotak et al. *Int J Cancer* **132**:1032, 2012; Polidarova et al. *Am J Physiol Gastrointest Liver Physiol* **306**: G346, 2014; Sotak et al. *Ann Med* **46**: 221, 2014).
- Department of Genetics of Model Diseases collaborates with six other departments within the Institute by providing internationally recognized expertise in **rat genetics and genomics** (BXH/HXB recombinant inbred strains), as documented by a number of studies published so far (e.g., Houstek et al. *Physiol Genomics* **44**: 487, 2012; Langley et al. *Cardiovasc Res* **97**: 653, 2013; Houstek et al. *Physiol Genomics* **46**: 671, 2014) and joint projects (e.g., Centre of Applied Genomics, ERC CZ project, MITOCENTRUM project of excellence).

Figure 3. ***Metformin – an anti-diabetic drug with potential benefits in cardiac patients***



- Departments of Bioenergetics, Adipose Tissue Biology, and Cardiovascular Morphogenesis, in collaboration with the Institute of Clinical and Experimental Medicine, conducted a study testing the **potential of metformin**, an established anti-diabetic drug, in improving cardiac function (Benes et al. *Clin Sci (Lond)* **121**: 29, 2011); see Fig. 3.

(f) National and international collaboration

The Institute of Physiology maintains extensive collaborations, both locally and internationally. It is placed in the largest biomedical research campus in the country, comprising five institutes at the outskirts of Prague and employing ~1,500 research staff. The location in the capital also facilitates cooperation with Faculties of Medicine (including their teaching hospitals), Science, Mathematics and Physics of the ***Charles University, Institute of Chemical Technology, Institute of Clinical and Experimental Medicine***, as well as a number of ***institutes of the Czech Academy of Sciences***, in particular Institutes of Organic Chemistry and Biochemistry, Macromolecular Chemistry, Molecular Genetics, and Microbiology.

National research collaboration was significantly stimulated by Institute's participation in a number of network grants locally referred to as *"**Research Centres (of Excellence)**"* and funded by the Ministry of Education. These included Centres for Cardiovascular Research, Neuroscience, Yeast Research, Fluorescence Microscopy in Biomedical Research. The Institute is currently involved in their current variant *("**Centres of Excellence**"),* namely the "***Neurosciences***" centre (since 2012) and Mitochondrial Biology and Pathology (***MITOCENTRE***) since 2014.

A completely new, EU-funded Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (***BIOCEV***) includes six research teams of the Institute and will offer opportunity to exploit the cutting-edge core facilities (http://www.biocev.eu/en/).

The Institute also participated in a number of ***EU-funded integral (multilateral) projects and other international collaborations***. These included:

- ***BIOCLAIMS***: identification of biomarkers of the effect of food on health (11 groups from 7 countries)
- ***DIABAT***: mechanisms of increasing energy expenditure in adipose tissue and thus preventing body fat accumulation (21 groups from 12 countries)
- ***EUCLOCK***: entrainment of the circadian system (27 groups)
- ***EURATRANS***: rat functional genomics for translational research of ortholog genetic determinants in humans
- ***LipiDiDiet***: therapeutic and preventive impact of nutritional lipids on neuronal and cognitive performance in ageing, Alzheimer´s disease and vascular dementia (19 groups from 7 countries)
- ***PHOTOLYSIS***: exploring a combined approach using electrophysiology and optical imaging in neurobiology (7 groups from 6 countries)
- ***COST*** **(**European Cooperation in Science and Technology**)** included three projects: (i) nutritional optimization of mitochondrial function to increase disease resistance; (ii) adipose tissue as a key target for prevention of the metabolic syndrome; and (iii) molecular machineries for ion translocation across biomembranes.

Internationalisation and increasingly interdisciplinary nature of research also required ***bilateral cooperation among the teams from the Institute, the Czech Republic and elsewhere*** (see Fig. 4). The Institute has maintained close research ties with a number of research institutions such as:

USA
- Howard Hughes Medical Institute
- Natnl. Inst. Child Health Human Develop., Univ Calif. San Francisco
- Univ. Minnesota

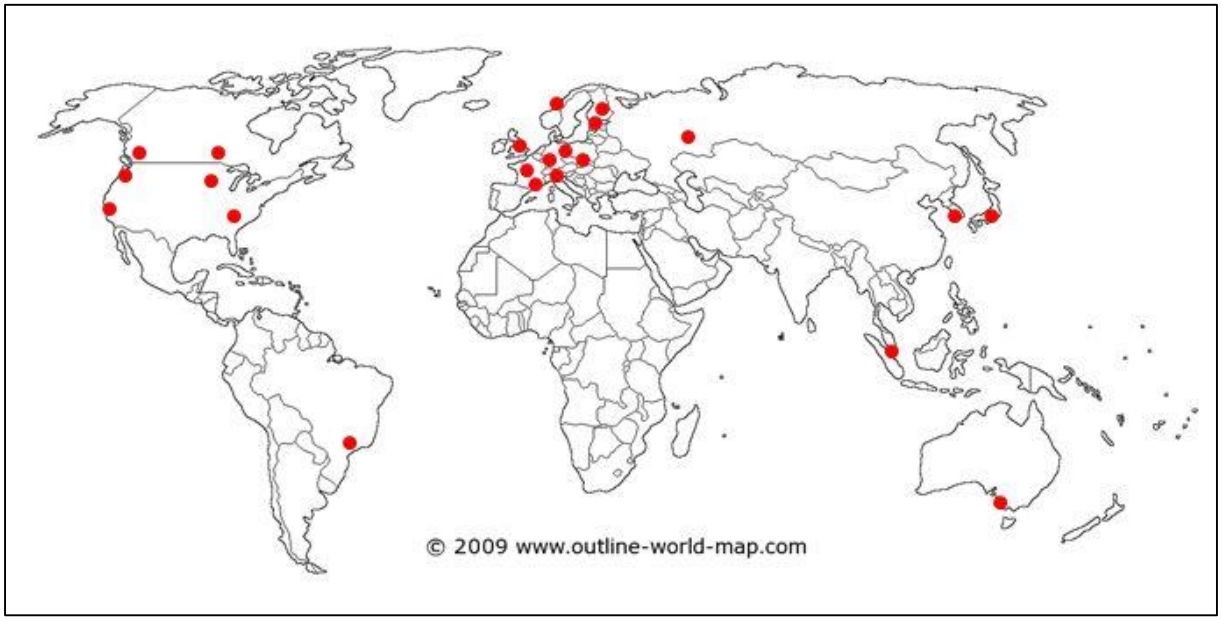
Europe

- MRC Laboratory of Molecular Biology (Cambridge)
- University of Oxford and Imperial College (London)
- Max-Delbrück Ctr. Mol. Medicine (Berlin) and Univ. Erlangen
- INRA Versailles, France
- Instit. Normal & Pathol. Physiol. & Inst. Heart Res., Slovak Acad. Sci.
- Kazan branch of the Russian Academy of Sciences and Kazan Medical University

(and many others; see reports of the individual teams)

The above examples document the breadth of Institute's international collaboration, and we hope to capitalize on it also from the perspective of identifying and recruiting able young researchers to our teams, irrespective of the country of origin. Two ***Marie Curie Initial Training Networks*** (*Cornucopia*, *ImResFun*), which brought together several European partners including our Institute are good examples of this endeavour.

Figure 4. ***Selected international links of the Institute of Physiology.***



(g) Applied outputs

As mentioned elsewhere within the present document (Parts 1b,c,d above) and reports from individual research teams, Institute's fundamental research often has a direct or indirect ***clinical impact***. The most important examples of such ***translational research*** are summarized below:

- Rare ***inherited mitochondrial diseases*** investigated at Department of Bioenergetics. Two of four known nuclear gene mutations (TMEM70 and ATP5E) causing defects of ATP synthase (Havlickova et al. *Biochim Biophys Acta* **1797**: 1124, 2010; Mayr et al. *Hum Mol Genet* **19**: 3430, 2010) were successfully identified. Another collaborative project resulted in the development of a non-invasive protocol for diagnosting human mitochondrial diseases. It is based on the use of ***isolated lymphocytes*** (Pecina et al. *BBA Clinical* **2:** 62, 2014). All these results have a major impact on ***diagnosing the diseases.***
- A randomized clinical trial on ***type 2 diabetic patients*** has been completed in collaboration between the Department of Adipose Tissue Biology and the Institute of Clinical and Experimental Medicine (Prague), as an extension of our findings about the mechanisms underlying beneficial effects of *n*-3 fatty acids, namely *omega-3* (Kus et al. *Plos One* **6**: e27126, 2011; Flachs et al. *Diabetologia* **54**: 2626, 2011). It has shown that a combination

of omega-3 and anti-diabetic drugs from the thiazolidinedione family improves patients' post-prandial lipid metabolism, thus ***improving the therapeutic strategy*** (Veleba et al. *Clinical Sci*., submitted, 2015).

- Using transgenic SHR rats expressing human C-reactive protein (CRP) developed at Department of Genetics of Model Diseases we have demonstrated a significant role of ***increased human CRP in pathophysiology of the metabolic syndrome*** (Pravenec et al. *Hypertension* **57**: 731, 2011).
- Our pioneering work on the detection of clock gene expression in the buccal mucosa in patients with a rare genetic ***Smith-Magenis syndrome*** has demonstrated that aberrations in the temporal regulation of melatonin, typical for this disease, are likely caused by the aberrant circadian system (Nováková et al. *J Clin Endocrinol Metab* **97:** E312, 2012).
- An effective diagnosis and treatment of human diseases requires reliable ***biomarkers of disease and its progression***. High-frequency oscillations in brain activity have been proposed as a marker of epileptogenic tissue. Detection and analysis of these oscillations significantly ***improve presurgical examination and epilepsy surgery*** (Cho et al. *Epilepsia* **55**: 1872-1883, 2014; http://isarg.feld.cvut.cz/).
- Evidence-based ***novel treatment strategies*** are typically following carefully controlled experimental studies. Our teams have elaborated many potential treatment procedures. We have shown in rats, macaques and patients that medical carbogen containing 5% CO_2 can be used as an acute ***anticonvulsive treatment in epilepsy*** (Tolner et al. *Epilepsia* **52**: 104-114, 2011).
- Experimental work of Department of Membrane Transport Biophysics yielded new compounds suitable for ***photodynamic therapy in oncology patients***. Hydrophobic phtalocyanine combined with a liposomal carrier is easily transferred to cancer tissue where it is efficiently accumulated (*Canadian Patent No*.2665762).
- Department of Biomaterials & Tissue Engineering improved existing synthetic ***tissue replacements*** by introducing into them cellular and other biological components. Completely new, 'hybrid' replacements based on a combination of synthetic materials and cells have been obtained. Some of the findings have been already patented (CZ304445, EU299687).
- An active search for ***new biological targets*** and their context dependency. Jointly with Institute of Organic Chemistry & Biochemistry CAS and Psychiatric Centre Prague, we participate in drug development and testing. The combined team has successfully synthetized and patented several neuroactive steroids (CZ303037, CZ303443, US8575376, WO/2010/136000); several of them have been successfully tested in models of ***neuropsychiatric disorders (neurodegeneration, epilepsy, stroke***) and are now considered by the National Institutes of Health (NIH at Bethesda, MD, USA) to be included in their preclinical testing program. Derivatives of prolactin-releasing peptide (US14/598160 patent application) are highly promising for treating ***obesity, diabetes*** and some ***neurodegenerative disorders***. High translation potential has been identified in ***pain*** research, especially the inhibition of TRPV1 receptors (Uchytilova et al. *Molecular Pain* **10**: 67, 2014).

Effective ***technology transfer*** makes our hi-tech methods and material accessible. In the context of scientific exchange the technology transfer at the Institute constantly occurs through the transfer of biological material, know-how or provision of individual animals or animal lines. In addition, we are aiming for the commercial exploitation of research results which are developed at the Institute. In these results appropriate Intellectual Property rights are set and commercialization strategy is followed to facilitate rapid and efficient transfer into the practice. Some commercial subjects have expressed interest in Institute's expertise, namely the Velaz company hoping to obtain its transgenic rats of specific-pathogen-free (SPF) quality (a joint project TA02010013 to create models of specific human diseases). Department of Biological Controls is a laboratory compliant with the principles of good manufacturing practice (GMP)

and certified by the State Institute for Drug Control (the Czech answer to the Food & Drug Agency in the USA) to carry out “Quality control testing of human medicinal products“. As such, it has a technology-transfer potential worth exploration.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the ASCR, v. v. i.

Scientific team	Experimental Hypertension

RESEARCH FOCUS

The ***Department of Experimental Hypertension*** (Institute of Physiology CAS, Prague) is investigating the pathophysiological mechanisms of hypertension in various experimental models, namely in spontaneously hypertensive rats (SHR) characterized by sympathetic hyperactivity, transgenic Ren-2 rats (TGR) with angiotensin II-dependent hypertension and Dahl rats with salt hypertension. Our attention is mainly focused on hemodynamic changes, the role of principal vasoactive systems, alterations of vascular contraction, ion transport abnormalities, cell calcium handling and defects of cell signaling in vascular smooth muscle. Further important research topics of our team are hypertensive end-organ damage, abnormal lipid metabolism, enhanced formation of reactive oxygen species, pathogenetic mechanisms of obesity-related hypertension and pathophysiology of neurogenic pulmonary edema following acute CNS trauma. Our traditional research topics are the ontogenetic aspects of hypertension development and its treatment (Zicha and Kuneš *Physiol Rev* **79**: 1227, 1999) with a special respect to the developmental windows (critical periods of development) in which the organism is highly sensitive to environmental stimuli (nutrition, stress, etc). Increased susceptibility of immature rats to high salt intake is a typical example (Zicha et al. *Hypertension* **8**: 1096, 1986; Zicha et al. *Physiol Res* **61** (Suppl 1): S35, 2012). At present this is complemented with our increasing research interest in epigenetic mechanisms participating in hypertension and/or obesity development (Kuneš and Zicha *Clin Sci* **111**: 295, 2006; Vaněčková et al. *J Endocrinol* **223**: R63, 2014).

PERSONNEL

Our department has been founded in early 60´s by Dr. Jiří Jelínek and was subsequently headed by Dr. Jaroslav Kuneš (1985-2006) and Dr. Josef Zicha (2007-2015). In 2010 Dr. Ivana Vaněčková from the Institute of Clinical and Experimental Medicine (Prague) has joined our department and is currently trained to be the Head since 2016. Actually our team consists of five senior investigators (Vaněčková, Dobiášová, Kuneš, Rauchová and Zicha, H-index = 13, 17, 29, 16 and 26, respectively), four junior scientists (Behuliak, Hojná, Kadlecová and Vokurková, H-index = 10, 6, 6 and 7, respectively), one postdoctoral fellow (Řezáčová), three PhD students (Bencze, Loučková and Mikulášková), two MSc students (Brunová and Misárková) and three laboratory assistants (Charvátová, Kopecká and Nahodilová). Two Ph.D. students are expected to defend their Theses in the near future (Bencze 2016, Loučková 2017). In addition, two Slovak post-doctoral fellows, whose Ph.D. training was partially (Dr. Líšková) or entirely (Mgr. Pintérová-Surovcová) accomplished in Prague, have left our lab to work abroad. It should also be noted that some of the team members are only part-time working so that the average size of our team corresponds to six full-time scientists.

KEY RESULTS

Our research team had four major topics within the evaluated period 2010-2014. All four topics were studied mainly in conscious instrumented rats in order to avoid the artifacts resulting from anesthesia (Bencze et al. *Physiol Res* **62**: 471, 2013). Nevertheless, we tried to investigate these topics simultaneously at different levels, ranging from molecular and cellular levels up to isolated organs and whole animals.

a) Calcium influx and calcium sensitization in spontaneously hypertesive rats (SHR)

This topic represents the analysis of our primary finding that calcium influx through L type voltage-dependent calcium channels (L-VDCC) is proportional to the basal blood pressure (BP) level and/or vascular wall tension (Paulis et al. *Acta Physiol Oxf* **191**: 255, 2007). Subsequently, we have brought a unique evidence that the inactivation of upregulated inhibitory G proteins by pertussis toxin treatment lowered BP of SHR by the attenuation of adrenergic vasoconstriction dependent on calcium entry through L-VDCC (Pintérová et al. *J Hypertens* **28**: 969, 2010). Later, we have demonstrated that not only adrenergic but also angiotensin II-dependent BP components are proportional to nifedipine-sensitive BP component. Thus, all three major vasoactive systems (sympathetic nervous system – SNS, renin-angiotensin system – RAS and nitric oxide – NO) modulate BP via nifedipine-sensitive calcium entry (Pintérová et al. *Physiol Res* **58** (Suppl 2): S43, 2009; Zicha et al. *Physiol Res* **63**: 13, 2014). Moreover, enhanced calcium entry through L-VDCC seems to be related to a decreased efficiency of vasodilator action of voltage-dependent (K_V) and/or calcium-activated (BK_{Ca}) potassium channels in SHR compared to WKY (Pintérová et al. *Physiol Res* **63**: 275, 2014) (grants 1M0510 ME CR, GACR 305/08/0139).

Furthermore, our attention was focused on calcium sensitization mediated by RhoA/Rho kinase pathway because greater BP reduction by nifedipine in SHR might result not only from the blockade of enhanced calcium entry through L-VDCC in this hypertensive strain but also from the inhibition of normal calcium entry under the conditions of increased calcium sensitization (Zicha et al. *Physiol Res* **63** (Suppl 1): S19, 2014). We were the first who demonstrated that basal calcium sensitization is attenuated in adult SHR with established hypertension (Behuliak et al. *J Hypertens* **31**: 2025, 2013). The reduction of calcium sensitization seems to be a compensation of enhanced calcium entry through L-VDCC, both abnormalities being present already in prehypertensive SHR (Behuliak et al. *J Hypertens* in revision) (grants IGA ASCR IAA50110902, GACR 304/12/0259).

b) Impaired vasodilator mechanisms in experimental hypertension

The attention has been paid to various models of experimental hypertension including SHR, hereditary hypertriglyceridemic rats, salt hypertensive Dahl rats and rats with NO-deficient hypertension elicited by chronic L-NAME treatment. This topic was investigated in a cooperation with several labs in Bratislava (Slovakia, see below) when the experiments with conscious cannulated rats and nearly all myographic experiments were done by the members of our team in Prague lab.

A comparison of the efficiency of three major vasodilator systems (nitric oxide, prostacyclin and calcium-activated potassium channels) in selected forms of experimental hypertension (SHR, Dahl, NO-deficient) indicated surprising compensatory activation of vasodilator function of prostanoids and BK_{Ca} channels, whereas NO-dependent vasodilation was not enhanced in any hypertensive model (Behuliak et al. *Hypertens Res* **34**: 968, 2011). Further attention was paid to the mechanisms modulating

arterial contraction in normotensive and hypertensive rats (Pintérová et al. *Physiol Res* **58** (Suppl 2): S43, 2009). Three consecutive papers indicated the importance of calcium-activated (BK_{Ca}) potassium channels (Líšková et al. *J Am Soc Hypertens* **4**:128, 2010), EDCF (Líšková et al. *Eur J Pharmacol* **667**: 265, 2011) and calcium-activated chloride channels (Líšková et al. *Biomed Res Int* **2014**: 289361, 2014) in adrenergic vascular contraction, the alterations of which are increasing with aging and/or hypertension development (grants 1M0510 ME CR, GACR 305/08/0139 and 304/12/0259).

c) Age-dependent differences in the pathogenesis of salt hypertension in Dahl rats

It is well known that high salt intake causes the development of severe self-sustaining form of salt hypertension in immature Dahl rats, whereas a less severe form of salt hypertension is induced by high salt intake applied in adult animals. Both age groups of salt hypertensive Dahl rats differ not only in SNS contribution to BP maintenance but also in the role of NO-dependent vasodilation and superoxide production (Zicha et al. *J Hypertens* **19**: 247, 2001; Dobešová et al. *J Hypertens* **20**: 925, 2002). In this evaluation period we have tried to elucidate the age-dependent effects of high potassium intake, endothelin A receptor stimulation and superoxide production in young and adult salt-sensitive Dahl rats. Three interesting original findings were published by our team.

First of all, we have demonstrated that preventive but not therapeutic dietary potassium supplementation attenuates salt hypertension development in Dahl rats. This antihypertensive effect, which is due to the attenuation of sympathetic hyperactivity, is present only in young but not in adult salt hypertensive Dahl rats (Zicha et al. *Acta Physiol (Oxf)* **202**: 29, 2011). Second, the participation of endothelin-1 (ET-1) in the pathogenesis of salt hypertension is age-dependent because chronic endothelin A receptor blockade attenuated salt-induced rise of blood pressure (BP) only in adult but not in young Dahl rats. This BP reduction was due to a decrease in sympathetic vasoconstriction, indicating ET-1 involvement in the central mechanisms controlling sympathetic tone. In contrast, the contribution of circulating ET-1 to BP maintenance was similar in young and adult salt hypertensive Dahl rats (Zicha et al. *Acta Physiol (Oxf)* **205**: 124, 2012). Finally, we have demonstrated major differences in the role of reactive oxygen species in salt hypertension of young or adult Dahl rats. Chronic administration of tempol (SOD mimetic) attenuated hypertension development in adult but not in young Dahl rats. In addition, increased superoxide production and resulting enhanced lipoperoxidation correlated with high blood pressure in adult rats (Vaněčková et al. *Acta Physiol (Oxf)* **208**: 340, 2013). Significantly greater effects of chronic tempol administration in adult salt hypertensive Dahl rats are at variance with our earlier observation of smaller BP effects of acute tempol injection in these animals (Dobešová et al. *J Hypertens* **20**: 925, 2002). (grants 1M0510 ME CR, GACR 305/08/0139, 305/09/0336 and 304/12/0259).

Our results obtained in Dahl rats were summarized in a recent review (Zicha et al. *Physiol Res* **61** (Suppl 1): S35, 2012) in which they were evaluated in the context of current international research performed in this experimental model.

d) ***Interaction of renin-angiotensin and endothelin systems in the pathogenesis of hypertension and renal damage in Ren-2 transgenic rats***

Aliskiren, a relatively new drug in antihypertensive therapy, is a direct renin inhibitor intervening the RAS at its rate-limiting step. We found that its BP lowering effect in Ren-2 transgenic rats (TGR) persisted for almost two weeks after its withdrawal and this effect was accompanied by the

reduced proteinuria due to the improved glomerular arrangement (Rakušan et al. *Am J Physiol* **299**: F758, 2010). When the effects of aliskiren were compared with angiotensin receptor blocker losartan, only aliskiren decreased albuminuria at similar BP decrease (Vaňourková et al. *Physiol Res* **59**: 339, 2010). This topic has been partly investigated in cooperation with IKEM (see below) under the supervision of Dr. Vaněčková who joined our team in 2010 (grant 1M0510 ME CR).

Based on our previous study with a single application of antisense against AT_1 receptor (Vaněčková, *Vascul Pharmacol* **47**: 63, 2007) we evaluated the effects of its repeated delivery in young and adult Ren-2 transgenic rats. We found that it retarded hypertension development and cardiac hypertrophy in young animals, and this BP-lowering effect was due to the reduced sympathetic vasoconstriction, while in the adult TGR, its effect was due to the attenuation of angiotensin II-dependent vasoconstriction (Vaněčková et al. *Hypertens Res* **35**: 761, 2012) (grant IGA ASCR IAA50110902).

Our previous studies (Vaněčková et al. *Hypertension* **46**: 969, 2005; Opočenský et al. *Hypertension* **48**: 965, 2006) demonstrated the beneficial effects of selective endothelin receptor A blockade (atrasentan) over nonselective ET_A/ET_B blockade with bosentan. We analyzed the mechanisms contributing to antihypertensive effects of atrasentan and showed that it is mediated by the reduced calcium influx through voltage-dependent calcium channels (L-VDCC) due to the attenuated angiotensin II-dependent vasoconstriction and missing ET_A vasoconstriction (Vaněčková et al. *J Hypertens* **33**: 161, 2015) (grant GACR 304/12/0259).

INTERNAL COLLABORATION (within the Institute of Physiology CAS, Prague)

Genetic analysis of spontaneous hypertension

A traditional cooperation with the *Department of Genetics of Model Diseases* (Dr. Pravenec), which started with BP determination in the unique set of Prague recombinant inbred (RI) strains (Pravenec et al. *J Hypertens* **7**: 217, 1989) and continued with a later radiotelemetric analysis of the participation of principal vasoactive systems in BP control of these rat RI strains (Kuneš et al. *Hypertens Res* **31**: 1659, 2008), yielded further results. Using human-rat comparative genomics we explored the transcriptional mechanisms mediating the effects of genes revealed by human genome-wide association studies. Extensive conservation of trans-regulated genes and their master regulators was shown in both rat and human hypertension (Langley et al. *Cardiovasc Res* **97**: 653, 2013). Our team participated in the determination of hemodynamic data essential for the above mentioned analysis (grant 1M0510 ME CR).

Mitochondrial energetic metabolism

One of our principal long-lasting cooperation with the *Department of Bioenergetics* (Dr. Drahota) is focused on the cell energetic metabolism, especially on FAD-linked glycerol-3-phosphate dehydrogenase (GPDH) localized on the outer surface of the inner mitochondrial membrane. We compared the inhibition of glycerol-3-phosphate and succinate-dependent consumption rates by digitonin treatment and the recovery of activity by cytochrome *c* and idebenone (hydrophobic synthetic analog of coenzyme Q) supplements (Rauchová et al. *Physiol Res* **61**: 259, 2012). In our recent joint paper (Rauchová et al. *Int J Biochem Cell Biol* **53**: 409, 2014) we showed that inhibition of glycerol-3-phosphate-dependent oxygen consumption by tocopheryl succinate (a suitable drug for cancer therapy)

was much higher than that on the oxidation rate of succinate-dependent one. All experimental data were provided by our team (grants GACR 303/09/0570 and 304/12/0259).

Long-term thyroid hormone level alterations

Our other long-lasting cooperation (Rauchová et al. *Horm Metab Res* **36**: 286, 2004) with the *Department of Functional Morphology* (Dr. Soukup) is based upon model experiments which are performed in rats with different thyroid states (euthyroid, hypothyroid and hyperthyroid). First, we confirmed that liver mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) activity is a useful marker for the evaluation of thyroid states also in chronic experiments lasting up to 12 months. In addition, we indicated a similar effect of altered thyroid hormone levels on the enzyme activity and protein amount of GPDH in female and male rats, these changes being more pronounced in females (Rauchová et al. *Horm Metab Res* **41**: 43, 2011). In our recent joint paper (Rauchová et al. *Horm Metab Res* **45**: 507, 2013) we tested how n-3 polyunsaturated fatty acids (PUFA) supplementation can change parameters, such as body and organ weights, thyroid hormone levels, blood glucose, plasma lipids and liver GPDH activity in rats with different thyroid states. However, we found no significant effects of 6-week-supplementation of PUFA administrated intragastrically at a dose of 200 mg/kg/day. GPDH activity and plasma lipids were measured by our team (grants GACR 303/09/0570 and 304/12/0259).

Other internal collaborations

We also cooperated with the *Department of Epithelial Physiology* (Dr. Pácha) in the study of local metabolism of glucocorticoids (Klusoňová et al. *Steroids* **76**: 1252, 2011; Vágnerová et al. *Steroids* **76**: 577, 2011) and with the *Department of Developmental Cardiology* (Dr. Ošťádalová) in the research concerning cardiac tolerance to ischemia in newborn SHR (Charvátová et al. *Physiol Res* **61** (Suppl 1): S145, 2012). We have also successfully cooperated with the *Department of Biomathematics* (Drs Karen and Vorlíček) in mathematical modeling of cardiovascular responses *in vivo* (Pintérová et al. *J Hypertens* **28**: 969, 2010; Behuliak et al. *J Hypertens* **31**: 2025, 2013) and *in vitro* (Líšková et al. *J Am Soc Hypertens* **4**:128, 2010; Líšková et al. *Eur J Pharmacol* **667**: 265, 2011; Líšková et al. *Biomed Res Int* **2014**: 289361, 2014) as well as in the sophisticated statistical evaluation of our biological results (Vaněčková et al. *Acta Physiol (Oxf)* **208**: 340, 2013).

DOMESTIC COLLABORATION (within the Czech Republic)

Institute of Clinical and Experimental Medicine (IKEM), Prague (Dr. L. Červenka)

Our long-lasting cooperation with the IKEM started within the project of *Cardiovascular Research Centre*. It continued with the project "*New pharmacological approaches in hypertension treatment: the combined interventions into renin-angiotensin and endothelin systems"*. This project was originally coordinated by Dr. Vaněčková, when she was the employee of IKEM, and the collaboration dates until now. Recently, a new common research project has been submitted to the Ministry of Health CR. The main topics were always **the interactions of major vasoactive systems** (RAS, ET system, metabolites of cytochrome P450) **in BP regulation and accompanying end-organ damage**.

Our cooperative research in the last five years was focused on the new possibilities in the treatment of chronic kidney disease. We used Ren-2 transgenic rats (TGR) a model of angiotensin II-dependent hypertension with 5/6 nephrectomy. Our first study determined the beneficial effects of RAS blockade on renal damage (Kujal et al. *Clin Exp Pharmacol Physiol* **37**: 1159, 2010). Further, we analyzed whether a combination of RAS blockade with endothelin A receptor blockade, which is known for its antiproteinuric effects, could have some additional effects. We found that although ET_A receptor blockade alone partially improved survival rate and decreased BP, it had no additional effects to RAS blockade if the treatment took 5 months (Vaněčková et al. *Kidney Blood Press Res* **35**: 382, 2012). However, the prolongation of the treatment to 10 months clearly showed additional positive cardio- and renoprotective effects of the combined therapy (Čertíková Chábová et al. *Life Sci* **118**: 297, 2014) (research grants of IKEM – IGA MH CR NS 9703 and NS 10500).

Further topics included the effects of metabolites of cytochrome P450 (HETEs and EETs) in renal damage (Čertíková Chábová et al. *Clin Sci* **118**: 617, 2010), the role of angiotensin-1-7 in hypertension development in rats with renal artery stenosis (Rakušan et al. *Kidney Blood Press Res* **33**: 476, 2010) and the effects of castration in Ren-2 transgenic rats (Vaněčková et al. *Kidney Blood Press Res* **34**: 46, 2011).

Institute of Organic Chemistry and Biochemistry, CAS, Prague (Dr. L. Maletínská)

The topic of **obesity-related hypertension** represents a relatively new field of our interest which is studied in the cooperation with IOCB CAS (Dr. Maletínská group). Recently, a unique set of neuropeptides with anorexigenic effects was tested. It was demonstrated that a lipidization of natural neuropeptides enabled to administer them peripherally (Maletínská et al. *Int J Obes (Lond)* 2015 in press). Since these neuropeptides could also have cardiovascular effects, their influence on blood pressure is studied as well (Vaněčková et al. *J Endocrinol* **223**: R63, 2014). Our team participates in this research by the study of physiological changes induced by above mentioned peptides in rats and mice. This program is a part of CVOL, TAČR (Center for Development of Original Drugs, Technology Agency of the Czech Republic) and some results were already patented. A common PhD student (Barbora Mikulášková) of Dr. Kuneš (IP CAS) and Dr. Maletínská (IOCB CAS) is currently working in our laboratory within this cooperation (grants IGA MH CR – NS 10024 and GACR 15-08679S).

Institute of Physiology, First Faculty of Medicine, Charles University, Prague (Dr. J. Koudelová)

Within the frame of this long-lasting cooperation on **lipid peroxidation in the brain** we observed that the acute hypobaric hypoxia applied during postnatal development of the rat induced lipid peroxidation damage in four different parts of brain (cortex, subcortex, cerebellum and medulla oblongata). Generally, young rats were more sensitive to oxygen changes than adult rats and males more than females, respectively. Rat pretreated with L-carnitine (and its derivatives) or phosphocreatine had significantly decreased lipid peroxidation damage in the brain (Rauchová et al. *Physiol Res* **61** (Suppl 1): S89, 2012) (grant GACR 304/12/0259).

Institute of Experimental Medicine, CAS, Prague (Dr. J. Šedý)

Neurogenic pulmonary edema (NPE) is a life-threatening complication of central nervous system (CNS) injuries such as brain trauma or spinal cord compression (current review of our results in Šedý et al. *J Neurotrauma* 2015 in press). A new model of NPE based upon spinal cord compression

under light isoflurane anesthesia has been developed in IEM CAS (Šedý et al. *Neurosci Lett* **423**: 167, 2007). Our research indicated that NPE develops due to the activation of specific CNS trigger zones located in the brainstem, leading to rapid sympathetic discharge, systemic blood pressure rise, consequent baroreflex-induced bradycardia, enhanced venous return, and pulmonary vascular congestion characterized by interstitial edema, intraalveolar accumulation of transudate and intraalveolar hemorrhage (Šedý et al. *J Neurotrauma* **24**: 1487, 2007). The degree and the type of anesthesia are crucial determinants for the extent of NPE development in experimental models due to their influence on the sympathetic nervous system activity. The deleterious effects of sympathetic hyperactivity are attenuated by endogenous nitric oxide because its absence is associated with the development of a more pronounced form of NPE (Šedý et al. *Am J Physiol* **297**: R1111, 2009). This sympathetic hyperactivity is caused by the major activation of ascending spinal pathways by spinal cord injury and/or by the activation of NPE trigger zones by increased intracranial pressure. The attenuation of sympathetic nerve activity or the abolishment of reflex bradycardia completely prevented NPE development (Šedý et al. *J Appl Physiol* **112**: 1, 2012). All the above mentioned results on hemodynamics, vasoactive system participation, blood volume redistribution etc. were obtained in our lab (grant 1M0510 ME CR).

INTERNATIONAL COLLABORATION

Endothelial dysfunction in experimental hypertension

Our long-term cooperation with the *Institute of Normal and Pathological Physiology (Slovak Academy of Sciences, Bratislava, Slovakia*) and the *Institute of Pathophysiology, Faculty of Medicine, Comenius University in Bratislava* has started in 2002. Endothelial dysfunction in NO-deficient hypertension is mediated not only by a reduced NO bioavailability but also by enhanced formation of endothelium-derived constricting factor (EDCF) and increased oxidative load (Paulis et al. *Hypertens Res* **31**: 793, 2008; Paulis et al. *J Hypertens* **28** (Suppl 1): S19, 2010; Paulis et al. *J Pineal Res* **48**: 102, 2010) (grant 1M0510 ME CR).

Lipid metabolism disturbances in human population

In the HDL-Atherosclerosis Treatment Study (*University of British Columbia, Vancouver, Canada* and *University of Washington, Seattle, USA*) we got the opportunity to examine the association between fractional rate of esterification of cholesterol (FER_{HDL}), atherogenic index of plasma (AIP), concentrations and size of lipoproteins and changes in coronary artery stenosis in participants who were treated with a combination of simvastatin, niacin and antioxidants. The changes in the FER_{HDL} and AIP corresponded with the findings on coronary angiography, reflected the actual composition of the lipoprotein spectrum and thus predicted both the cardiovascular risk and the effectiveness of therapy. Now AIP is generally available for the use in clinical practice as it can be readily calculated from the routine lipid profile (Dobiášová et al. *J Lipid Res* **52**: 566, 2011). Recent studies have shown the association between FER_{HDL} and *CILP2* polymorphism (Rašlová et al. *Physiol Res* **60**: 785, 2011) and the comparison of particular markers (AIP, ratio of apoB/apoA and HOMA-IR) in obese/overweight children (Vrablík et al. *Physiol Res* **63**: 743, 2014).

Key methodology and core facilities

Our laboratory is excellently equipped for the research of high blood pressure starting from the level of molecular biology (PCR amplification, quantitative real-time RT-PCR , Western blotting) over the measurements of vascular wall properties (using pressure myograph and four-channel wire myograph) up to whole organism experiments in conscious instrumented rats. Blood pressure measurement techniques cover both invasive BP measurements in carotid artery of cannulated rats as well as non-invasive tail plethysmography method in restrained conscious animals, up to telemetric BP monitoring, in which systolic, diastolic, mean BP as well as heart rate are measured over longer periods of time in conscious freely moving animals. This highly sophisticated method for telemetric blood pressure measurement is now complemented with the measurement of renal sympathetic nerve activity. The activity of major vasoactive systems is analysed using consecutive blockade of vasopressor (renin-angiotensin system, sympathetic nervous system) and vasodilator (nitric oxide, BK_{Ca} channels, prostanoids) systems contributing to blood pressure maintenance.

New cardiometabolic risk markers (FER_{HDL} and AIP) were developed and successfully tested within the frame of a long-term study of the interaction of lipids, especially cholesterol and phospholipids, with cell membranes and plasma lipoproteins. A special attention was paid to the role of plasma enzyme lecithin cholesterol acyltransferase (LCAT) in the pathogenetic mechanisms of atherosclerosis (Dobiášová *Advances in Lipid Research* 20: 107, 1983; WOS **97 citations**). A ^{3}H-cholesterol radioassay for measuring the fractional rate of esterification of cholesterol (FER_{HDL}) in HDL plasma free of lipoproteins containing apoprotein B was developed by Dobiášová and Frohlich (*Methods in Molecular Biology. Lipoprotein Protocols* 1998: 217-230). We have revealed that FER_{HDL} is significantly elevated in people at risk for atherosclerosis, e.g. males, hypertensives, diabetics type 2 and in those already suffering from cardiovascular disease (Dobiášová et al. *Arterioscler Thromb* 11: 64, 1991; Dobiášová et al. *J Lipid Res* 33: 1411, 1992; WOS ***137 citations*** together). FER_{HDL} depends only slightly on the concentration of plasma cholesterol but considerably correlates with a rise in triglyceride (TG) levels and a fall in HDL cholesterol (HDL-C). FER_{HDL} is controlled by the relative occurrence of large and small HDL particles in which the LCAT reaction occurs and is also highly correlated with atherogenic small LDL particles. We found that FER_{HDL} and therefore the presence of differently sized subpopulations of lipoproteins is directly proportional to the ratio of molar concentration of TG and HDL-C in plasma (Dobiášová and Frohlich *Clin Biochem* **34**: 583, 2001; Frohlich and Dobiášová *Clin Chem* **49**: 1873, 2003; Dobiášová *Clin Chem* **50**: 113, 2004; WOS ***330 citations*** together). We proposed that both FER_{HDL} and the logarithmically transformed ratio of TG/HDL-C (atherogenic index of plasma, AIP) may serve as markers of cardiometabolic risk. The validity of these potential biomarkers we tested in cooperation with four Czech and three international clinical research centers (Dobiášová et al. *J Lipid Res* **52**: 566, 2011).

Involvement in significant projects

Our laboratory together with several other Prague laboratories from the field of cardiovascular research (Department of Developmental Cardiology, Institute of Physiology, CAS; Institute for Clinical and Experimental Medicine; Second Faculty of Medicine, Charles University) participated in two

multidisciplinary projects devoted to the health-threatening human diseases – *Centre for Cardiovascular Research*, founded by the Czech Ministry of Education, Youth and Sports (2000-2004; 2005-2011). This project gathered more than 90 basic and clinical physiologist (including more than 30 PhD students) from Prague scientific institutes. The main topic was the study of mechanisms of cardiovascular diseases, with a special focus on the risk factors, such as hypertension and atherosclerosis. Particular attention was paid to the ontogenetic and sex-dependent factors.

SUMMARY

Our research team investigated pathophysiological mechanisms of experimental hypertension with a special focus on hemodynamic changes, the role of principal vasoactive systems, alterations of vascular contraction, cell calcium handling and defects of cell signaling in vascular smooth muscle. In addition, we also studied hypertensive end-organ damage, abnormal lipid metabolism, and enhanced formation of reactive oxygen species. As far as ontogenetic aspects of hypertension development are concerned, we demonstrated several major age-dependent differences in the mechanisms of salt hypertension. High potassium intake attenuated the development of salt hypertension only in immature Dahl rats, whereas chronic blockade of endothelin A receptors or chronic antioxidant treatment by tempol had beneficial effects only in adult salt hypertensive Dahl rats. Our research on the influence of RAS and ET systems on the organ damage demonstrated renoprotective and cardioprotective effect of aliskiren (direct renin inhibitor) in heterozygous Ren-2 transgenic rats (TGR), as well as the effect of atrasentan (endothelin receptor A antagonist) applied simultaneously with RAS blockade on proteinuria in adult TGR rats with chronic kidney disease. The attention was also paid to vasoconstrictor and vasodilator mechanisms of arterial contraction. Our findings indicated that the impairment of vasodilator mechanisms is mainly due to decreased formation and/or action of nitric oxide than due to alterations of other vasodilator systems. Enhanced sympathetic vasoconstriction in spontaneous hypertension is exerted under the conditions of enhanced calcium entry and reduced calcium sensitization. In the future we shall pay more attention to i) CNS mechanisms involved in BP control, ii) interactions between major vasoactive systems, and iii) molecular mechanisms responsible for arterial contraction in the development of experimental hypertension.

RESEARCH IMPACT

As far as the **research impact of our work** is concerned, we have published 45 papers in the last 5 years, 26 of them being corresponded from our department. There are four papers describing our original findings which perhaps contributed to the progress in the field of cardiovascular research.

Behuliak et al. (*J Hypertens* **31**: 2025, 2013) described a new approach to the determination of basal calcium sensitization in conscious rats in which BP response to acute L-VDCC opening elicited by BAY K8644 is recorded at two different levels of calcium sensitization, i.e. in the presence of existing Rho kinase activity and after its inhibition by fasudil pretreatment. This original method demonstrated that basal calcium sensitization (measured after the acute blockade of RAS and SNS) is attenuated in adult SHR with established hypertension. Using this approach we have further revealed a fundamental difference between genetic hypertension (SHR, TGR) and salt-induced hypertension (Dahl rats) because

basal calcium sensitization is reduced in genetic hypertension but enhanced in salt hypertension. These findings open new research possibilities for the analysis of vascular contractile mechanisms in various forms of hypertension.

Zicha et al. (*Acta Physiol Oxf* **202**: 29, 2011) analyzed the antihypertensive action of high K^+ intake in salt hypertensive Dahl rats. It was demonstrated that high K^+ intake had greater preventive than therapeutic effects and that these preventive effects were observed only in immature animals but not the adult ones. Since the antihypertensive effects of high K^+ intake in young Dahl rats were based upon the reduction of sympathetic vasoconstriction, the hypothesis on the influence of monovalent cation imbalance on central mechanisms governing sympathetic outflow should be further tested.

Rakušan et al. (*Am J Physiol* **299**: F758, 2010), who compared the effects of direct renin inhibitor aliskiren and angiotensin receptor blocker losartan in young and adult TGR rats, demonstrated similar antihypertensive effects of both drugs but a more pronounced antiproteinuric effects of aliskiren. This effect persisted for almost two weeks after treatment withdrawal. The same was true for blood pressure lowering effects and cardioprotective effects of aliskiren. It should be noted that the antiproteinuric effects of aliskiren went beyond its blood pressure lowering effects. According to our data, the antiproteinuric effect was mediated by the normalization of glomerular morphology. This might be of principal importance for patients suffering chronic kidney disease.

Dobiášová et al. (*J Lipid Res* **52**: 566, 2011) examined the association between FER_{HDL} , AIP, concentrations and size of lipoproteins in relation to the changes in human coronary artery stenosis. The changes in the FER_{HDL} and AIP corresponded to the findings on coronary angiography, reflect the actual composition of the lipoprotein spectrum and thus predict both the cardiovascular risk and the effectiveness of therapy. AIP is available for the use in clinical practice as it can be readily calculated from the routine lipid profile.

We hope that our research on the pathophysiological mechanisms of experimental hypertension brought some new findings which might be helpful for better understanding of the pathophysiology of human hypertension. The findings on human lipid metabolism were directly applicable to human cardiovascular diseases.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Bioenergetics

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

Main focus of the Department of Bioenergetics lies in the research into the physiology of mitochondria, cell organelles responsible for most of the energy production at the molecular level. As mitochondria accommodate number of key metabolic pathways, it is not surprising that their dysfunction has been recognised as an important determinant of a variety of human pathologies, ranging from OXPHOS disorders to common complex diseases (diabetes, cancer). Many of these aspects are also studied at our department. In particular, our research is focused on:

- Assembly of mitochondrial protein complexes and supercomplexes.
- Human diseases caused by mutations in assembly factors of these enzyme complexes.
- Mitochondrial reactive oxygen species production
- Role of mitochondria in the pathophysiological presentation of common diseases

To achieve these goals, we utilise animal models, cells derived from patients harbouring various mitochondrial disorders as well as knockdown and knock-out cellular models derived in our laboratory.

Department of Bioenergetics has a long history at the Institute of Physiology. During the period covered by this report, it had undergone change in leadership, as the long standing head of the department, Josef Houstek, MD, DSc. retired from the position of group leader in 2013. Tomas Mracek, PhD was appointed as the new head from the beginning of 2014. Inevitably, this will cause some changes in the departmental focus in the longer run. But given the fact that Dr Mracek and his scientific topics were already well established within the group, it changes little in terms of the main research areas outlined above.

ii. PERSONNEL

At present, six **senior scientists** represent the core of the department. Most of them were originally educated within the department but gained significant further expertise during their international postdoc appointments. Given their varying scientific backgrounds, they form a team able to cope with the multidisciplinary approaches required in today's science.

RNDr. Tomáš Mráček, PhD. (group leader), age 38, H-index 14– cell physiology, ROS production, biogenesis of OXPHOS complexes
MUDr. Josef Houštěk, DrSc., age 67, H-index 32 – biochemistry, ATP synthase, mitochondrial diseases
RNDr. Zdeněk Drahota, DrSc., age 82, H-index 29 – enzymology, oxygraphy, biochemistry of mitochondrial glycerol-3-phosphate dehydrogenase
Mgr. Petr Pecina, PhD., age 35, H-index 18 – oxygraphy, assembly factors for OXPHOS complexes, biology of cytochrome c oxidase

RNDr. Alena Pecinová, PhD., age 37, H-index 18 – biophysical methods, measurement of mitochondrial membrane potential, energetics of lymphoid cells
RNDr. Marek Vrbacký, PhD., age 41, H-index 11 – chemistry, protein mass spectrometry, ATP synthase

Junior scientist:
Mgr. Vilma Kaplanová, PhD., age 43, H-index 7 – molecular biology, transgenic models, mtDNA genetics

Postdoctoral fellows:
Mgr. Vendula Karbanová, PhD., age 31, H-index 5 – molecular biology, ATP synthase
Mgr. Alena Vrbacká, PhD., age 33, H-index 5 – mitochondrial diseases, ATP synthase

PhD/MSc/BSc **students** (4/0/2), laboratory **technicians** (2)

Several other students defended their theses during the period of this report and continue with their professional career outside of the department.

2) KEY RESULTS

Disorders of oxidative phosphorylation apparatus

Inborn disorders of the energetic function of mitochondrial respiratory chain result in severe metabolic diseases in paediatric population. They are caused by genetic defects of mitochondrial biogenesis, due to mutations in nuclear or mitochondrial (mtDNA) genes. Isolated defects of the key enzymes of the mitochondrial energy metabolism, ATP synthase and cytochrome c oxidase (COX) are among the most severe metabolic disorders manifesting early after birth. Our group focuses on the elucidation of molecular mechanisms underlying such defects. Such studies are made possible by the collaboration by several laboratories spanning from clinical research (group of Prof Zeman, General University Hospital in Prague) through molecular genetics (group of Ing Kmoch, Institute of Inherited Metabolic Diseases, 1st Faculty of Medicine, Prague) to biochemistry (our group) and subsequent development of animal models of the disease (group of Ing. Pravenec, our institute; group of Prof Sedláček, Institute of Molecular Genetics, CAS). This was further strengthened by receiving the support in the form of Centre of Excellence for study of mitochondrial biology (MITOCENTRE – see below). In the period 2010-2014, main topics involved the role of TMEM70 protein in ATP synthase biogenesis, mutations in the subunit ATP5E of ATP synthase and biogenesis of COX as further outlined below.

Role of TMEM70 in ATP synthase biogenesis
Research into biogenesis of ATP synthase has in the department particularly long tradition, going back into the 1990's and also very well documents the fruitful collaboration between the three groups mentioned above. In 1999 we described the first patient with an ATP synthase deficiency of nuclear origin (Houstek et al., *Hum Mol Genet* **8**: 1967, 1999) and in 2008 finally characterised the disease causing gene – TMEM70 (Cizkova et al., *Nat Genet* **40**: 1288, 2008). Most of the period 2010-2014 was then devoted to functional characterisation of TMEM70 as a novel ancillary factor of mammalian ATP synthase. We confirmed its localisation in inner mitochondrial membrane and processing into a 21kDa mature form and demonstrated, that mature protein has a hairpin structure with the N- and C- termini oriented towards the mitochondrial matrix (Figure 1). Because TMEM70 does not directly interact with mature ATP synthase, it behaves as a bona fide assembly factor. We found no TMEM70 protein in cells and

isolated mitochondria from patients with ATP synthase deficiency due to TMEM70 c.317-2A>G mutation, confirming that the mutation prevents TMEM70 biosynthesis (Hejzlarova et al., *Physiol Res* **63 Suppl 1**: S57, 2014; Hejzlarova et al., *Biochim Biophys Acta* **1807**: 144, 2011; Kratochvilova et al., *Mitochondrion* **15**: 1, 2014).

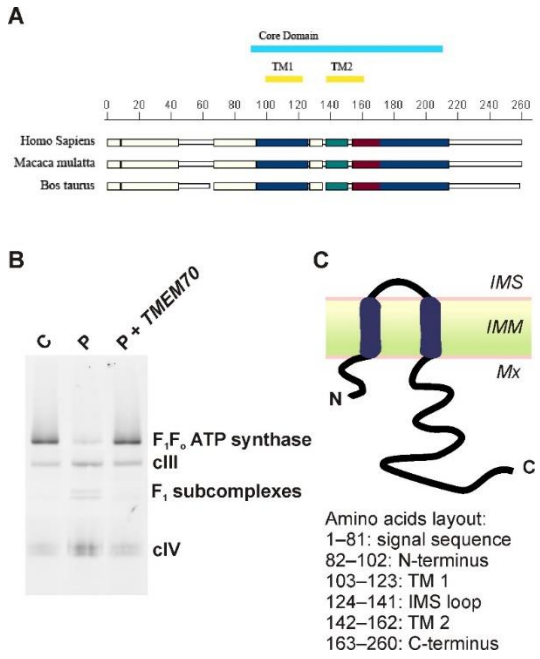


Figure 1: *Structure of TMEM70 protein A - Sequence alignment denoting putative trans-membrane regions. B - Decrease of assembled ATP synthase in patient's cells C - Structure and orientation of mature TMEM70 protein in the inner mitochondrial membrane.*

Apart from biochemical features of TMEM70, we also focused on physiological adaptations caused by TMEM70 mutations in patients' fibroblasts. In this respect, we demonstrated, that cells respond to the metabolic disbalance caused by insufficient mitochondrial ATP production by compensatory increase of respiratory chain complexes III and IV occurring at posttranscriptional level (Havlickova Karbanova et al., *Biochim Biophys Acta* **1817**: 1037, 2012). Finally, we also took part in retrospective multi-site survey of patients with mutation in the TMEM70 gene. The team comprised six European laboratories dealing with patients with ATP synthase deficiency. Resulting paper summarises clinical features of patients and gives recommendations, when diagnostics of TMEM70 mutations should be considered (Honzik et al., *Arch Dis Child* **95**: 296, 2010).

Supported by Grant Agency of the Czech Republic (GA CR) (GAP303/11/0970, GAP303/12/1363, GB14-36804G), Grant Agency of the Ministry of Health of the Czech Republic (GA MH) (NT14050, NT12370, NS9759) and Ministry of Education, Youth and Sports of the Czech Republic (MEYS) (LL1204, 1M0520).

Subunit ATP5E and ATP synthase biogenesis

One of patients with ATP synthase deficiency screened in our previous study (Cizkova et al., *Nat Genet* **40**: 1288, 2008) had a distinct clinical phenotype and was negative for mutation in TMEM70 protein. In a joint project with Paediatric department of Prof Sperl (Paracelsus Medical University in Salzburg) who diagnosed this patient, subsequent sequencing uncovered a unique missense mutation in ATP5E gene, encoding structural subunit ε of ATP synthase and this new type of mitochondrial disorder was characterised in detail. ATP5E is the smallest subunit of the catalytic part of the enzyme and the only subunit without a homolog in bacterial or chloroplast enzyme. Its mutation leads to a replacement of a highly conserved tyrosine 12 with cysteine. While this does not affect a biochemical function of the ATP synthase, it leads to a strong inhibition of enzyme's biosynthesis. This is accompanied by a unique accumulation

of subunit c, the main component of ATP synthase's proton channel. RNA interference of ATP5E in HEK293 cell line recapitulated the same phenotype – ATP synthase deficiency and accumulation of subunit c. Results on patient cells and our knock-down experiments point to a novel, regulatory role in ATP synthase biogenesis of the epsilon subunit. ATP5E is thus yet another gene responsible for the isolated ATP synthase deficiency, in addition to TMEM70 (see above) and ATP12 genes. Ultimately, this represents the first described mutation in a nuclear-encoded ATP synthase subunit, followed just recently by mutation subunit α described by others (Havlickova et al., *Biochim Biophys Acta* **1797**: 1124, 2010; Mayr et al., *Hum Mol Genet* **19**: 3430, 2010).
Support GA MH (NS9759) and MEYS (1M0520).

Cytochrome c oxidase biogenesis
Here we predominantly focussed on the role of SURF1 protein in COX biogenesis. We have demonstrated that the mitochondrial biogenetic machinery is capable of responding to energetic defects of COX by a compensatory up-regulation of several other respiratory chain complexes. The mechanism of adaptation mechanism is elicited at a post-transcriptional level and points to a selective regulation in the synthesis of individual mtDNA-encoded proteins. We have also shown, that while absence of SURF1 affects biogenesis of COX holoenzyme, all fully assembled COX complexes are then present as components of supercomplexes (Kovarova et al., *Biochim Biophys Acta* **1822**: 1114, 2012). We have also participated in the study predominantly performed by Prof Zeman's group, looking into the role of intramitochondrial ATP-dependent proteases. Here it was shown that the YME1L protease participates in a quality control of newly formed subunits of respiratory chain complexes I and IV (Stiburek et al., *Mol Biol Cell* **23**: 1010, 2012).

Non-invasive diagnostics of mitochondrial OXPHOS disorders
As today biochemical diagnostics of mitochondrial diseases mostly depend on muscle biopsy or establishing of fibroblast cell culture, we attempted to develop alternative approach based on the use of isolated lymphocytes. In collaboration with clinicians from 1st Medical faculty Charles University and Thomayer hospital in Prague we established and tested complex methodology for detection and evaluation of respiratory chain disorders using high resolution analysis of mitochondrial respiration in lymphocytes from peripheral blood. This represent a non-invasive, reliable and fast approach for diagnostics and screening of OXPHOS disorders in paediatric patients which also allows for repeated sampling and can thus be utilised in future longitudinal studies. We have also verified that it is suitable for diagnosis of SURF1 and TMEM70 patients as well as patients with defects of complex I (Pecina et al., *BBA Clinical* **2**: 62, 2014).
Support: GA CR (GAP303/11/0970, GB14-36804G), GA MH(NT12370, NS9759) and MEYS (1M0520).

Flavin dehydrogenases of the mitochondrial respiratory chain

Reactive oxygen species production by mitochondrial flavin dehydrogenases
At the Department of Bioenergetics, we focus on several aspects of reactive oxygen species (ROS) biology. One of the long term interests lies in the ROS production by mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), one of the flavin dehydrogenases in the mitochondrial respiratory chain. Indeed, we originally described this enzyme as a source of ROS back in 2002 (Drahota et al., *J Bioenerg Biomembr* **34**: 105, 2002) and since then we try to pinpoint molecular mechanisms behind this leak. When studied in intact mitochondria, it is difficult to assess the net ROS production by individual complexes, as electrons may flow

between them and the actual site of electron leak may lie either upstream or downstream due to the electron transfer between complexes through Coenzyme Q pool (see Figure 2).

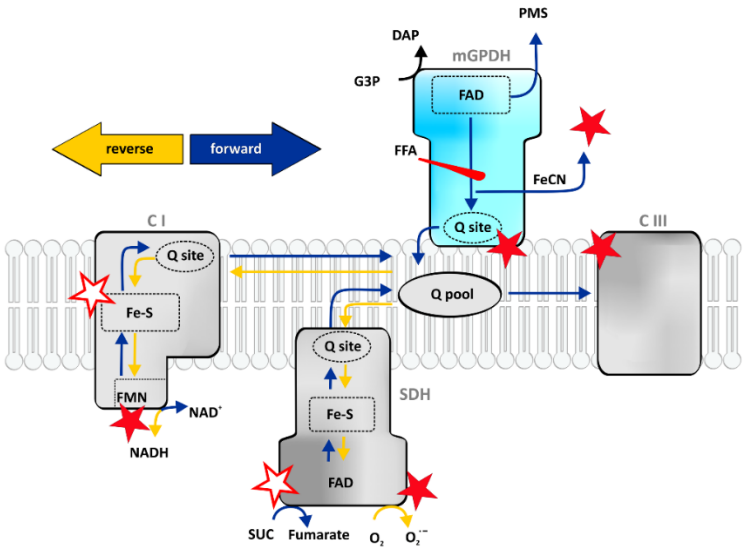


Figure 2: *Possible sites of glycerol-3-phosphate-dependent ROS generation (marked with red stars) and electron flow in the respiratory chain.*

Therefore, to define the propensity to electron leak and the actual site of it for two prominent flavin dehydrogenases of the respiratory chain, mGPDH and succinate dehydrogenase (SDH) we utilised the model of enzymes solubilised by mild non-ionic detergents to study the individual complexes in isolation. By this approach, we revealed flavin as the most likely source of electron leak in SDH under in vivo conditions, while we proposed coenzyme Q as the site of ROS production in the case of mGPDH. We also managed to directly demonstrate that isolated mGPDH itself is capable of ROS production (Mracek et al., *Biochim Biophys Acta* **1827**: 401, 2013; Mracek et al., *Biochim Biophys Acta* **1837**: 98, 2014; Rauchova et al., *Physiol Res* **61**: 259, 2012).
Support: GA CR (GPP303/10/P227) and MEYS (LL1204).

Native forms of flavin dehydrogenases
By using mild detergents, we also focussed on native organisation of flavin dehydrogenases in the membrane. In this respect, we demonstrated that mGPDH associates into homooligomers as well as high molecular weight supercomplexes, which represent native forms of mGPDH in the membrane. However, none of the supramolecular forms of mGPDH associated with other OXPHOS complexes and thus does not play role in facilitating electron transfer to oxygen (Mracek et al., *Biochim Biophys Acta* **1837**: 98, 2014). In other study we also examined the native organisation of SDH, whose presence in respiratory supercomplexes is mired by controversy. Using very mild separation conditions, we demonstrated the existence of high molecular weight forms (≈600 kDa) of SDH that represented catalytically active functional form of the enzyme. While these complexes clearly depend on the functional oxidative phosphorylation, similar to the situation with mGPDH, they do not represent association with C III or IV of the respiratory chain and thus do not function as respiratory supercomplexes. To the contrary, at least part of SDH SC proved to represent association with ATP synthase and presumably forms part of the mitochondrial ATP-sensitive K+ channel (Kovarova et al., *PLoS One* **8**: e71869, 2013).
Support GA CR (GPP303/10/P227), GA MH (NT12370) and MEYS (LL1204).

Inhibitors of flavin dehydrogenases as anti-tumour agents

We joined the team of Prof Neuzil (Institute of Biotechnology CAS) in a collaborative effort to characterise the properties of anti-tumour agent, a mitochondrially targeted analogue of α-tocopheryl succinate (TOS) - MitoVES. Both TOS and MitoVES were described by the group of Dr Neuzil as inhibitors of SDH and inducers of ROS production. In the common study we demonstrated, that one of the properties of MitoVES is its antiangiogenic action exerted through ROS induced apoptosis in proliferating cells (Rohlena et al., *Antioxid Redox Signal* **15**: 2923, 2011). Further elaborating on this topic, we also decided to study TOS properties on mGPDH. Rather surprisingly, we found that TOS acts as an even better inhibitor of mGPDH than is the case for its established target – SDH (IC_{50} 10 μM vs. 80 μM). As many tumours show high activity of mGPDH, its inhibition could also play a role in TOS-induced growth suppression in neoplastic cells (Rauchova et al., *Int J Biochem Cell Biol* **53**: 409, 2014).

Support: MEYS (1M0520).

(Patho)physiological aspects of reactive oxygen species production

ROS modulation by membrane potential

One important paradigm of mitochondrial ROS production modulation states that high levels of mitochondrial membrane potential ($\Delta\Psi_m$) increase ROS generation in the respiratory chain. In a common project with laboratory of Prof Cannon (The Wenner-Gren Institute, Stockholm, Sweden) we addressed this hypothesis on a model of brown adipose tissue (BAT) mitochondria, where $\Delta\Psi_m$ is dissipated by the action of uncoupling protein 1 (UCP1). We compared the ROS production in BAT mitochondria isolated from wild-type mice or from UCP1−/− mice (with a high membrane potential) and found only ROS production supported by exogenously added succinate was affected by the presence of active UCP1. ROS production supported by any other tested substrate (including endogenously generated succinate) was not affected by $\Delta\Psi_m$. This indicates that UCP1 is not involved in control of ROS production in BAT mitochondria, possibly indicating that also under other situations membrane depolarization would not decrease physiological relevant ROS production (Shabalina et al., *Biochim Biophys Acta* **1837**: 2017, 2014).

Support: GA CR (GB14-36804G) and MEYS (LL1204).

Epileptogenesis and mitochondrial ROS production

Department of Developmental epileptology from the Institute of Physiology (group of Dr Kubova) has a long tradition of research into the mechanisms of epileptogenesis. Together we focused on the potential involvement of mitochondrial ROS production in this process. On a model of seizures induced by DL-homocysteic acid and we found marked 60% decrease in mitochondrial complex I activity, which persisted for up to 5 weeks and was neither associated with changes in the size of the assembled complex I nor its content. For the whole period we observed significant increase in markers of oxidative damage (3-nitrotyrosine, 4-hydroxynonenal, protein carbonyls) as well as by the increase in antioxidant defence enzymes (Cu/ZnSOD, MnSOD and glutathione peroxidase). The decrease of complex I activity was substantially alleviated by the pre-treatment with free radical scavengers. Thus, we could conclude that the persisting inhibition of complex I leads to the enhanced production of ROS and/or nitrogen species and contributes to neuronal injury and epileptogenesis (Folbergrova et al., *Neurochem Int* **56**: 394, 2010; Folbergrova et al., *Int J Dev Neurosci* **31**: 123, 2013).

Support: MEYS (LL1204, 1M0520).

Mitochondrial component in the pathogenesis of metabolic syndrome

Mitochondria apparently play a significant role in pathogenesis of polygenic diseases like type 2 diabetes or cardiovascular diseases. However, whether their dysfunction is cause or

consequence of the disease remains to be established. We addressed this problem in several projects, mainly in collaboration with the group of Dr Pravenec.

Mito-nuclear epistasis in development of metabolic phenotypes

Interaction of the mitochondrial and nuclear genomes is critically involved in coordinating metabolic energy production. The importance of mito-nuclear crosstalk in influencing disease-related phenotypes stems from the central roles of the mitochondrial and nuclear genomes in regulating energy metabolism, redox balance, and other determinants of cellular function. Conplastic strains which share nuclear background and differ in mitochondrial genome represent valuable tool for dissecting contribution of mtDNA haplogroups into the whole body physiology. In laboratory rats, four major mtDNA haplogroups can be defined - BN, F344, LEW, and SHR and group of Ing Pravenec (Institute of Physiology) has established a unique model of conplastic strains carrying all those 4 haplotypes on SHR nuclear background. Foundations for their analysis were laid by comparison of BN and SHR animals (Pravenec et al., *Genome Res* **17**: 1319, 2007) and during 2010-2014 period, we focused on the two remaining strains.

SHR-LEW animals (with amino acid substitutions in subunits ND1, ND2 and ND4 of complex I) had reduced oxidative and nonoxidative glucose metabolism in skeletal muscle and resistance to insulin stimulated incorporation of glucose into adipose tissue lipids (Houstek et al., *Physiol Genomics* **44**: 487, 2012). Speaking about the SHR-mtF344 conplastics analysis of OXPHOS in heart left ventricles (LV), muscle and liver revealed reduced activity and content of several respiratory chain complexes which was associated with significantly increased relative ventricular mass. Those animals also exhibited reduced insulin sensitivity in skeletal muscle (Houstek et al., *Physiol Genomics* **46**: 671, 2014).

Summary of phenotypic effects associated with transfer of major mtDNA haplotypes on SHR genetic background

Traits	SHR vs. Conplastic Strains		
	SHR-mt^{BN}	SHR-mt^{LEW}	SHR-mt^{F344}
Body weight	↓	↑	↔
Serum triglycerides	↔	↔	↓
Glucose during OGTT	↑	↔	↔
Insulin during OGTT	↑	n.d.	↑
Muscle glycogenesis	↓	↓	↓
Left ventricular mass	n.d.	n.d.	↑
Fractional shortening	n.d.	n.d.	↓
RCH complexes CI, CII, CIII, CIV, CV activity and/or content	CIV ↓	CI ↓, CII ↓, CIII ↓, CIV ↓, CV ↓	CI ↓, CII ↓, CIII ↓, CIV ↓
Tissue specificity of RCH complexes decrease	liver	muscle>>heart>liver	heart>>muscle>liver

↑, ↓, and ↔ symbols denote significantly increased, decreased, and not significantly different, respectively, values in conplastic strains when compared with the SHR. n.d., Not determined; RCH, respiratory chain; CI–CV, complexes I–V.

Table 1: *Differences in metabolic parameters between SHR-mt^{BN}, SHR-mt^{LEW} and SHR-mt^{F344}*

Key differences between conplastic strains are summarised in Table 1. Together, they provide evidence that inherited alterations in mitochondrial genome, in the absence of variation in the nuclear genome and other confounding factors, can influence various physiological parameters associated with metabolic syndrome.

For evaluation of mitochondrial parameters in conplastic animals we also had to adapt our methodology. Use of tissue homogenates proved to be superior to mitochondrial isolation in terms of material requirements and speed, which we reported in our methodological publication in Mitochondrion (Pecinova et al., *Mitochondrion* **11**: 722, 2011).

Support: GA CR (GAP303/11/0970, GB14-36804G), GA MH (NS9759) and MEYS (LL1204, 1M0520, OC08017).

Mitochondria and cardiac function

We contributed the expertise in measurement of mitochondrial metabolism in two studies looking for cardiometabolic phenotypes. In the first, groups of Ing Pravenec and Prof Kolář examined the effect of CD36 fatty acid translocase dysfunction in heart. Study was performed on SHR rats which harbour a deletion variant of CD36 gene and SHR-CD36 transgenes. CD36 was found to play an important role in modulating the incidence and severity of ischemic and reperfusion ventricular arrhythmias and myocardial infarct size induced by coronary artery occlusion (Neckar et al., *Physiol Genomics* **44**: 173, 2012). During our work on CD36 model we also pinpointed potential problem with using an established CD36 inhibitor sulfo-N-succinimidyl oleate (SSO) as we found that SSO is also a potent and irreversible inhibitor of mitochondrial respiratory chain complex III (Drahota et al., *Biochem Biophys Res Commun* **391**: 1348, 2010). In the other study, conducted with the Institute of Clinical and Experimental Medicine (Dr Melenovský) we looked for the potential utility of metformin in improvement of cardiac function. On rat volume-overload model, metformin induced enhancement of myocardial fatty acid oxidation but had a neutral effect on cardiac function, mitochondrial metabolism and survival, meaning that previously reported cardioprotective effects of metformin may require AMPK activation or ATP depletion. (Benes et al., *Clin Sci (Lond)* **121**: 29, 2011).

Support: MEYS (1M0520, OC08017).

3) INTERNAL COLLABORATIONS

Most prominent collaboration exists with groups of Dr Pravenec (**Dep. of Genetics of Model Diseases**) and Dr Kopecký (**Dep. of Adipose Tissue Biology**) which is also formalised within the MITOCENTRE consortium (see below). Common projects were pinpointed in the Key Results section.

Dep. of Developmental Cardiology (Prof Kolář, Dr Neckář) – studies on cardiac mitochondria during development, their role in cardioprotection, sexual dimorphism. (Drahota et al., *J Bioenerg Biomembr* **44**: 309, 2012; Drahota et al., *Physiol Res* **61 Suppl 1**: S165, 2012; Drahota et al., *Physiol Res* **63**: 1, 2014; Neckar et al., *Physiol Genomics* **44**: 173, 2012)

Dep. of Cardiovascular Morphogenesis (Prof Sedmera) – newly established collaboration on characterisation of TMEM70 -/- embryos.

Dep. of Experimental Hypertension (Dr Rauchová) - effects of tocopheryl succinate on mitochondria. (Rauchova et al., *Physiol Res* **61**: 259, 2012; Rauchova et al., *Int J Biochem Cell Biol* **53**: 409, 2014)

Dep. of Developmental Epileptology (Dr Folbergrová) – role of mitochondria in epileptogenesis. (Folbergrova et al., *Neurochem Int* **56**: 394, 2010; Folbergrova et al., *Int J Dev Neurosci* **31**: 123, 2013)

Dep. of Analysis of Physiologically Active Compounds (Dr Mikšík) – HPLC analyses of nucleotides, MS analyses of proteins.

4) DOMESTIC COLLABORATION

1st Faculty of Medicine, Charles University (Dr Kmoch) and **General University Hospital** in Prague (Prof Zeman) – long lasting collaboration in the area of inherited mitochondrial diseases, number of common grant projects both in the past and current. Both groups are also members of MITOCENTRE. (Cizkova et al., *Nat Genet* **40**: 1288, 2008; Havlickova Karbanova et al., *Biochim Biophys Acta* **1817**: 1037, 2012; Hejzlarova et al., *Biochim Biophys Acta* **1807**: 144, 2011; Honzik et al., *Arch Dis Child* **95**: 296, 2010; Houstek et al., *Hum Mol Genet* **8**: 1967, 1999; Kovarova et al., *Biochim Biophys Acta* **1822**: 1114, 2012; Kratochvilova et al., *Mitochondrion* **15**: 1, 2014; Pecina et al., *BBA Clinical* **2**: 62, 2014)

Thomayer Hospital, Prague (Assoc. Prof Houšťková, Dr Janota) – noninvasive diagnostics of mitochondrial diseases (Pecina et al., *BBA Clinical* **2**: 62, 2014)
Institute of Clinical and Experimental Medicine (IKEM), Prague (Dr Cahová, Dr Kazdová, Dr Melenovský) – metformin effects on mitochondria (Benes et al., *Clin Sci (Lond)* **121**: 29, 2011; Drahota et al., *Physiol Res* **63**: 1, 2014; Drahota et al., *Biochem Biophys Res Commun* **391**: 1348, 2010)
Institute of Physiology, Medical Faculty Hradec Králové (Prof Červinková) – mitochondrial function in hepatotoxicity. (Drahota et al., *J Bioenerg Biomembr* **44**: 309, 2012; Drahota et al., *Physiol Res* **61 Suppl 1**: S165, 2012; Drahota et al., *Physiol Res* **63**: 1, 2014; Garnol et al., *Physiol Res* **63**: 271, 2014)
Institute of Biotechnology, CAS (Prof Neužil) – biochemistry of succinate dehydrogenase (Kovarova et al., *PLoS One* **8**: e71869, 2013; Rohlena et al., *Antioxid Redox Signal* **15**: 2923, 2011)

5) INTERNATIONAL COLLABORATION

Faculty of Medicine, Goethe-University, Frankfurt, Germany (Dr Wittig) – mitochondrial proteomics, student exchange. (Kratochvilova et al., *Mitochondrion* **15**: 1, 2014)
Paracelsus Medical University, Salzburg, Austria (Prof Sperl) – defects of ATP synthase (Mayr et al., *Hum Mol Genet* **19**: 3430, 2010)
Department of Pediatrics, Ghent University Hospital, Ghent, Belgium (Dr Van Coster) – ATP synthase disorders (Honzik et al., *Arch Dis Child* **95**: 296, 2010)
Department of Metabolic Diseases, Endocrinology and Diabetology, Children's Memorial Health Institute, Warsaw, Poland (Prof Pronicka) – mitochondrial diseases (Kovarova et al., *Biochim Biophys Acta* **1822**: 1114, 2012).
The Wenner-Gren Institute, Stockholm, Sweden (Prof Cannon) – ROS production in brown adipose tissue. (Shabalina et al., *Biochim Biophys Acta* **1837**: 2017, 2014)

6) KEY METHODOLOGY AND CORE FACILITIES

Department is very well equipped for the functional analyses of cellular bioenergetics. We have at our disposal three Oroboros 2k oxygraphs, as well as Seahorse XFe-24 bioanalyzer for measurements of respiration in cells or mitochondria. For mitochondrial membrane potential ($\Delta\Psi_m$) measurements, we have two TPP^+ electrode setups, one directly coupled to O2k oxygraph, one independent. For $\Delta\Psi_m$ evaluation in intact cells, we use Partec PAS III flow cytometer, located and serviced by our department. For enzymology assays, we operate several spectrophotometers and ISS PC1 spectrofluorometer with photon counting detector. Recently, we started to transfer some of our measurements into the high-throughput format, for which we utilise monochromator based Tecan M200 plate-reader at the department or bunch of other plate-readers located at the institute.
At the departmental premises, we have well equipped cell culture rooms (class II), which allow for all necessary knockdown / knockout experiments on cell models. For animal breeding we team up with the department of Dr Pravenec.
In recent years we have invested heavily into human resources to be able to perform MS analysis of mitochondrial membrane proteins at the institutional proteomic facility (Dr Miksik). At present we can perform LFP quantification, SILAC quantification as well as AQUA quantification for stoichiometry evaluation.

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

Josef Houstek is the main coordinator of the GA CR Centre of Excellence project MITOCENTRE (GB14-36804G), which joins three groups from the Institute of Physiology (Dr Mracek, Dr Kopecký, Dr Pravenec), two from the 1st Faculty of Medicine, Charles University

in Prague (Dr Kmoch, Prof Martásek), and one from General University Hospital, Prague (Prof Zeman).

8) OTHER RELEVANT INFORMATION

In 2012 Josef Houštěk was awarded by the **Minister of Health for extraordinary results** achieved during the grant project NS9759 - Genetic causes of mitochondrial diseases due to deficiency of ATP synthase. In 2014, Petr Pecina received the **Otto Wichterle Award** by the Czech Academy of Sciences. This was based on his excellent scientific record in the area of OXPHOS complexes biochemistry.

9) SUMMARY AND RESEARCH IMPACT

Dysfunction of mitochondria, the powerhouses of mammalian cells is deleterious to all functions of human organism and can lead to a broad spectrum of diseases. Major challenges are to identify disease-causing genes and to elucidate pathogenic mechanisms in the many conditions with mitochondrial component. This research does not only have high clinical significance – identification of new genes also contributes to the discovery of new components of mitochondrial metabolism and helps to dissect the basic mechanisms behind mitochondrial biogenesis.

Studies performed during 2010-2014 at the Dept. of Bioenergetics mainly contributed to:

a) **Elucidation of nuclear-genetic defects of ATP synthase**. We uncovered and functionally characterised the first mutation in nuclear encoded structural subunit of the enzyme – ATP5E. Also, we have substantially improved our understanding of biological properties of TMEM70 protein, which represents new ancillary factor required for ATP synthase biogenesis

b) **Improved diagnostics of mitochondrial disorders**. By establishing protocol for evaluation of mitochondrial function in lymphocytes from peripheral blood, we provided clinicians easily accessible and non-invasive method for screening of paediatric patients with suspected mitochondrial disease.

c) **Establishing the effect of mtDNA background on metabolic phenotypes**. Our studies on conplastic rats clearly documented that naturally occurring non-pathogenic polymorphisms in mtDNA can influence parameters such as insulin sensitivity, glucose metabolism in muscle or left ventricle mass, commonly associated with metabolic syndrome.

d) **Mechanisms of mitochondrial ROS production**. We have demonstrated, that electron leak in mGPDH occurs at the level of coenzyme Q. While this in itself is a result of basic research, it can have further clinical significance, as documented e.g. by research on anticancer agents α tocopheryl succinate and MitoVES, which interact with Coenzyme Q site of flavin dehydrogenases.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Analysis of Biologically Important Compounds

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

Instrumentation progress in the area of separation methods
Methodological progress and development of new separation methods applicable to the estimation of physiologically important compounds (capillary electrophoresis and HPLC/MS).
Posttranslational changing of structural proteins (mainly connective tissue)
Developing of separation methods for study of proteins and peptides

ii. PERSONNEL (profesní struktura)

- Senior scientist(s)
  - Prof. Ing. Ivan Mikšík, DrSc. (team leader), analytical chemist, age 54, H-index 24
  - Mgr. Adam Eckhardt, Ph.D., expert in protein analysis, age 43, H-index 13
- Junior scientist(s):
  - Mgr. Kateřina Lacinová, Ph.D., expert in posttranslational modifications of proteins, age 37, H-index 8 (on maternity leave)
  - Ing. Jana Svobodová, Ph.D., expert in capillary electrophoresis, age 39, H-index 9
  - Mgr. Pavla Sedláková, Ph.D., expert in capillary electrophoresis, age 34, H-index 7
  - Mgr. Michal Jágr., Ph.D., expert in planar electrophoresis, age 37, H-index 2
- Research assistant:
  - Ing. Statis Pataridis
- PhD/MSc/BSc students (1/0/0), laboratory assistants (1)

2) KEY RESULTS

Instrumentation progress in the area of separation methods

Developing of new separation methods applicable to the estimation of physiologically important compounds (capillary electrophoresis and HPLC/MS).

The main topic in the area of development of new separation methods was made at capillary electromigration methods when these works were supported by Czech Science Foundation (GACR) (GA203/08/1428, GA203/09/0675;

GAP206/12/0453; GA13-17224S) and include cooperation with Institute of Organic Chemistry and Biochemistry, Czech Academy of Science (Dr. V. Kašička) as well as Institute of Chemical Technology, Department of Analytical Chemistry, Prague (Dr. D. Sýkora, Prof. V. Král).

In this research we developed new separation methods using affinity ligands as well as multidimensional approaches. Task of our group was used a newly developed ligands (prepared at Institute of Chemical Technology) for the analysis of real biological samples.

One of the topic of this research was developing and using gold nanoparticles for capilary electromigration methods (we wrote joint review article also (Sykora et al., *Journal of Separation Science* **33**: 372, 2010)). We used them for proteomic analysis, e.g. separation of complex peptide mixtures arising from tryptic digestion of native and glycated albumin and transferrin, and for investigation of glycation (nonenzymatic glycosylation) of these proteins (Miksik et al., *Journal of Separation Science* **35**: 994, 2012), as well as for separation of polyaromatic hydrocarbons (Rezanka et al., *Journal of Separation Science* **35**: 73, 2012; Rezanka et al., *Journal of Nanoparticle Research* **13**: 5947, 2011).

Affinity capillary approach was used also for the examination of enzyme inhibitory activity (Hlavacek et al., *Analytical Biochemistry* **467**: 4, 2014) – we (IM) made real analytical experiments as well as discussed about analytical procedures.

There was developed and studied a new possibility for coupling of capillary zone electrophoresis with mass spectrometry using non-volatile buffers. For example, this approach should be useful in area forensic analysis (Gottardo et al., *Electrophoresis* **33**: 599, 2012). There was also studied applicability of microemulsion electrokinetic chromatography for drug analysis (Liotta et al., *Forensic Science International* **220**: 279, 2012). All these forensic-applicable methods were made in cooperation with Institute of Forensic Medicine, University of Verona (Italy) (Prof. F. Tagliaro). I. Mikšík contributed on this research at Verona's laboratory as expert and visiting professor.

All developed separation methods were used for study of proteins and peptides as well as other physiologically important compounds. Many of them were applied at other cooperation/analysis for other Departments or Institutes (see below – parts 3, 4 and 5).

Posttranslational changing of proteins

We studied physiologically important posttranslational modification of proteins - glycation by modern analytical methods (nanoLC, CE and coupling with high-resolution MS). Besides well-known carboxymethyl lysine, new modifications were determined – creating mass shifts of 78 and 218. In addition, a mass shift of 132 belonging to a Schiff base was also identified

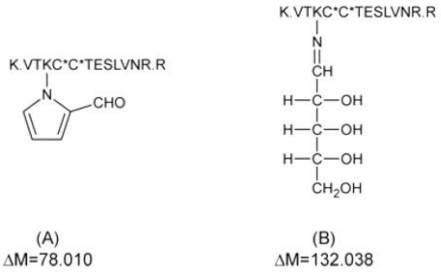


Figure 1. Schematic drawings of proposed structures of VTKC*C*TESLVNR peptide modified with (A) pyrraline-derived advanced glycation product (C_5H_2O), ΔM = 78.010 and (B) Schiff base ($C_5H_8O_4$), ΔM = 132.038. C* indicates carboxymethyl cysteine. Please note that the *N* in the cyclic structure originates from lysine (K) in the peptide structure.

Also reactive sites at structure of the albumin were determined. This research demonstrates the importance of modern analytical methods for understanding processes involving in modifications of proteins. (Lacinova et al., *Advances in Molecular Mechanisms and Pharmacology of Diabetic Complications* 2010; Pataridis et al., *Electrophoresis* **34**: 1757, 2013; Zmatlikova et al., *Journal of Chromatography A* **1217**: 8009, 2010). These researches were made on our department only.

In the aim to determine long-time modification of proteins we studied the natural mummy of prince Cangrande, Lord of Verona, Italy (1291–1329 AD). Two samples were taken: rib bone and muscle. These samples were cleaved with trypsin and analysed by liquid chromatographic methods coupled to mass spectrometry (Q-TOF, ion-trap). Special attention was devoted to nonenzymatic protein modification—the deamidation of asparagine and glutamine. A huge amount of collagen was determined in the tissues of the mummy (covering over 80 % of the sequence)—collagen type I was identified in the rib bone and collagen types I and III in the muscle. A high overall percentage of asparaginyl and glutaminyl residues were deamidated (up to 92 %). In agreement with the literature we can suppose that the deamidation of really old samples (at least 100-years-old) is mainly dependent on the burial conditions and/or thermal age and cannot serve as a precise “molecular clock”. On this research collaborated colleagues from Verona that supplied samples, in our department we made all analysis.

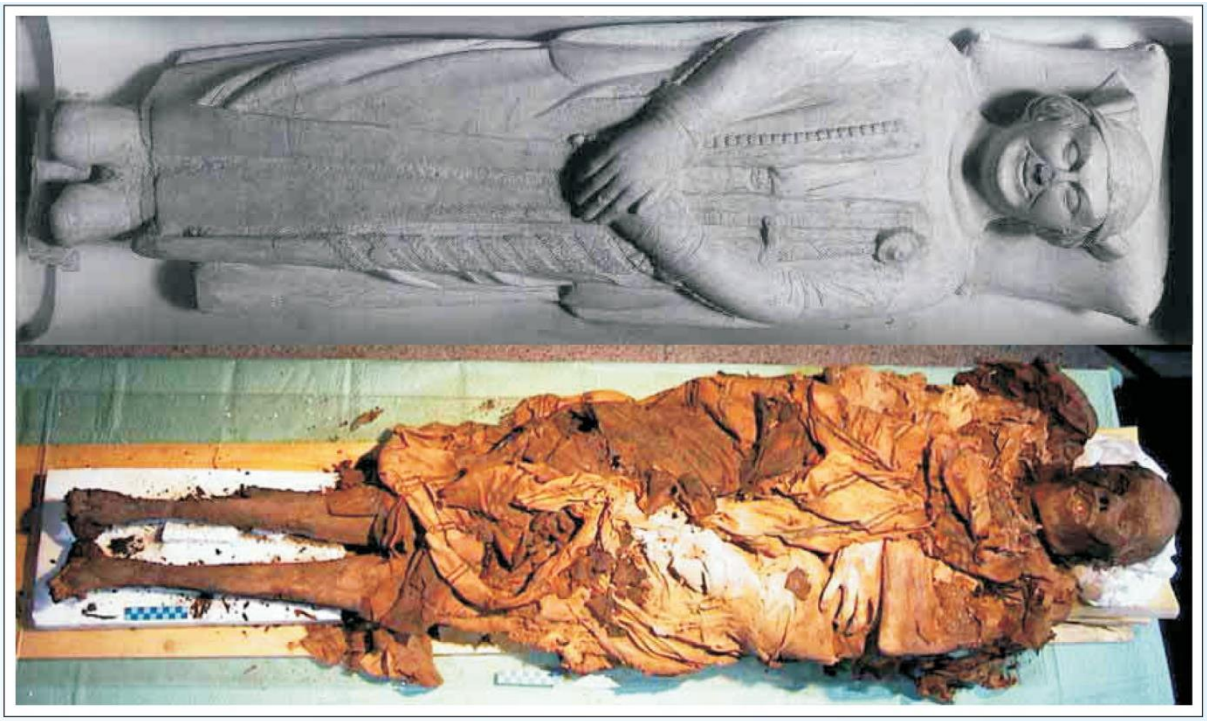


Figure 2. The sarcophagus and the mummy of Cangrande della Scala

(Miksik et al., *Chromatographia* **77**: 1503, 2014)

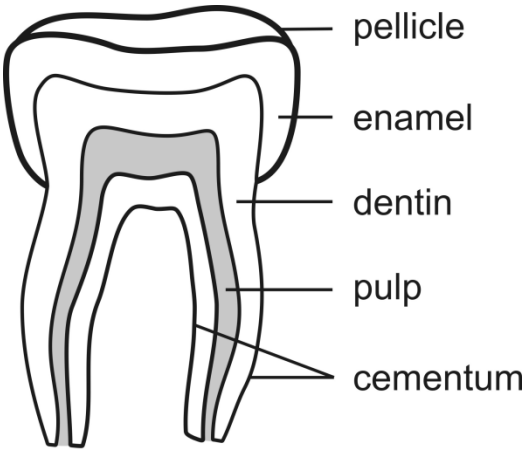
Protein analysis

We analysed a plethora of tissues in the view of proteomic analysis/determination of proteins (frequently in cooperation with other departments and Institutes – see parts 3, 4 and 5). Two main topics of our department were human teeth and eggshell proteins.

Teeth proteins

In this mission we have common project (grant) from the Ministry of Health (NT14324) with the General University Hospital in Prague and Institute of Clinical and Experimental Dental Medicine, First Faculty of Medicine, Charles University, Prague (Prof. Z. Broukal). Our task is a proteomic analysis as well as sample preparation of samples (teeth, saliva) obtained from dentists (i.e. cooperated institute.

Figure 3. Detailed structure of human tooth.



Teeth have been a focus of interest for many centuries – due to medical problems with them. They are the hardest part of the human body and are composed of three mineralized parts – enamel, dentin and cementum, together with the soft pulp. However, saliva also has a significant impact on tooth quality. Proteomic research of human teeth is now accelerating, and it includes all parts of the tooth. Some methodological problems still need to be overcome in this research field – mainly connected with calcified tissues.

In this context we used modern proteomic methods (2-D electrophoresis, nLC-MS) including sophisticated sample preparation method.

The detailed protemic analysis of human dental pulp tissue was made when 342 proteins were identified. The identified tooth pulp proteins have a variety of functions: structural, catalytic, transporter, protease activity, immune response, and many others. In a comparison with dentin and blood plasma, 140 (pulp/dentin) shared proteins were identified, 37 of which were not observed in plasma. In the connection with previously determined dentin proteome in our department we improved current understanding of the composition and functions of human teeth. In this case we demonstrate the medical application of analytical methods used in our department.(Eckhardt et al., *Journal of Endodontics* **40**: 1961, 2014; Jagr et al., *Physiological Research* **63**: 2014; Jagr et al., *European Journal of Oral Sciences* **120**: 259, 2012)

Eggshell proteins

The eggshell is a barrier that plays an important role in the defence of the egg against microbial and other infections; it protects the developing bird against unfavourable impacts of the environment and is essential for the reproduction of birds. The avian eggshell is a complex structure that is formed during movement along the oviduct by producing a multilayered mineral-organic composite. It is proposed that proteins present in the eggshell play an important role in the antimicrobial protection of egg.

The extractable proteins of avian eggshells have been studied extensively and many of them identified, however the insoluble (non-extractable) proteins have been sparsely studied.

We studied EDTA-insoluble proteins by gradual decalcification of eggshell. The insoluble proteinaceous films were chemically treated with cyanogen bromide and the arising mixtures of large fragments were gradually precipitated with salt. The separated fractions were digested with trypsin and analyzed by HPLC-MS/MS (ion trap mass spectrometer). The analysis of the entire matrix of eggshell (without precipitation steps) enabled to determine 6 proteins only (ovocalyxins 32 and 36, ovocleidin 17 and 116, clusterin and ovalbumin). The using of pre-treatment of individual eggshell layers and gradual precipitation with salt markedly increased the number of proteins identified – 28 proteins were determined. We identified for the first time collagens I (two chains) and III in the eggshell matrix, and Kunitz-like protease inhibitor as a major shell matrix protein. Besides the above mentioned proteins we can also mention EDIL3, fibronectin, sulfhydryl oxidase, tubulin alpha 1, lysozym, Dickkopf-related protein 3, keratins and ovotransferrin. The relative abundances of proteins were determined using the exponentially modified protein abundance index (emPAI) in all eggshell layers. 7 proteins were identified in the cuticle layer, whereas 16 proteins were described in the palisade layer and 23 in the mammillary layer. (Miksik et al., *Analytical and Bioanalytical Chemistry* **397**: 205, 2010)

In one more study we examined the EDTA-insoluble proteinaceous film from the cuticle layer of eggshell. This film consists of three main areas: spots (cca 300 μm diameter), blotches (small spots with diameter only tens of μm) and the surroundings (i.e. the area without spots and blotches) where spots contain a visible accumulation of pigment. These areas were cut out of the membrane by a laser microdissection, proteins were cleavaged by trypsin and the peptides were analysed by nLC/MS (Q-TOF). We identified 29 proteins whereas further 8 were determined by less specific “cleavage” with semitrypsin. The relative abundances of these proteins were determined using the exponentially modified protein abundance index (emPAI) where the most dominant proteins were eggshell-specific ones such as ovocleidin-17 and ovocleidin-116. Individual areas of the cuticle membrane differ in their relative proportions of 14 proteins, where significant differences between the three quantification criteria (direct, after normalization to ovocledin-17 or to ovocledin-116) were observed in four proteins. (Miksik et al., *Analytical and Bioanalytical Chemistry* **406**: 7633, 2014). In our department proteomic analysis were made (I. Mikšík) and in another department (P. Ergang, J. Pácha) microdissection was made.

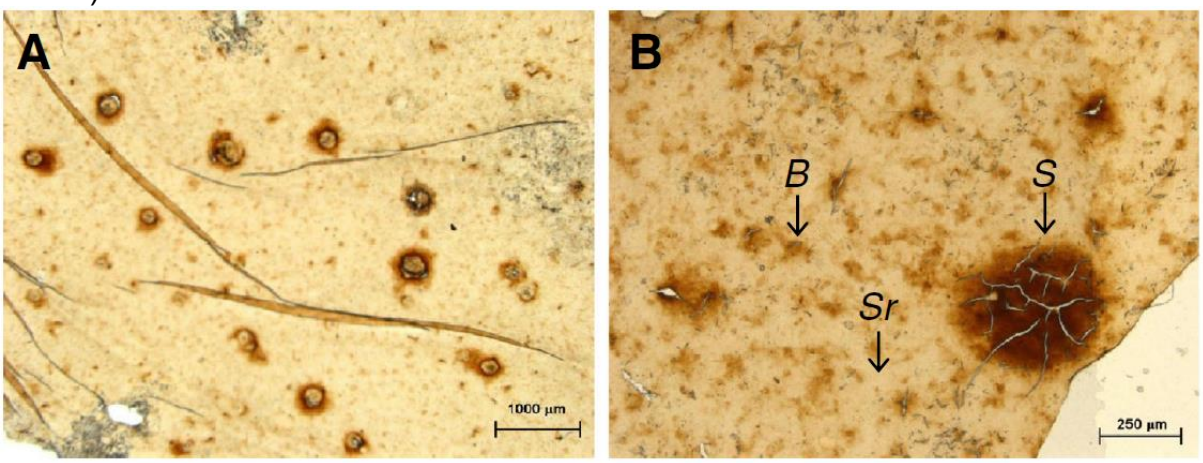


Figure 4. Photo of cuticle membrane from laser microdissection microscope. Panel A: view at the lower resolution, B at higher magnification (see scale). S: spot, B: blotch (small spot) and Sr (surroundings, i.e. area without spots or blotches. Spot (S) has an area of cca 166 800 μm^2

Me

The eggshell pigment deposition (biliverdin, protoporphyrin) together with protein composition has recently been considered as a mechanism to resist pathogen penetration into the egg. In the presented paper (Fargallo et al., *Evolutionary Ecology* **28**: 627, 2014) we showed no evidence of a detrimental effect of the reduction of eggshell pigments on egg hatchability, mortality of the chicks during the nesting period, nestling body condition, nestling local immune response to PHA antigen or probability of recruitment. In this study we analysed pigments and proteins, Spain colleagues made "field" experiments; this study will be prolonged when Spain group obtained financial support by Spanish Ministry of Economy and Competitiveness (Eggshell pigmentation to prevent pathogens, 2015-2017)

Eggshell pigments

Special attention was paid to study of pigment concentration in various avian eggshells. In these studies we analysed in our department pigments (biliverdin, protoporphyrin). However these research were made in the context of big „Ornithological group" (see part International collaboration) when this "Group" provided various eggs for studies (e.g. various species, breeding etc.).

3) **INTERNAL COLLABORATION (within the Institute)** – only collaboration with scientific paper(s) are mentioned.

Adipose Tissue Biology (Dr. J. Kopecký)
In our department we done analysis of nucleotides.(Jelenik et al., *Diabetes* **59**: 2737, 2010)
Bioenergetics (Dr. T. Mráček, Dr. J. Houštěk)
We made some proteomic analysis as well as analysis of nucleotides.(Houstek et al., *Physiological Genomics* **44**: 487, 2012; Houstek et al., *Physiological Genomics* **46**: 671, 2014)
Biomaterials and Tissue Engineering (Doc. L. Bačáková, dr. E. Filová)
We compared proteins from pericard and heart valve and observe differences in modified pericard (the aim is the research of possible substitution of heart valve).
Another topic was to analysis of cell adhesion by proteomic approach.(Grausova et al., *PLoS ONE* **6**: 2011; Maxova et al., *Cellular Physiology and Biochemistry* **25**: 615, 2010)
Cardiovascular Morphogenesis (Prof. D. Sedmera)
We compare proteom (mainly collagens) of healthy and ill heart. Research leads to treatment of ill hearts and understanding of heart development.
Developmental Cardiology (Prof. F. Kolář, Prof. B. Ošťádal)

We are making proteomic analysis of cells from the left and right heart ventricle with the aim to research gender differences.
Another topic was the analysis of malondialdehyde in heart samples.(Neckar et al., *Canadian Journal of Physiology and Pharmacology* **90**: 1303, 2012)

Epithelial Physiology (Prof. J. Pácha, dr. P. Ergang)
We made analysis of steroid metabolism (mainly corticosterone and 11-dehydrocosrticosterone) but not only, by HPLC and radioactive or mass-spectrometric detection (Ergang et al., *Molecular and Cellular Endocrinology* **323**: 155, 2010; Ergang et al., *Journal of Steroid Biochemistry and Molecular Biology* **126**: 19, 2011; Klusonova et al., *Steroids* **76**: 1252, 2011; Vagnerova et al., *Steroids* **76**: 577, 2011)
Another subject was analysis of proteins of eggshell cuticle when in the Dpt. Epithelial Physiology the laser dissector was used and in our Dpt. proteomic analysis were perform (Miksik et al., *Analytical and Bioanalytical Chemistry* **406**: 7633, 2014)

Laboratory of Biochemistry of Membrane Receptors (Doc. P. Svoboda)
We made proteomic analysis (Ujcikova et al., *Proteome Science* **12**: 11, 2014)

4) **DOMESTIC COLLABORATION (within the country)** - only collaboration with scientific paper(s) are mentioned.

Institute of Organic Chemistry and Biochemistry, Czech Academy of Science (dr. V. Kašička)
We cooperate on many projects by Czech Science Foundation (GACR) (GA203/08/1428, GA203/09/0675; GAP206/12/0453; GA13-17224S) on the development of new capillary electrophoretic methods for the analysis of various compounds. We have also many common papers.

Institute of Chemical Technology, Department of Analytical Chemistry, Prague (Dr. D. Sýkora, Prof. V. Král)
We also cooperate on many projects by Czech Science Foundation (GACR) (GA203/09/0675; GAP206/12/0453) on the development of new capillary electrophoretic methods for the analysis of various compounds when in this Department colleagues prepare (synthetize) various ligands/nanoparticles for affinity separations. We have also some common papers.

Institute of Organic Chemistry, Czech Academy of Science (dr. Jana Brabcova, Dr. Irena Valterova
Proteomic analysis of lipase and serin protease from insect were performed at our department (Brabcova et al., *Journal of Molecular Catalysis B-Enzymatic* **98**: 62, 2014; Brabcova et al., *Archives of Insect Biochemistry and Physiology* **82**: 117, 2013)

General University Hospital in Prague and Institute of Clinical and Experimental Dental Medicine, First Faculty of Medicine, Charles University, Prague (Prof. Z. Broukal)
We have common project from the Ministry of Health (NT14324) about comparative study of protein content of saliva, dental pellicle and teeth in relation to dental decay. Our task is proteomic analysis as well as sample preparation of samples (teeth, saliva) obtained from dentists. (Eckhardt et al., *Journal of Endodontics* **40**: 1961, 2014; Jagr et al., *Physiological Research* **63**: 2014; Jagr et al., *European Journal of Oral Sciences* **120**: 259, 2012)

Second Faculty of Medicine, Charles University, Prague (Prof. J. Herget, Dr. H. Maxová)
Our task was proteomic analysis of pulmonary arteries in the aim to elucidate pulmonary hypertension.(Grausova et al., *PLoS ONE* **6**: 2011)
Department of Orthopaedics, 1st Faculty of Medicine, Charles University, Prague (dr. M. Ošťádal)
We made proteomic analysis of the extracellular matrix in idiopathic pes equinovarus (paper was accepted and will be published 2015 in Molecular and Cellular Biochemistry).

5) **INTERNATIONAL COLLABORATION**

Institute of Forensic Medicine, University of Verona (Italy) (Prof. F. Tagliaro)
I. Miksik stayed there as visiting professor at years 2004 and 2010. There were done some works on the application of capillary electrophoresis for toxicological/forensic analysis (Gottardo et al., *Electrophoresis* **33**: 599, 2012; Liotta et al., *Forensic Science International* **220**: 279, 2012) as well as proteomic analysis of mummy (Miksik et al., *Chromatographia* **77**: 1503, 2014)
„Ornithological group“ (University of Adelaide, South Australia; University of London, UK; University of St Andrews, UK; City University of New York, USA; The Natural History Museum, Tring, UK)
Another topic of really international cooperation is analysis of pigments in avian eggshells, when in our Department we analysed/quantified these pigments from the samples (eggs) obtained from the ornithologists. These analysis were published in many papers (and still will be published) (Brulez et al., *Journal of avian biology* **45**: 94, 2014; Cassey et al., *Journal of avian biology* **43**: 503, 2012; Cassey et al., *Biological Journal of the Linnean Society* **106**: 657, 2012; Duval et al., *PLoS ONE* **8**: 2013; Duval et al., *Journal of Experimental Biology* **216**: 700, 2013; Duval et al., *General and Comparative Endocrinology* **208**: 146, 2014; Maurer et al., *Journal of Zoology* **285**: 194, 2011)
Departamento de Ecologia Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, Madrid, Spain (Juan A. Fargallo)
In our department we made the analysis of proteins and pigments in eggshells of kestrels (Fargallo et al., *Evolutionary Ecology* **28**: 627, 2014) however this cooperation will continue due the project by Spanish Ministry of Economy and Competitiveness (Eggshell pigmentation to prevent pathogens, 2015-2017)

6) **KEY METHODOLOGY AND CORE FACILITIES**

Proteomic laboratory
Laboratory is equipped for modern proteomic analysis: 1D and 2D electrophoresis including scanners and adequate software for data analysis of gels; nLC coupled to high-resolution mass spectrometry (Q-TOF) and necessary software for proteins analysis (including database and database search software)

Analytical instruments

Besides proteomic instruments (laboratory) there are other analytical instruments for the analysis of a broad spectrum of biologically (physiologically) important compounds:

- 2x HPLC (with UV-VIS, DAD, fluorescence, radioactive and evaporative light-scattering detectors)
- ion-trap MS (besides high-resolution mass spectrometry Q-TOF in proteomic laboratory) coupled to HPLC, including electro-spray (ESI) and chemical ionization (API-CI) and necessary software for analysis of compounds
- capillary electrophoresis (1 new and 2 very old) with possibility to connect to MS

7) **INVOLVEMENT IN SIGNIFICANT PROJECTS**

not applicable

8) **OTHER RELEVANT INFORMATION**

not applicable

9) **SUMMARY AND RESEARCH IMPACT**

Our department works in methodical and application areas of analytical chemistry when we are focused on the biological (physiological) research. Our main research tasks and research impact at the last five years were:

- developing of new separation methods (capillary electromigration techniques)
  - affinity electromigration methods
  - application of gold nanoparticles in capillary electromigration methods
  - forensic application (including coupling CE-MS)
- proteomic analysis of biological samples (teeth, eggshell etc.) when many new proteins were identified, for example:
  - we described proteom of human teeth
  - we described insoluble proteins from the avian eggshell, especially cuticle
- analysis of posttraslational modifications of proteins
  - determination of arising compounds in glycation reaction as well as sites of modifications
  - identification of age-related changes in proteins - deamidation of proteins in mummy
- proteomic facility of Institute of Physiology CAS
  - cooperation with many Departments and Institutes
- analysis of a broad spectrum of biologically important compounds in cooperation with many departments of our Institutes, various Czech Institutes as well as international cooperation

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the ASCR, v. v. i.

Scientific team	Membrane Transport Biophysics

2.1. RESEARCH FOCUS

Our research group focuses on the role of mitochondria in physiological and pathophysiological processes of the cell and organism. We belong to pioneer researchers in the mitochondria integrated physiology and applications of three-dimensional super-resolution microscopy to studies of mitochondrial network, nucleoids and cristae morphology in relation to diseases related to the oxidative stress, type 2 diabetes and cancer.

Because the work in our laboratory is targeted towards the understanding of mitochondrial physiology and mitochondrial ROS homeostasis, one of our long term goals is to uncover the mechanisms by which mitochondrial lipids and lipid-derived compounds participate in the **cytoprotective signalling**. Our laboratory has recently pointed to a synergic role of mitochondrial phospholipase iPLA2γ and mitochondrial uncoupling protein UCP2 in attenuation of mitochondrial ROS production, and we aim to elucidate the role of mitochondrial phospholipase iPLA2γ in cellular antioxidant protection and cytoprotective signalling.

In addition, chronic **oxidative stress** accompanies number of pathophysiological disorders including neurodegenerative diseases, type 2 diabetes and pulmonary hypertension, the etiology of which we study in our laboratory. In case of irreversible damage, mitochondria must be removed by specific pathway of autophagy, called **mitophagy**. This process is crucial for mitochondrial quality control process, disruption of which is accompanied by a number of diseases. Moreover, mitochondria are semiautonomous organelles for they have mitochondrial DNA (mtDNA). **Genetic manipulation of the mtDNA** is rather complicated and the methods of gene silencing or quantification of genetic mutations in mtDNA are either not satisfactory or not developed. Therefore, the development of such techniques is one of our goals.

Because mitochondria are rather small organelles and resolution of commonly available light and fluorescence microscopy is not sufficient to study certain aspects of their morphology, we employ special **super-resolution fluorescence microscopy**. The prototype of such microscope we have recently purchased for our department. We develop further methodologies of “nanoscopy” to study mitochondrial morphology and function. We focus on reflection of physiological and pathological situations by mitochondrial morphology and *vice versa*.

Finally, we study the role of **mitochondrial signalling in cancer cells** and cancer-specific enzymatic pathways, study of which could be essential for the development of future anticancer drugs.

Concerning applied research, we have also developed novel drug carriers to transport specific anticancer drugs called photosensitizers into cancer tissues. Our findings have been patented in Industrial Property Office of the Czech Republic, patent No. CZ 298 978 and by PCT (PCT/CZ2007/ 000107) **Patents since 2010:** Canadian patent No.2.665.762 and Norwegian patent No. 20091595 and patent filed for the European

Patent Office No.07817403.4, “Liposomal gel phthalocyanine preparation for photodynamic therapy of tumours and its manufacturing”.

2.2. PERSONNEL

Senior scientists:

Petr Ježek, PhD, DSc (team leader), biophysicist and physiologist, age 58, H-index 36
Martin Jabůrek, PhD, biophysicist and biochemist, age 45, H-index 16
Lydie Plecitá – Hlavatá, PhD, cell biologist, age 38, H-index 12

Junior scientists:

Jaroslav Zelenka, PhD, biochemist, age 34, H-index 9
Andrea Dlasková, PhD, biochemist, age 39, H-index 7
Tomáš Olejár, MD, PhD, pathologist, age 46, H-index 6
Hana Engstová, PhD, biophysicist, age 44, H-index 2
Katarína Smolková, PhD, biochemist, age 33, H-index 9
Michal Růžička, PhD, biochemist, age 47, H-index 6

Postdoctoral fellows:

Jitka Šantorová – Špačková, PhD, biochemist, age 39, H-index 7
Jan Ježek, PhD, biochemist, age 36, H-index 6
Tomáš Špaček, PhD, biochemist, age 39, H-index 5
Jan Tauber, PhD, biochemist, age 31, H-index 2
David Pajuelo Reguera, PhD, biochemist, age 33, H-index 5
PhD/MSc/BSc students (in 2010: 6/1/1; 2014:2/1/0), laboratory assistants (4)

2.3. KEY RESULTS

Channel character of uncoupling protein-mediated transport (Jezek et al., *FEBS Lett* **584**: 2135, 2010) – Here we review the evidence showing that mitochondrial uncoupling proteins (UCPs) are sole anion transporters, which mediate uniport of dissociated free fatty acids (FA). Protonated FAs then diffuse back across the lipid bilayer by spontaneous flip-flop. An existence of sole proton channel in UCPs is excluded by the equivalent flux-voltage dependencies for uniport of FAs and halide anions, which are best described by the Eyring barrier variant with a single energy well in the middle of two peaks. Experiments with FAs unable to flip and alkylsulphonates also support this view. We hypothesise that phylogenetically, UCPs took advantage of the common FA-uncoupling function of SLC25 family carriers and dropped their solute transport function.
Team’s contribution: Supported by the Academy of Sciences (grant # AV0Z501 10509, PI: Petr Ježek), Czech Science Foundation (grant # 303/07/0105, PI: Martin Jabůrek), Czech Ministry of Education (grant # ME09018, PI: Martin Jabůrek). In collaboration with Keith D. Garlid, Department of Biology, Portland State University, who contributed by writing a particular section of the manuscript.

Role of the transmembrane potential in the membrane proton leak (Rupprecht et al., *Biophys J* **98**: 1503, 2010) – The molecular mechanism responsible for the regulation of the mitochondrial membrane proton conductance is not clearly understood. This study investigates the role of the transmembrane potential using planar membranes together with reconstituted isolated recombinant uncoupling proteins. We provide evidence that tight regulation of proton conductance by the transmembrane potential together with the regulation of membrane concentration of fatty acids determines cellular respiration and metabolism.

Team's contribution: Supported by the Academy of Sciences (grant # AV0Z501 10509, PI: Petr Ježek), Czech Science Foundation (grant # 303/07/0105, PI: Martin Jabůrek), Czech Ministry of Education (grant # ME09018, PI: Martin Jabůrek). M.J. and P.J. participated by preparing isolated recombinant UCP1 and UCP2 suitable for the study and by editing the manuscript. The laboratory of E.E. Pohl (University of Veterinary Medicine, Vienna) performed the majority of experiments.

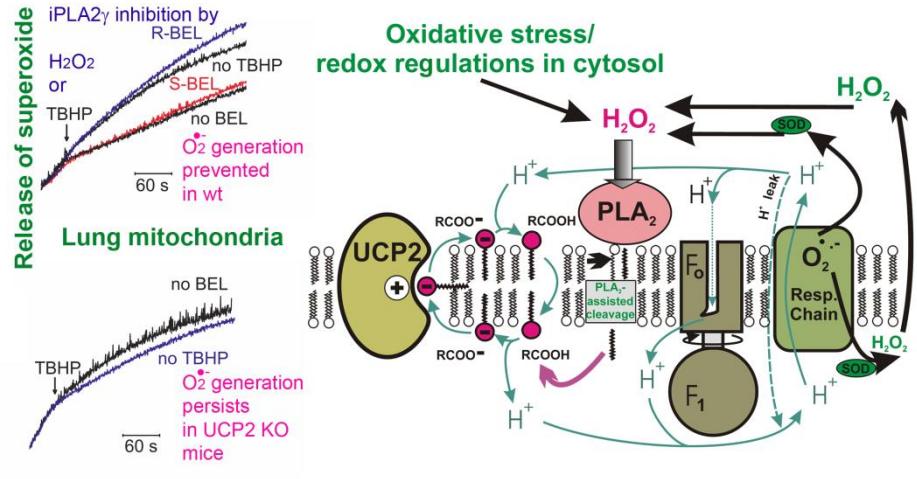
Fatty acids are key in 4-hydroxy-2-nonenal-mediated activation of uncoupling proteins 1 and 2 (Malingriaux et al., *PLoS One* **8**: e77786, 2013) – Mitochondrial uncoupling proteins were hypothesized to be activated by hydroxynonenal (HNE), a product of lipid peroxidation, but the results supporting this hypothesis are highly controversial. Here we demonstrate that HNE does not directly activate either UCP1 or UCP2, but may bind to positively charged amino acid residues in both proteins, altering its protonophoretic activity in the presence of fatty acids.
Team's contribution: Supported by the Czech Science Foundation (grant # P302/10/0346, PI: Petr Ježek). Our team (MJ, PJ) contributed by performing auxiliary experiments and data analysis. The laboratory of E.E. Pohl (University of Veterinary Medicine, Vienna) performed the majority of experiments.

Mitochondrial phospholipase A2 activated by reactive oxygen species in heart mitochondria induces mild uncoupling (Jezek et al., *Physiol Res* **59**: 737, 2010) – Homeostasis of reactive oxygen species (ROS) in cardiomyocytes is critical for elucidation of normal heart physiology and pathology. Mitochondrial calcium-independent phospholipases A2 have been previously suggested to be activated by ROS. Here, we investigated the hypothetical activation of mitochondrial phospholipases iPLA2 by reactive oxygen species (ROS) and attempted to propose a physiological role of such activation using isolated rat heart mitochondria. We show that the ROS-induced activation of iPLA2 leads to increased H^+ fluxes mediated by the mitochondrial adenine nucleotide translocase and by uncoupling protein(s). We conclude that ROS-induced function of iPLA2 contribute to the iPLA2-dependent cardioprotective signalling.
Team's contribution: Supported by the Academy of Sciences (grant # AV0Z501 10509, PI: Petr Ježek), Czech Science Foundation (grant # 303/07/0105, PI: Martin Jabůrek), Czech Ministry of Education (grant # ME09018, PI: Martin Jabůrek) and Academy of Sciences (grant KJB500110902, PI: Jan Ježek). This work was initiated and completed solely by members of our team. All authors contributed to formulating hypotheses, performing the experiments, analyzing data and writing the manuscript.

Antioxidant activity by a synergy of redox-sensitive mitochondrial phospholipase A2 and uncoupling protein-2 in lung and spleen (Jaburek et al., *Int J Biochem Cell Biol* **45**: 816, 2013) – Mitochondrial uncoupling protein-2 (UCP2) has been suggested to participate in the attenuation of the reactive oxygen species production, but the mechanism of action and the physiological significance of UCP2 activity remain controversial. In this pioneer work, we tested the hypothesis that mitochondrial uncoupling protein 2 (UCP2) provides feedback downregulation of oxidative stress in vivo via synergy with an H_2O_2-activated mitochondrial phospholipase. We demonstrate that UCP2 is regulated by redox-activated phospholipase iPLA2 gamma and consequent increase in mitochondrial H^+ flux protects cell against mild oxidative stress.

Team's contribution: Supported by the Czech Science Foundation (grant # P302/10/0346, PI: Petr Ježek; grant # P303/11/P320, PI: Jan Ježek), Czech Ministry of Education (grant # ME09018, PI: Martin Jabůrek), and by the Academy of Sciences (grant # AV0Z501 10509 and RVO67985823, PI: Petr Ježek). This work was initiated and completed solely by members of our team. All authors contributed to formulating hypotheses, performing the experiments, analyzing data and writing.

Fig. 1. Redox-activated iPLA2γ acts in synergy with UCP2 to prevent oxidative stress



Antioxidant and Regulatory Role of Mitochondrial Uncoupling Protein UCP2 in Pancreatic beta-cells (Jezek et al., *Physiological Research* **63**: S73, 2014) – This review summarizes the contribution of our group to the field of mitochondrial uncoupling protein transport mechanism and its regulation for the last two decades. We review the evidence that the mechanism of proton transport catalyzed by mitochondrial uncoupling proteins is fully dependent on non-esterified free fatty acids and review the physiological roles of the mitochondrial uncoupling protein 2 emphasizing its roles in pancreatic beta-cells.
Team's contribution: Supported by the Czech Science Foundation (grant # P302/10/0346, PI: Petr Ježek, and grant # P304/10/P204, PI: Andrea Dlasková, grant # P305/12/1247, PI: Martin Jabůrek). This work was initiated and completed solely by members of our team. All authors contributed to formulating hypotheses and writing the manuscript.

Redox homeostasis in pancreatic beta cells (Jezek et al., *Oxid Med Cell Longev* **2012**: 932838, 2012) – Redox cell homeostasis is inevitable part of insulin release signalling of β cells in Langerhans islets. Induction and development of type II diabetes is considered as the result of dysregulation of redox homeostasis with progressive self-accelerating oxidative stress. Here we review out current understanding of detailed mechanisms that determine redox homeostasis and redox signalling in association with metabolic regulation, which might be beneficial for proper design of anti-diabetic drugs.
Team's contribution: Supported by the Czech Science Foundation (grant # P302/10/0346, PI: Petr Ježek, and grant # P304/10/P204, PI: Andrea Dlasková). This work was initiated and completed solely by members of our team. All authors contributed to formulating hypotheses and writing the manuscript.

Mitochondrial reactive oxygen species: which ROS signals cardioprotection? (Garlid et al., *Am J Physiol Heart Circ Physiol* **305**: H960, 2013) – This work focuses on the role of mitochondria as the major effectors of cardioprotection and aims to identify reactive oxygen species (ROS) responsible for the activation of mitochondrial protein kinase C epsilon – dependent cardioprotective signalling. Our results support the conclusion that the cardioprotective ROS message is carried by a product of lipid peroxidation.
Team's contribution: Supported by the Czech Ministry of Education (grant # ME09018, PI: Martin Jabůrek). In collaboration with the laboratory of Keith D. Garlid, Department of Biology, Portland State University. MJ contributed to the conception and design of experiments, performing selected experiments, analyzing data, preparing figures, writing the first draft of the manuscript and revising the manuscript.

Dehydrosilybin attenuates the production of ROS in rat cardiomyocyte mitochondria with an uncoupler-like mechanism (Gabrielova et al., *J Bioenerg Biomembr* **42**: 499, 2010) – Reactive oxygen species (ROS) originating from mitochondria are perceived as a factor contributing to cell signalling and aging. Silybin and dehydrosilybin, two polyphenolic compounds, display a plethora of biological effects generally ascribed to their known antioxidant capacity. Here we investigated the cytoprotective effects of the two natural polyphenolic compounds using the primary cell cultures of neonatal rat cardiomyocytes and isolated rat heart mitochondria. We show that dehydrosilybin interacts with mitochondria and uncouples respiration and we infer that this property is the basis for its ability to modulate reactive oxygen species in cells. We also propose a hypothesis on natural ischemia preconditioning by natural polyphenols.
Team's contribution: Supported by the Czech Science Foundation (grant # 303/08/0658, CO-PI: Martin Jabůrek) with a major involvement of JJ. In collaboration with the Institute of Medical Chemistry and Biochemistry, Palacky University, Olomouc, that contributed by studies on primary cell cultures and the Institute of Microbiology, AS CR, Prague, that contributed by preparation of tested compounds.

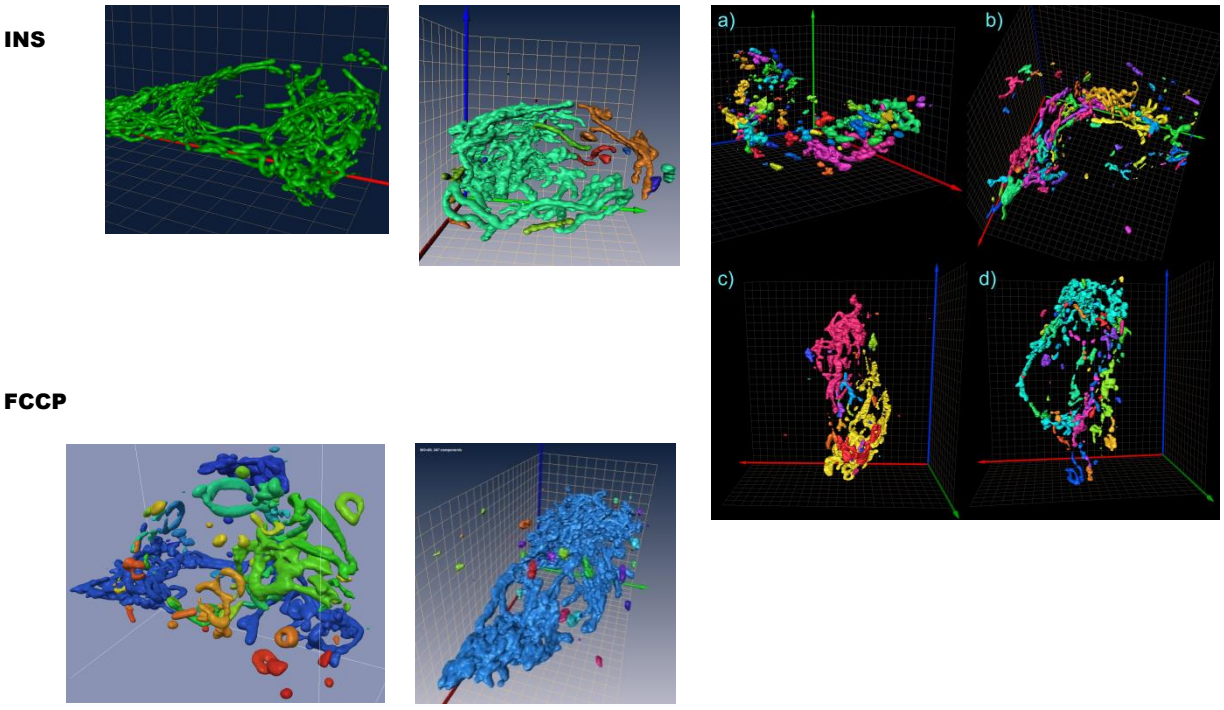
Distribution of mitochondrial nucleoids upon mitochondrial network fragmentation and network reintegration in HEPG2 cells (Tauber et al., *Int J Biochem Cell Biol* **45**: 593, 2012) – Mitochondrial DNA (mtDNA) is organized in nucleoids in complex with accessory proteins, proteins of mtDNA replication and gene expression machinery. A robust mtDNA genome is represented by hundreds to thousands of nucleoids in cell mitochondrion. Detailed information is lacking about the dynamics of nucleoid distribution within the mitochondrial network upon physiological and pathological events. Therefore, in this study, we used confocal microscopy to study mitochondrial nucleoid redistribution initiated by mitochondrial fission and following reintegration of the mitochondrial network. Our observations suggest that both mitochondrial network fission and reconnection of the disintegrated network are initiated in the proximity of nucleoids. Analyses of these morphological icons provide a basis for a future mitochondrial morphology diagnostics.
Team's contribution: Supported by the Czech Academy of Sciences (grant # IAA500110701, PI: Petr Ježek; grant # AV0Z50110509, PI: Petr Ježek), Czech Science Foundation (grant # 302/10/0346, PI: Petr Ježek; grant # P304/10/P204, PI: Andrea Dlasková; grant # P305/12/1247, PI: Martin Jabůrek). This work was initiated

and completed solely by members of our team. All authors contributed to formulating hypotheses, performing the experiments, analyzing data and writing the manuscript.

Import of desired nucleic acid sequences using addressing motif of mitochondrial ribosomal 5S-rRNA for fluorescent in vivo hybridization of mitochondrial DNA and RNA (Zelenka et al., *J Bioenerg Biomembr* **46**: 147, 2014) – In order to provide fluorescence hybridization of the matrix-addressing sequences of mitochondrial ribosomal 5S-rRNA, we constructed an import system for in vivo targeting of mitochondrial DNA or RNA. We present a proof-of-principle for mitochondrial in vivo hybridization and mitochondrial nucleic acid import.
Team's contribution: Supported by the Czech Science Foundation (grant # P305/12/1247, PI: Martin Jabůrek; grant # P305/12/P388, PI: Jaroslav Zelenka); and the research projects RVO67985823 and BIOCEV—Biotechnology and biomedicine Centre of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund. This work was initiated and completed solely by members of our team. All authors contributed to formulating hypotheses, performing the experiments, analyzing data and writing the manuscript.

4Pi microscopy reveals an impaired three-dimensional mitochondrial network of pancreatic islet beta-cells, an experimental model of type-2 diabetes (Dlaskova et al., *Biochimica Et Biophysica Acta-Bioenergetics* **1797**: 1327, 2010) – Pathology of diabetic beta-cells might be reflected by the altered morphology of mitochondrial network. Its characterization is however hampered by the complexity and density of the three-dimensional (3D) mitochondrial tubular networks in these cell types. Here, we present a pioneer study utilizing 4Pi super-resolution microscopy to detect mitochondrial network morphology in fixed cells. We present a quantitative approach to describe these networks in insulinoma INS-1E cells and primary beta-cells in Langerhans islets. We propose that standardization of the patterns of mitochondrial network may lead to development of morphological diagnostics for Langerhans islets and for the assessment of beta-cell condition prior to their transplantation.
Team's contribution: Supported by the Czech Academy of Sciences (grant # IAA500110701, PI: Petr Ježek; grant # AV0Z50110509, PI: Petr Ježek), Czech Ministry of Health (grant # NR/7778, PI: Petr Ježek), and Czech Ministry of Education (grant # ME09029, PI: Petr Ježek) with a major contribution from AD, who became the first author, and TŠ, JŠ, and LPH, who performed particular experiments. In collaboration with The Jackson Laboratory, Bar Harbor, which contributed by 4Pi microscopy technique and Institute of Clinical and Experimental Medicine, Prague, which helped with the isolation of Langerhans islets.

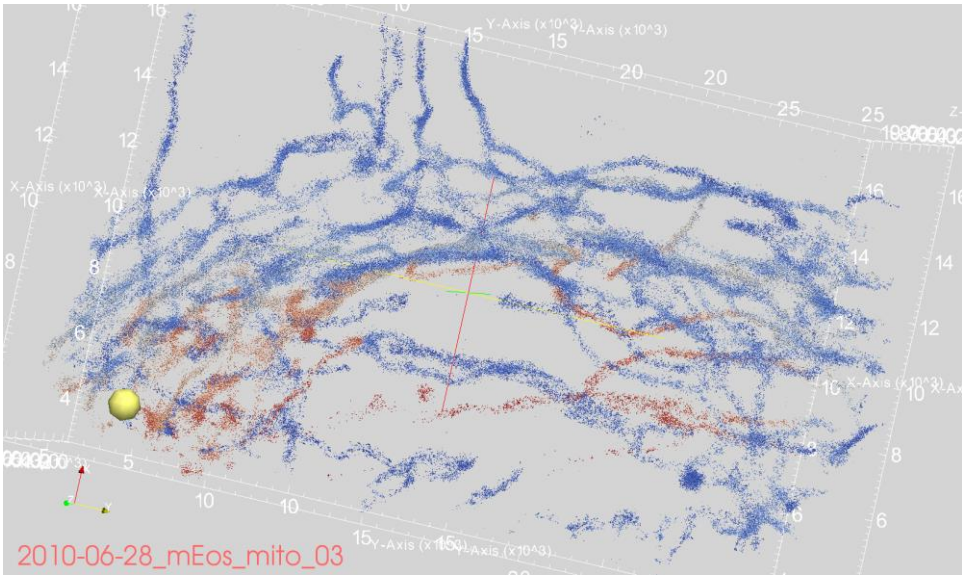
Fig. 2. Goto Kakizaki islet β-cells (a-d) unlike control β-cells of Wistar rats (gray background) possess disintegrated mitochondrial network due to oxidative stress and accelerated mtDNA degradation. 3D images by high (100 nm) resolution 4Pi microscopy. For comparison, network of INS1E cells intact (INS) and disintegrated by uncoupling (FCCP) is also shown.



Sample drift correction in 3D fluorescence photoactivation localization microscopy (Mlodzianoski et al., *Opt Express* **19**: 15009, 2011) – This study provides an approach that corrects for three-dimensional drift in images of fixed samples using diffraction-unlimited far-field fluorescence microscopy without the requirement for fiduciary markers or instrument modifications. We demonstrate the performance of the proposed drift correction algorithm with different simulated structures. By imaging mitochondria with Biplane FPALM, we show the algorithm's feasibility in a practical application.

Team's contribution: Supported by the Czech Academy of Sciences (grant # AV0Z50110509, PI: Petr Ježek), Czech Science Foundation (grant # 302/10/0346, PI: Petr Ježek), Czech Ministry of Education (grant # ME09029, PI: Petr Ježek). Members of our team (KS, AD, JS, PJ) contributed by providing biological samples used in the study and by consulting the manuscript. In collaboration with The Jackson Laboratory, Bar Harbor, which contributed by providing the fluorescence microscopy technique.

Fig. 3. BiplaneFPALM imaging of mitochondrial network



Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells (Smolkova et al., *Int J Biochem Cell Biol* **43**: 950, 2010) – Here we propose a hypothesis of dynamic waves of gene expression that promote metabolic changes during carcinogenesis. We discuss the bioenergetically relevant functions of oncogenes, the involvement of mitochondrial biogenesis/degradation in carcinogenesis, the unexplained Crabtree effect of instant glucose blockade of respiration, and also the relevant metabolic signalling.
Team's contribution: Supported by the Czech Academy of Sciences (grant # IAA500110701, PI: Petr Ježek; grant # AV0Z50110509, PI: Petr Ježek), Czech Ministry of Health (grant # NR/7778, PI: Petr Ježek), and Czech Ministry of Education (grant # ME09029, PI: Petr Ježek), with a major involvement of KS, who became the first author, and in collaboration with INSERM, Bordeaux, that contributed by writing selected portions of the manuscript.

Distinctions and similarities of cell bioenergetics and the role of mitochondria in hypoxia, cancer, and embryonic development (Jezek et al., *Int J Biochem Cell Biol* **42**: 604, 2010) – In this review, we compare situations under which the major cellular role of mitochondria, oxidative phosphorylation, is transiently suppressed. We discuss our current knowledge of the role of mitochondria in hypoxia, cancer, and embryonic development and also discuss a hypothesis, which predicts repetitive conversions to a transient glycolytic mode after a meal and concomitant insulin signalling, which relates to type 2 diabetes.
Team's contribution: Supported by the Czech Academy of Sciences (grant # IAA500110701, PI: Petr Ježek; grant # AV0Z50110509, PI: Petr Ježek), Czech Ministry of Health (grant # NR/7778, PI: Petr Ježek), and Czech Ministry of Education (grant # ME09029, PI: Petr Ježek) in collaboration with INSERM, Bordeaux, that contributed by consulting the manuscript.

Mitochondrial bioenergetic adaptations of breast cancer cells to glycemia and hypoxia (Smolkova et al., *J Bioenerg Biomembr* 42: 55, 2010) – Breast cancer cells can survive and proliferate under conditions of nutrient deprivation, and we hypothesized that such environments trigger metabolic adaptations of mitochondria, which promote tumour progression. We mimicked glycaemia and hypoxia in vitro and

compared the mitochondrial and cellular bioenergetic adaptations of human breast cancer and non-cancer cells. Our data demonstrate that cancer cells adapt their bioenergetics differently than normal cells to microenvironmental conditions.
Team's contribution: Supported by the Czech Academy of Sciences (grant # IAA500110701, PI: Petr Ježek; grant # AV0Z50110509, PI: Petr Ježek), Czech Ministry of Education (grant # ME09029, PI: Petr Ježek), and the Grant Agency of the Ministry of Health (grant # NR7778, PI: Petr Ježek). With a major involvement of KS, who became the first author, and LPH and PP, who consulted the research. In collaboration with INSERM, Bordeaux, France and Oroboros, Austria, that contributed by consulting the project.

Evaluation of Topical Photodynamic Therapy of Mammary Carcinoma with an Experimental Gel Containing Liposomal Hydroxyl-aluminium Phthalocyanine (Sutoris et al., *Anticancer Res* **32**: 3769, 2012) – Photodynamic therapy is a clinically accepted approach for the treatment of many types of cancer. This study focuses on the treatment of mammalian carcinoma by topical administration of hydroxyl-aluminium phtalocyanine (AlOH-PC) and compares its affects to a clinically approved photosensitizer. We show that AlOH-PC gel is potentially suitable for photodynamic therapy of mammalian carcinoma.
Team's contribution: Supported by the TA CR grant agency (grant # TA01010781, PI: Petr Ježek). Members of our team contributed by preparing the experimental gel. In collaboration with the First and the Third Faculty of Medicine, Prague, that provided animal models of mammalian carcinoma.

2.4. INTERNAL COLLABORATION (within the Institute) – Our team has an informal collaboration with several other departments within the Institute concerning the methodology and techniques applied to particular research projects. These include the Department of Bioenergetics (the methodology concerning bioenergetics experiments), the Department of Membrane Transport (preparing lectures for the CBV tutorial "Function and structure of cellular membranes), the Department of Biomathematics (confocal microscopy experiments and image processing) and others.

2.5. DOMESTIC COLLABORATION (within the country) – Our domestic collaborations include: Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic (group of Martin Modrianský, PhD); Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic (Eva Gabrielová, PhD); Centre of Biocatalysis and Biotransformation, Institute of Microbiology AS CR, Prague (laboratory of Vladimír Křen, DSc); Laboratory of Pancreatic Islets, Institute of Clinical and Experimental Medicine, Prague, Czech Republic (laboratory of František Saudek, MD, DSc); Department of Transgenic Models of Diseases, Institute of Molecular Genetics AS CR, Prague (head of the department: Radislav Sedláček, PhD); Charles University, Third Faculty of Medicine, Prague (K. Sutoris, MD); Charles University, First Faculty of Medicine, Prague (L. Vítek, MD, P.Poučková, MD); Institute of Macromolecular Chemistry, Prague (D. Horák, PhD); Wake Ltd., development of liposomal gels for photodynamic therapy of tumours.

2.6. INTERNATIONAL COLLABORATION – Our group has established collaborations with the groups of: J. Bewersdorf, PhD, Department of Cell Biology, Yale

School of Medicine, New Haven, CT, USA; K.D. Garlid, MD, PhD, Department of Biology, Portland State University, Portland, OR, USA; E.E. Pohl, PhD, Institute of Physiology, Pathophysiology and Biophysics, University of Veterinary Medicine, Vienna, Austria; M.R. Wieckowski, PhD, Nencki Institute of Experimental Biology, Warsaw, Poland; R. Rossignol, PhD, INSERM CR1, Universite Victor Segalen, Bordeaux, France; K.R. Stenmark, MD, School of Medicine, University of Colorado, CO, USA; D. Siemen, PhD, Klinik für Neurologie, Universitaet Magdeburg, Magdeburg, Germany; W.F. Graier, PhD, Center of Molecular medicine, Medical University of Graz, Graz, Austria; P. Di Mascio, PhD, Department of Biochemistry, Sao Paulo University, Sao Paulo, Brazil; M. Cagalinec, PhD, Centre for Disease Models and Biomedical Imaging, University of Tartu, Estonia

2.7. KEY METHODOLOGY AND CORE FACILITIES

The Department of Membrane Transport Biophysics was formed in the Institute of Physiology in 1991. Since its formation, it has succeeded in building completely new laboratories for biofluorescence methods, molecular biology and 3D superresolution PALM microscopy, employing cell cultures silenced for selected genes and several knockout mice models. The laboratories are equipped with necessary instrumentation for biochemistry, biophysics and molecular biology which are needed for a successful completion of the projects. Besides the basic laboratory equipment, the facility contains Olympus fluorescent microscope with semi-confocal capability and a full confocal Olympus microscope, a diode-array spectrophotometer Milton Roy, Spectronics 3000, and fluorometers Perkin-Elmer LS50B, Shimadzu RF5301 PC and a unique high-resolution Fluorolog 322 fluorometer (Spex-Jobin&Yvon-Horiba). The Oroboros oxygraph 2k is available for sensitive respiratory assays. Jouan IG 750 multigas CO_2 incubator is available for incubation of cell cultures at different concentrations of oxygen plus Scitive N hypoxic workstation.

2.8. INVOLVEMENT IN SIGNIFICANT PROJECTS

Biocev – Biotechnology and Biomedicine Center of the Academy of Sciences and Charles University
CBV – Centre of Biomedical Research of the Institute of Physiology AS CR

2.9. SUMMARY AND RESEARCH IMPACT

All our past and future work concerns with mitochondria-integrated physiology.
Recently, as a new unexpected feedback antioxidant cytoprotection, we discovered a novel antioxidant synergy of mitochondrial phospholipase iPLA2γ and uncoupling protein UCP2. In 2011 we achieved the world priority in the first imaging of biological samples – mitochondrial network and nucleoids, by BiplaneFPALM, i.e., by 3D superresolution PALM microscopy (Mlodzianoski et al., *Opt Express* **19**: 15009, 2011; 37 times cited). In 2010 we have revealed disintegration of mitochondrial network in diabetic pancreatic beta cells of diabetic Goto Kakizaki rats possessing only 25% of mitochondrial DNA (Dlaskova et al., *Biochimica Et Biophysica Acta-Bioenergetics* **1797**: 1327, 2010; 12 times cited). In cooperation with French laboratory of Dr. Rossignol, we described the mixed Warburg (aerobic glycolysis) and oxidative phosphorylation phenotype in breast carcinoma (Smolkova et al., *J Bioenerg Biomembr* 42: 55, 2010; 33 times cited).

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Adipose Tissue Biology

1) TEAM DESCRIPTION

i. Research focus

We investigate physiological regulations of metabolism and their disorders in obesity, which are clustered in the so-called metabolic syndrome (namely dyslipidemia, impaired glucose homeostasis and hypertension). These adverse metabolic conditions lead to severe diseases such as type 2 diabetes and cardiovascular disease, which are of major concern for the health care systems in affluent societies. We also focus on a possible use of *n*-3 fatty acids of marine origin (**omega-3**), namely eicosapentaenoic (**EPA**) and docosahexaenoic (**DHA**) acid, in prevention and treatment the diseases associated with obesity. As a general strategy, experimental findings from studies in mice are complemented by mechanistic studies *in vitro*, and they are further explored in clinical studies. Collaborations with industrial and biotech partners are essential for the effective translational research.

The complex etiology of metabolic syndrome is the reason why multiple molecular targets should be affected to optimise the prevention and treatment strategies. In agreement with this notion, a detailed characterisation of the effects of omega-3 used in various combined interventions, for instance with calorie restriction or with anti-diabetic drugs, represents an important aspect of our studies. Findings from the animal studies are verified in human subjects, and, inversely, detailed mechanims of the effects of various interventions found in humans are being further explored in the animal experiments. A part of our efforts is focused on basic mechanisms of regulation of metabolism in major insulin-responsive tissues, namely adipose tissue, liver and muscle, as well as on the systemic effects of changes in tissue metabolism. This complex approach is supported by the use of a large reportoire of advanced techniques, ranging from the methods of cell biology and the complex panel of complementary methods for the whole body phenotyping in mice to the use of targeted metabolomics (see section 6).

Before the start of the reported period in 2010, we have introduced a model of obesity induced in mice by a high-fat feeding in order to study the effects of dietary interventions with omega-3 on various components of the metabolic syndrome (Flachs et al., *Clinical Sciences* **116**: 1, 2009; Rossmeisl et al., *Obesity* **17**: 1023, 2009; Ruzickova et al., *Lipids* **39**: 1177, 2004), and namely we found that omega-3 could induce mitochondrial biogenesis and fatty acid oxidation (Flachs et al., *Diabetologia* **48**: 2365, 2005) , >137 citations, and adiponectin (Flachs et al., *Diabetologia* **49**: 394, 2006), >179 citations, in adipose tissue of the mice, while reducing cellularity of the tissue (Ruzickova et al., *Lipids* **39**: 1177, 2004), >111 citations. That omega-3 could augment the effects of other interventions was described in mice, specifically in case of the combined use of omega-3 and the anti-diabetic drug rosiglitazone (Kuda et al., *Diabetologia* **52**: 941, 2009), >51 citations,

and also in the frame of a collaborative clinical study in which omega-3 augmented the effect of very low calorie diet in reducing obesity (Kunesova et al., *Physiol Res* **55**: 63, 2006), >55 citations. In collaboration with Pronova Biopharma (Norway), chemical derivatives of omega-3 DHA with anti-obesity properties were developed (Rossmeisl et al., *Obesity* **17**: 1023, 2009), >24 citations. A related patent (Bryhn et al., US Patent 7,550,613 B2, 2009) triggered a milestone payment to the Institute.

ii. **Personnel**

Head of the Department/team leader:

Jan Kopecky, MD, DSc (graduated from medical faculty UK, Prague in 1978; head of the Department since 1992; experience abroad: Dept. of Biochemistry, University of Ottawa, Canada – 6 mo, 1985-86; Roche Inst. Molecular Biology, NJ, USA – 1 year, 1989-90; The Jackson Laboratory, ME, USA – 1 year, 1990-91; age 64, H-index 31).

Senior scientists:

Martin Rossmeisl, MD, PhD (Biochemistry and pathobiochemistry, 1999) - nutritional interventions in mice, glucose metabolism - hyperinsulinemic-euglycemic clamps in mice, and *in vivo* phenotyping; experience abroad: University of Barcelona, Spain – 6 mo, 1995; The Jackson Laboratory, ME, USA – 6 mo, 1996-97; Pennington Biomed. Res. Center, LA, USA – 3 years, 1999-2002; CNRS, Université Paul Sabatier, Toulouse, France – 2 mo, 2003; age 46, H-index 23.

Pavel Flachs, PhD (Molecular and Cellular biology, 2002) - nutritional interventions and cold exposure in mice, energy metabolism and related phenotyping in mice; experience abroad: CNRS, Université Paul Sabatier, Toulouse, France - 8 mo, 1998-2002; Wageningen University, Netherlands – 5 mo, 2004; age 40, H-index 20.

Junior scientists:

Ondrej Kuda, PhD (Biochemistry, 2009) - adipose tissue metabolism, metabolipidomics; experience abroad: Dept. of Nutrition, Washington University in St. Louis, MO, USA - 2 years, 2009-2011; age 34, H-index 13).

Olga Horakova, PhD (Biochemistry and Pathobiochemistry, 2006) - nutrition, biochemistry, translational medicine; age 37, H-index 7.

Petra Janovska, Ing., PhD (Biochemistry and Pathobiochemistry, 2014) - adipose tissue metabolism, translational medicine; age 41, H-index 12.

Postdoctoral fellow(s): none

PhD/MSc/BSc students (5/4/2), laboratory assistants/animal character/secretary (3/1/1).

Former PhD students:

Tomas Jelenik, PhD (Biochemistry and Pathobiochemistry, 2010) - currently at the German Diabetes Center, Düsseldorf,Germany.

Zuzana Macek Jilkova, PhD (Biochemistry and Pathobiochemistry, 2010) - currently at the Institut Albert Bonniot, Grenoble, France.

Dasa Medrikova, PhD (Biochemistry and Pathobiochemistry, 2011) - currently at the German Cancer Research Center, Heidelberg, Germany.

Vladimir Kus, PhD (Animal Physiology, 2011) - currently at the biotech company SOTIO a.s., Prague, Czech Republic.

Michal Hensler, PhD (Molecular and Cellular Biology, 2012) - currently at the biotech company SOTIO a.s., Prague, Czech Republic.

Michaela Svobodová, PhD (Molecular and Cellular Biology, 2015) - maternity leave.

Petr Zouhar, PhD (Animal Physiology, 2015) postdoc at the University of Stockholm.

2) KEY RESULTS

i. Combined interventions using omega-3 in dietary obese mice and related clinical studies: interactions with thiazolidinediones and calorie restriction

The effects of the combined use of omega-3 with the anti-diabetic drugs from the thiazolidinedione (**TZD**) family (rosiglitazone and pioglitazone; see below) were further explored in dietary-obese mice. These results demonstrated that omega-3 could augment the insulin-sensitizing, hypolipidemic and anti-inflammatory effects of both TZDs, while specifically supporting the anti-steatotic effect of pioglitazone in the liver (Kus et al., *Plos One* **6**: e27126, 2011). In the analogous study also performed in dietary obese mice, it was demonstrated that the combined use of omega-3 and rosiglitazone stimulated the whole-body metabolic flexibility, while activating the switch between the glycolytic and oxidative muscle fibres in skeletal muscle (Horakova et al., *Plos One* **7**: e43764, 2012).

The above mentioned studies as well as the previous results (Kuda et al., *Diabetologia* **52**: 941, 2009)) suggested that the combined use of omega-3 and TZDs could improve the efficacy of the therapy of obese and diabetic patients. Therefore, in a collaboration with the Diabetes Centre (IKEM, Prague) a randomized clinical trial was perfomed during 2010-2013, using 69 patients with type 2 diabetes (all treated also with metformin). Patients were randomly assigned to a 24-week-intervention using: (i) corn oil (5 g/day; Placebo), (ii) pioglitazone (15 mg/day; Pio), (iii) EPA+DHA concentrate (5 g/day; Omega3), or (iv) pioglitazone and EPA+DHA concentrate (Pio+Omega3). Primary endpoints were changes from baseline in response to the intervention in insulin sensitivity evaluated using hyperinsulinemic-euglycemic clamp, and in triacylglycerol clearance assessed using a meal test. Omega3 and Pio+Omega3 increased EPA+DHA content in serum phospholipids. Pio and Pio+Omega3 increased body weight and adiponectin levels. Both fasting glycemia and HbA1c were increased by Omega3, but were unchanged by Pio+Omega3. Insulin sensitivity was not affected by Omega3, while it was improved by Pio+Omega3. Postprandial clearance of NEFA and triacylglycerols was additively improved by Pio+Omega3. Thus, besides preventing a modest negative effect of Omega3 on glycemic control, the combination of pioglitazone and EPA+DHA can be used to increase insulin sensitivity and postprandial lipid clearance in patients with type 2 diabetes on stable metformin therapy (these results were submitted to *Clinical Science* in April 2015).

Team's contribution: *Supported by the Ministry of Health of the Czech Republic (grant # NT13763-4; 2012-2015, PI: J. Kopecky), with a significant involvement of PJ, OH and OK, in collaboration with Diabetes Centre, IKEM, Prague (co-PI: T. Pelikanova). On all publications, the corresponding author (J.K.) and the first authors are from the Dept. of Adipose Tissue Biology, where all animal work was done.*

Decreased calorie intake is an assential part of the therapy of obese patients, and only in the combination with a decreased calorie intake, modest anti-obesity effects of omega-3 could have been unmasked also in human patients in several studies, including ours (see above). To understand the underlying mechanisms, we have studied in mice the effects of a combination treatment using 10% calorie restriction while replacing 9% of lipids in corn oil-based high-fat diet by omega-3. Our results demonstrated the additive effects of combination treatment in prevention of obesity and preservation of metabolic health, as well as the induction of mitochondrial

fatty acid oxidation, quite specifically in abdominal white fat depot. Metabolic changes in white fat were also associated with synergistic up-regulation of anti-inflammatory lipid mediators in response to the combined intervention (Flachs et al., *Diabetologia* **54**: 2626, 2011). In more recent experiments, we aimed to characterise: 1) role of dietary carbohydrates in the effects of the combined intervention; 2) the biochemical basis of energy expenditure induced in white adipose tissue by combined intervention; 3) the integrating role of lipid mediators in the effects of the combined intervention; and 4) the effects of a combined use of a natural calorie restriction mimetics, like resveratrol, and omega-3. While the results on the use of resveratrol were largely negative (van Schothorst et al., *Food Res Int* **65**: 95, 2014), our studies strongly suggest namely the induction of futile metabolic cycle based on hydrolysis of triacylglycerols and re-esterification of fatty acids in adipocytes is essential for the maintenance of the lean phenotype induced by this combination treatment. These metabolic changes in white adipose tissue are independent on thermogenesis mediated by mitochondrial uncoupling protein 1, and require oxidative phosphorylation in adipocytes. This new concept regarding the role of white fat in energy metabolism and metabolic flexibility was described in our review article (Flachs et al., *Biochim Biophys Acta* **1831**: 986, 2013). Our twin review article describing the role of lipid mediators in modulation of white fat metabolism was published within an international collaboration (Masoodi et al., *Biochim Biophys Acta* **1851***: 503,* 2015).

Team's contribution: *Supported by the Czech Science Foundation (grant # 13-00871S; 2013-2017, PI: Jan Kopecký), with a significant involvement of PF and 3 PhD students. Except the articles by van Schothorst et al. and Masoodi et al. (see above), the first (P.F.) and corresponding (J.K.) author are from the Dept. of Adipose Tissue Biology.*

ii. Basic mechanisms underlying the effects of thiazolidinediones and omega-3

The insulin-sensitizing effect of TZDs is thought to result from the activation of peroxisome-proliferator activated receptor γ (**PPARγ**), and it is associated with the induction of adiponectin and stimulation of AMP-activated protein kinase (**AMPK**). Our data suggest that the mechanisms underlying the effects of sub-optimal doses of TZDs on insulin sensitivity may depend on a change of plasma lipid profile resulting from modulation of hepatic lipid metabolism by TZDs, independent of the adiponectin-AMPK axis. Thus, the major goal of the current studies is to characterize the dose-dependent mechanisms of TZDs action using mice with genetically disrupted AMPK (a breeding colony of these mice was established through our participation in the EU FP6 project EXGENESIS, 2005-2010). Characterization of the mechanisms behind the dose-dependent effects of TZDs may help to improve combination treatment strategies for patients with type 2 diabetes (see above).

Team's contribution: *Supported by the Czech Science Foundation (grant # P301/11/0226; 2011-2015, PI: P. Flachs) with a significant involvement of 2 PhD students, in collaboration with 1st Medical Faculty, Charles University, Prague (co-PI: E. Tvrzicka).*

The metabolic and anti-inflammatory effects of omega-3 reflect multiple mechanims involved, which are, however, only partially described. In our studies in mice, we focus on several of these mechanisms, as decribed below. Thus, using the mice with genetically disrupted AMPK, we could demonstrate the role of AMPK in the preservation of hepatic insulin sensitivity by dietary omega-3, as well as in the reduction of hepatic steatosis in response to omega-3; these results were published in the "best journal in the field" (Jelenik et al., *Diabetes* **59**: 2737, 2010).

We have shown in mice fed a high-fat diet that metabolic effects of omega-3 were stronger when supplied as phospholipids as compared with triacylglycerols. These effects were associated with a more efficient modulation of major endocannabinoid molecules in white adipose tissue (Rossmeisl et al., *Plos One* **7**: e38834, 2012). These results thus demonstrate that the benefical metabolic effects are mediated in part by normalization of endocannabinoid system activity, since its activity is augmented in obesity. We have demonstrated *(Rossmeisl et al., Biochim Biophys Acta* **1841**: 267, 2014) that the hepatic effects of omega-3 administered as phospholipids are associated with an integrated inhibition of biosynthetic pathways in the liver – in collaboration with the University of Wageningen (cDNA array analyses). Further studies within this project are focused on characterization of: 1) omega-3-induced changes in the content of EPA, DHA in lipid fractions from white adipose tissue, liver and plasma, and their relationship to the levels of endocannabinoid molecules and the metabolic profile during obesity development; 2) mechanisms, by which omega-3-phospholipids affect insulin sensitivity; 3) changes in lipid metabolism of hepatocytes due to the modulation of the endocannabinoid system in adipose tissue by omega-3-phospholipids; 4) metabolic effects of omega-3 in transgenic mice carrying human PPARα. Our results will help to clarify, how modulation of the endocannabinoid system by omega-3 contributes to their beneficial effects in obesity and associated disorders.

Team's contribution: *Supported by the Czech Science Foundation (grant # 14-09347S; 2014-2016, PI: M. Rossmeisl) with a significant involvement of 2 PhD students. On all publications, the corresponding (J.K.) and first (M.R.) author are from the Dept. of Adipose Tissue Biology, where all animal work was done.*

Obesity is associated with a low-grade inflammation of adipose tissue when pro-inflammatory macrophages negatively affect adipocyte metabolism. We have shown that omega-3-derived lipid mediators, eicosanoids and docosanoids (oxylipins) released from adipose tissue, exert beneficial metabolic effects in obese mice, but the molecular mechanisms and contribution of either adipocytes or macrophages are unknown. Our hypothesis is that omega-3 specifically stimulate production of pro-resolving lipid mediators *via* a pathway integrating CD36—GPR120—MAP kinases—phospholipase A2 and the enzymes of oxylipin metabolism; see also our review article (Masoodi et al., *Biochim Biophys Acta* **1851**: 503, 2015). Our aims are to characterize 1) transport and signaling events triggered by omega-3 with relation to oxylipin production; 2) contributions of the enzymes involved in oxylipin metabolism; 3) cooperation between adipocytes and macrophages in oxylipin production by means of molecular biology and mass spectrometry. Our results will be important for a better understanding of the metabolic role of omega-3 in adipose tissue. These studies depend in large on the metabolomics approach (see section 6).

Team's contribution: *Supported by the Czech Science Foundation (grant # 13-04449P; 2013-2015, PI: O. Kuda) with a significant involvement of 1 MSc and 1 PhD student. A related project focused on fatty acid re-esterification in adipose tissue and a role of lipid mediators is supported by Ministry of Education, Youth and Sports (grant # LH14040; 2014-2016, PI: O. Kuda).*

iii. Role of mitochondria in the development of metabolic syndrome phenotypes

Obesity is associated with compromised function of mitochondria in muscle, liver as well as in adipose tissue. Our special interest in the role of adipose tissue was triggered by a discovery that the induction of energy expenditure in white adipose

tissue could counteract obesity (Kopecky et al., *Journal of Clinical Investigation* **96**: 2914, 1995), >310 citations, also consistent with our findings of the involvement of futile fatty acid cycling and mitochondrial oxidative phosphorylation in the anti-obesity effect of the combined intervention using omega-3 and calorie restriction in mice (see above). The precise biochemical basis of this mechanism and its control in white fat is studied in depth, taking advantage of the obesity-prone and obesity-resistant strains of mice (C57BL/6J vs. A/J strain) exposed to cold as a new model for differential induction of the above mentioned mechanism in abdominal fat; a new NMR-based method has been developed to assess in vivo triglyceride turnover in white fat of mice (collaboration with the University of Coimbra).
Team's contribution: *Supported by the Czech Science Foundation (grant # 13-00871S; 2013-2017, PI: J. Kopecky; see above), with involvement of PF and 3 PhD students.*

To assess the role of liver and muscle mitochondria in the interindividual differences in propensity to obesity, mice of the obesity-prone and obesity-resistant strains (see above) are studied during postnatal development and aging, while being fed a low- or high-fat diet. Large cohorts of individually caged mice (about 150 mice of each strain) have been already used in the experiment, while their obesity-related phenotypes (energy metabolism, insulin sensitivity, plasma metabolome) were recorded. In mice exhibiting extreme phenotypes, the expression of selected metabolic genes and genes for mitochondrial proteins will be analysed.

Team's contribution: *This study was initiated in the frame of the EU FP7 project no. 244995 (BIOCLAIMS; 2010-2015, co-PI: J. Kopecky), and currently it represents the key activity of the Department within the MITOCENTRUM project (see below).*

iv. Applied research - omega-3 and lignin

As described above, numerous animal and human studies are being performed using omega-3 concentrates, either in the form of triacylglycerols or phospholipids. All these studies were/are supported by various companies in Norway (e.g. Pronova BioPharma, Epax, Olympic Seafood AS, and Silentia AS), which either donated the omega-3 products or they were even directly involved in designing the studies, the analysis of the results and writing the collaborative publications (Flachs et al., *Diabetologia* **48**: 2365, 2005; Flachs et al., *Diabetologia* **49**: 394, 2006; Horakova et al., *Plos One* **7**: e43764, 2012; Jelenik et al., *Diabetes* **59**: 2737, 2010; Jilkova et al., *Int J Obes (Lond)* **38**: 216, 2014; Kuda et al., *Diabetologia* **52**: 941, 2009; Kus et al., *Plos One* **6**: e27126, 2011; Rossmeisl et al., *Obesity* **17**: 1023, 2009; Rossmeisl et al., *Plos One* **7**: e38834, 2012; Rossmeisl et al., *Biochim Biophys Acta* **1841**: 267, 2014; Ruzickova et al., *Lipids* **39** 1177, 2004).

More recently, in collaboration with a biotech company in the Czech Republic (VIDIA Inc., Vestec; http://www.vidia.cz), we aim to learn whether modified lignin, which can be isolated from a by-product of paper and cellulose production, could be applied in biomedicine as a food supplement with potential antioxidant effects, and which could also protect organism against negative environmental factors and diseases of affluence. Effects of lignin are being characterized 1) in vitro by testing cytotoxicity, genotoxicity, antioxidant effects, and occurrence of DNA modifications, as well as 2) in vivo using an animal experimental model of diet-induced obesity and metabolic syndrome to reveal potential protective effects on tissue damage, low-grade chronic inflammation, and oxidative stress. The effects of lignin on lipid and glucose homeostasis are also assessed by quantifying appropriate metabolites levels in plasma. The results should help to introduce new nutritional supplements for the protection of humans against obesity and associated diseases.

Team's contribution: *Supported by the Technology Agency of the Czech Republic (grant # TA03010581; 2013-2015, PI: M. Polakova, VIDIA Inc.), co-PI: M. Rossmeisl, with involvement of 1 PhD student.*

3) INTERNAL COLLABORATION (within the Institute)

Targeted metabolipidomics core - based on the techniques developed by O. Kuda, who is in charge of the CORE SERVICES, collaborations with:

- *Dept. of Bioenergetics* - the measurement of metabolomical profiles of cells and mitochondria;
- *Dept. of Genetics of Model Diseases* - lipidomical profiling of rat tissues;
- *Dept. of Developmental Cardiology* - lipidomical analysis of hearts;
- *Dept. of Epithelial Physiology* - measurements of steroids in rat tissues;
- *Dept. of Cardiovascular Morphogenesis* targeted analysis of phospholipids;
- *Dept. of Analysis of Biologically Important Compounds* - measurements of steroids in feathers; and
- *Dept. of Developmental Epileptology* - measurements of aminoacids in microdialysates.

OPPK project Biomodels (a centre for production, breeding and analysis of biomodels) - P. Flachs was responsible for establishing the systems for whole-body analysis of small laboratory animals using a CT/PET scanner and indirect calorimetry system at the Institute – several collaborations.

MITOCENTRE (a centre of excellence supported by the Czech Science Foundation; grant # GB14-36804G; 2014-2018; see also the Institution report) - collaborations with:

- *Dept. of Bioenergetics*; and
- *Dept. of Genetics of Model Diseases* in several tasks within the project.

Other collaborations:

- *Dept. of Developmental Epileptology* – tissue morphology and morphometry;
- *Dept. of Neurophysiology of Memory* - behavioral analysis; and
- *Dept. of Developmental Cardiology* – studies in mice with genetic disruption of AMPK.

4) DOMESTIC COLLABORATION (within the country)

Collaborations with other institutes of ASCR:

- *Institute of Molecular Biology ASCR* - measurements of eicosanoids in immune cells (P. Draber); and
- *Institute of Organic Chemistry and Biochemistry ASCR* - synthesis of lipid standards labeled with stable isotopes, synthesis of branched fatty acid esters, synthesis of sulfo-N-succinimidyl oleate (E. Kudova,); proteomical analysis of posttranslational protein modifications (J. Cvacka); analysis of membrane phospholipid composition in murine adipose tissues (I. Valterova)

Collaborations with biomedical research centers and universities*:*

- *Diabetes Centre, IKEM* - clinical trials on diabetic patients (T. Pelikanova); and

- *First faculty of Medicine, Charles University in Prague* - fatty acid composition in serum/plasma and tissue samples from mice and humans (E. Tvrzicka); collaborations within the MITOCENTRE (J. Zeman, S. Kmoch - see above).

5) INTERNATIONAL COLLABORATION

Projects supported by EU:

- EU FP7 project no. 244995 *BIOCLAIMS* (2010-2015; 11 partners; *co-PI: J. Kopecky*; see http://bioclaims.uib.es) – identification of biomarkers of health/disease; and
- EU FP7 project *DIABAT* (2011 – 2015; 20 partners; *co-PI: J. Kopecky*; see http://www.diabat.org/content/index_eng.html) - description of mechanisms that could increase energy expenditure in adipose tissue and thus prevent body fat accumulation; especially the DIABAT project corresponds well with the long-term strategy of the Department to understand the role of adipose tissue metabolism in propensity to obesity, and maintenance of the lean phenotype, respectively; both projects involve dietary and/or pharmacological interventions in laboratory mice.

Other intl. collaborations:

- J. Keijer (*Wageningen University, Netherlands*) - microarray analyses, detection of cytokines in plasma samples from mice, and the use of dietary polyphenols;
- P. Calder (*University of Southampton, UK*) - metabolomics analysis and detection of inflammation-related molecules in human serum/plasma samples;
- K. Kristiansen (*University of Copenhagen*) - lipidomics analyses, cold-exposure experiments in obesity-prone C57BL/6J and obesity-resistant A/J mice, as well as the analysis of intestinal microbiota;
- S. Cinti (*University of Ancona, Italy*) - (immuno)histological analyses of adipose tissue;
- J. Jones (*University of Coimbra, Portugal*) - NMR-based analysis of triglyceride turnover primarily in adipose tissue; and
- N. Abumrad (*Washington University in St. Louis, USA*) - the role of CD36 in lipid metabolism.

For the collaboration with industries in Norway, see section iv above.

6) KEY METHODOLOGY AND CORE FACILITIES RESPONSABILITIES

In vitro techniques - 3T3-L1 cell line, primary cultures of brown and white adipocytes, isolated hepatocytes and macrophages.

In vivo phenotyping - a complex panel of complementary methods for whole-body phenotyping in mice includes:

- indirect calorimetry system (Somedic, Sweden), comprising 8 measurement units, and enabling the assessment of whole-body metabolism, as well as physical activity and core body temperature by telemetry;
- evaluation of glucose homeostasis by hyperinsulinemic-euglycemic clamps using radiolabeled glucose (whole-body glucose turnover, glycolysis and

glycogen synthesis, hepatic glucose production, glycogen synthesis in tissues); and
- analysis of body composition using X-ray absorptiometry (a CT/PET- CORE FACILITY).

Animal facility - maintainance of breeding and experimental colonies of mice are enabled by the state-of-the-art animal facility belonging to the Department, which was established in 2003; it was the first facility of its kind at the whole Institute, which includes 5 animal rooms with stable temperature and humidity, and with a total capacity of ~1500 mice; a room with indirect calorimetry equipment (see above), a surgical unit, as well as a laboratory for performing hyperinsulinemic-euglycemic clamps (see above) and routine biochemical analyses is also located here.

Metabolomics CORE FACILITY (see also section 3) - targeted metabolomics based on a panel of LC-MS analytical methods was developed by O. Kuda, a junior scientist at our Department; the methods include:
- complex metabolomical approach (~200 polar analytes);
- analysis of lipid mediators such as eicosanoids, docosanoids, and endocannabinoids (~ 150 analytes);
- shotgun as well as targeted analysis of phospholipids and acylglycerols;
- analysis of steroids; and
- analysis of acylcarnitines and amino acids.

Cell biology techniques - fluorescence and confocal miscroscopy, molecular biology techniques such as real-time qPCR, DNA microarrays, Western blotting, etc.

The above described complex methodological repertoire puts our Department in a unique position not only within the Institute, but in the whole country. It is also a vehicle for multiple collaborations (see sections 3 and 4).

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

Department of Adipose Tissue Biology was/is a partner in two EU projects (FP7 projects BIOCLAIMS and DIABAT; see above), both of which come to the end during 2015, and it is also a partner in MITOCENTRE (see above).

8) OTHER RELEVANT INFORMATION

Several of our research papers have been awarded by various institutions and/or professional organizations:
- one article was included on the list of the most significant papers of the Academy in the year 2011 (Flachs et al., *Diabetologia* **54:** 2626, 2011);
- two articles were selected within the institutional contest „Paper of the year“ in the year 2011 Flachs et al. (see above), and in the year 2013 (Kuda et al., *J Biol Chem* **288**:1554, 2013); and
- our article was one of the three papers of the Institute, which were selected by the R&D Council of the Czech Republic among the best 20% articles in the Pillar II – quality of publications in 2014 (Jelenik et al., *Diabetes* **59**: 2737, 2010).

In 2013 Jan Kopecky was awarded by the Minister of Education, Youth and Sports for extraordinary achievements in science and innovations, for the results of the studies focused on "*New mechanisms in the complex effects of omega-3 fatty acids: perspectives for health*", based on 12 articles published in prominent international scientific journals during 2009-2013. Based on these results, new therapeutical approaches for treatment of patients with type 2 diabetes were further explored in collaboration with Diabetes Centre at IKEM, Prague (see above).

In 2014, Ondrej Kuda was awarded the Otto Wichterle Award for young scientists, specifically for their remarkable contributions to the advancement of scientific knowledge in a given area of science.

9) SUMMARY AND RESEARCH IMPACT

Our experiments conducted in mice during 2010-2014 brought results demonstrating namely:

- systemic effects of adipose tissue metabolism (fat accumulation *vs*. lean phenotype), the role of mitochondrial energy metabolism and fatty acid re-esterification in the tissue, thus revealing new possibilities for prevention and treatment of obesity-related disorders by modulating metabolism of the tissue in response to nutritional supplementation using omega-3 and other life-style interventions (calorie restriction, cold), as well as pharmacological interventions;
- the role of lipid mediators in the interplay between adipocytes and macrophages, and uncovered part of the mechanisms controlling adipose tissue metabolism in lean and obese – these studies depend in large on the novel metabolomics approach, which is available, as other methodological approaches in the corresponding core facility, to researchers within as well as outside the Institute; and
- the key role of liver metabolism (and its control by AMPK), and also the involvement of endocannabinoid system, in the beneficial metabolic effects of omega-3, and the superior metabolic efficacy of omega-3 administered as marine phospholipids (as compared with fish oils, i.e. triacylglycerols) – these studies begin to focus on glucose homeostasis, depending on the use of hyperinsulinemic-euglycemic clamp technique for assessment of insulin sensitivity; the use of marine phospholipids seems to be a better choice for dietary Omega-3 supplementation.

The results showing additive benefits of the combined interventions using omega-3 and anti-diabetic drugs in mice triggered a clinical study in patients with type 2 diabetes, which verified in part the animal data and set a solid basis for further collaborative clinical studies.

Unique methodological approaches such as indirect calorimetry, hyperinsulinemic-euglycemic clamps, and microCT have been introduced/further developed, thus representing an important integrating aspect of our collaborative studies within the Institute. Results of our studies contributed to the characterization of the basic mechanisms underlying development of the diseases triggered by the metabolic syndrome, namely type 2 diabetes. They are also relevant for improving prevention and treatment strategies of these diseases.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Biomathematics

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

The main areas of the research are:

- 3D image analysis, stereology, spatial statistics, 3D reconstruction of biological samples
- First- and second-order stereological methods: study of variance of the methods, new methods and software development, consulting and tutorials
- 3D microscopy of biological specimens:
- confocal and two-photon excitation microscopy: data processing and analysis, application of modern fluorescence approaches and recent two-photon excitation trends, calibration services, deconvolution, service and maintenance of Leica confocal microscopes including pulse near-infrared laser, consulting and tutorials
- optical projection tomography, fluorescence and transmitted light microscopy of specimens at the submacroscopic level, optical clearing of specimens

The instruments are adopted for a wide range of projects, for example, bird brain anatomy, conduction system of the heart, capillary system of the human placenta, rat muscles and brain, extracellular matrix proteins – collagen and elastin, architecture of the spruce needle and its cells, for instance, structure of the nucleus and of the endoplasmic reticulum.

ii. PERSONNEL

Senior scientist(s)

- Jiří Janáček, Ph.D. (team leader), mathematician, age 53, H-index 15
- Martin Čapek, Ph.D., expert in programming and OPT, age 42, H-index 4
- Lucie Kubínová, Ph.D., mathematician specialized in stereology and confocal microscopy, age 55, H-index 18
- Martin Kundrát, Ph.D., developmental biologist and palaeontologist, age 45, H-index 6
- Jan Michálek, Dr.sc.techn.ETH, computer scientist, age 66, H-index 2
- Radek Pelc, DPhil., expert in optics, age 49, H-index 3
- Barbora Radochová, Ph.D., expert in stereology, age 41, H-index 5
- Petr Karen, Ph.D., expert in programming, age 68, H-index 9

Junior scientist

- Zuzana Burdíková, Ph.D., expert in microscopy and testate amoebae, age 36, H-index 4

PhD/MSc/BSc students (2/0/0), laboratory assistants (2)

2) KEY RESULTS

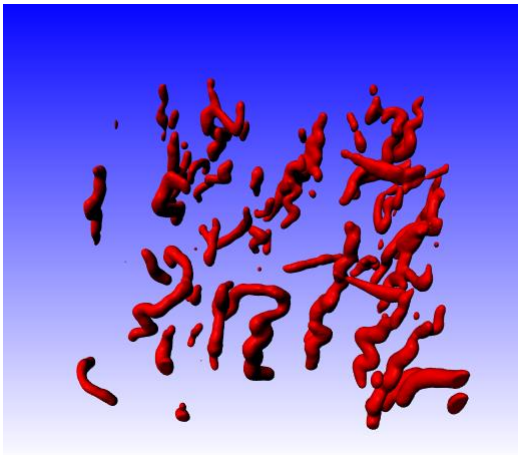
Capillary bed studies

The department was involved in several projects aimed on study of capillary bed in various tissues. The 3D architecture of the tissues was visualized by confocal microscopy. Description of changes in capillary bed often required development of new original methods for 3D image analysis.

Capillary bed in skeletal muscle

The capillary bed in skeletal muscle was studied in cooperation with Anatomical institute at University of Ljubljana, that contributed the biological topics and muscle samples. 3D image analysis methods provided improved measurement of muscle capillarity and other geometrical parameters of the capillary bed **measured from 3D images acquired by confocal microscopy** (Janáček et al., **Microvascular Research 81, 2, 2011). The capillary network is quantitatively described by the length of capillaries per unit volume of muscle tissue and the length of capillaries supplying individual muscle fibres per unit fibre length, per surface area and per volume. The course of capillaries in the muscle is characterized by the tortuosity, orientation and mean capillary length.**

Applying confocal microscopy and virtual 3D stereological grids, or tracing of capillaries in virtual reality, length of capillaries within a muscle volume or length of capillaries adjacent to a muscle fibre per fibre length, fibre surface area or fibre volume are evaluated and 3D models of capillaries and muscle fibres are produced. The described 3D methodology is applied to the anatomical remodelling of capillarity during acute denervation and early reinnervation in the rat soleus and extensor digitorum longus muscles (Eržen et al., **Physiological Research 60, 1, 2011**) and enabled measurement of aging effect on capillary bed (Cvetko et al., **Image Analysis and Stereology 32, 3, 2013**).



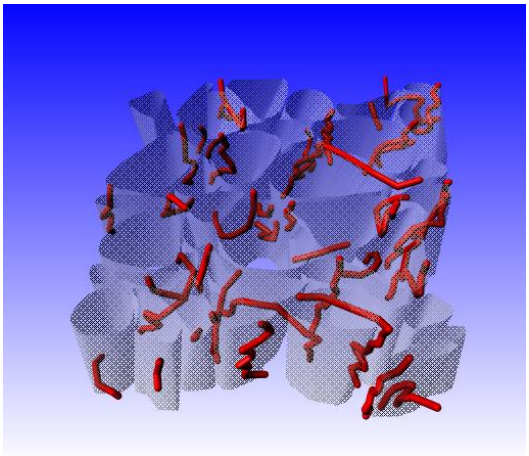


Figure 1. Capillary bed in human skeletal muscle (vastus lateralis). Left - surface rendering, right – skeleton of capillaries with fibres.

We also compared our 3D method of evaluation of capillary supply of individual skeletal muscle fibers where the mean length of capillaries around individual muscle fibers per fiber length (Lcap/Lfib) is measured from 3D images acquired by confocal microscopy with the traditional one using 2D images of thin transverse sections by the number of capillary profiles around a fiber (CAF) (Čebašek et al., **Microvascular Research 79, 1, 2010, corresponding author L. Kubínová). We showed on geometrical models that Lcap/Lfib was sensitive to different arrangements of capillaries, while CAF underestimated capillarization since it could not detect the increased length of capillary bed. In true muscle samples, we detected statistically significant differences in the capillary supply of control and denervated rat soleus muscles by both 2D and 3D methods. Lcap/Lfib was larger than CAF in control muscles reflecting their more complicated capillary bed.**

We studied the deformation in thick sections of the rat skeletal muscle from complete stacks of images captured by confocal microscope in order to avoid deformation artefacts in capillary measurement. Rescaling by the inverse of the axial scaling factor of the stack of optical slices in the direction of the microscope optical axis satisfactorily corrects the axial deformation of skeletal muscle samples (Janáček et al., **Journal of Microscopy 246, 2, 2012).**

The collaboration was supported by ministry of education, youth and sports (grant MEB090910). L. Kubínová participated by designing stereological methods, J. Janáček by development of 3D image analysis methods, PhD student A. Vyhnal by constructing models of capillary bed.

Muscle fibers classification and myosin composition

The same collaboration with Anatomical institute at University of Ljubljana yielded results concerning muscle fibers classification and myosin composition in masseter muscle. A study dealing with MyHC isoforms expression in edentulous persons with the aim to clarify to which extent the decreased functional load following teeth loss contributed to the changed muscle phenotype during ageing (Cvetko et al., **Journal of Oral Rehabilitation 39, 8, 2012).** The observed differences in the proportion of fibre types between denture wearers and dentate subjects cannot be ascribed to degenerative changes intrinsic to the ageing muscle, but to functional differences in muscle activity and to morphological alterations of stomatognathic system accompanying the complete teeth loss. Adult masseter muscles contain a substantial portion of MyHC-neo, which is coexpressed with mature MyHC isoforms (Cvetko et al., **Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology 295, 8, 2012)**. The diminished expression of MyHC-neo with age could point to a lower regeneration capacity of masseter muscle in the elderly.

The study describing the myosin heavy chain (MyHC) isoform composition of the sternocleidomastoid (SCM) muscle of presumably healthy young males was performed for the purpose of better understanding the contractile properties of the muscle as well as to help in evaluation of pathologically altered structure of the muscle. Autopsy samples were processed

immunohistochemically to reveal the MyHC isoform composition. In addition to the MyHC isoforms, characteristic of adult limb muscles, a very low percentage of muscle fibres (0.2–2.7%) expressed MyHC-neo, which is normally not found in adult limb muscles. Since the SCM muscle shares the same embryogenic potential as limb muscles, its distinct MyHC expression appears to be associated with twin innervation and with the intrinsic specialisation to perform multiple functions (Cvetko et al., **Annals of Anatomy-Anatomischer Anzeiger 194, 5).**

Our department participated on this results by implementing muscle fibre classification methods done by P. Karen.

Capillaries in brain

Capillary bed in brain was studied in collaboration with Loma Linda University (CA, USA) in connection with radiation effects of 56Fe ions (Mao et al., **Radiation Research 173, 2010, corresponding author L. Kubínová**). In contrast to the hippocampal cornu ammonis region 1 (CA1), in the dentate gyrus (DG), there was no significant difference in microvessel cell and length density between irradiated groups and age-matched controls.

Methods for measurement of capillary bed in 3D using automatic and interactive stereological methods were improved and tested for precision, applicability and workload (Kubínová et al., **Microscopy and Microanalysis 16, Suppl.2, 2010;** Kubínová et al., **Microscopy and Microanalysis 19, 4, 2013**). 3D confocal images of two different rat brain regions processed by three types of capillary length estimation methods: (1) Stereological methods based on a computer generation of isotropic uniform random virtual test probes in 3D, either in the form of spatial grids of virtual "slicer" planes, or spherical probes. (2) Automatic method employing a digital version of the Crofton relations using the Euler characteristic of planar sections of the binary image. (3) Interactive "tracer" method for length measurement based on a manual delineation in 3D of the axes of capillary segments. The presented methods were compared in terms of their practical applicability, efficiency and precision. Interactive methods can be used more widely and sometimes even with low quality images, but are more time consuming.

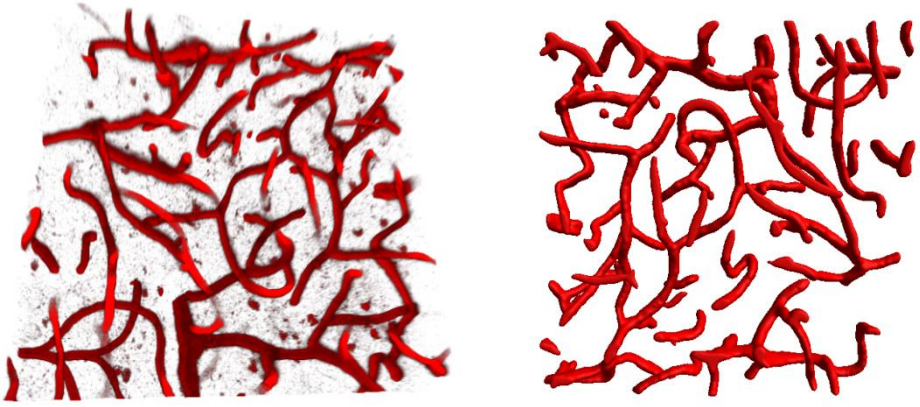


Figure 2. Capillaries in rat brain cortex stained by perfusion of biotinylated lectin and by FITC streptavidin. Left – volume rendering, right – surface rendering.

The collaboration was supported by ministry of education, youth and sports (grant ME09010). Department participated by pictures acquisition by confocal microscope. L. Kubínová participated by designing stereological methods, J. Janáček by development of 3D image analysis methods.

Capillaries in placenta

Placenta capillary bed branching pattern was described in cooperation with 1st. Faculty of medicine Charles University (Jirkovská et al., *Placenta* 33, 5, 2012). In comparison with normal villi, capillaries of hypovascular villi had a smaller diameter and displayed a markedly wavy course whereas in hypervascular villi numerous capillaries occurred in reduced stroma and often had a large diameter. The quantitative assessment of capillary branching has shown that villous capillaries are more branched in diabetic placentas. J. Janáček and L. Kubínová participated in stereological part of this research. This research was supported by the Czech Science Foundation (grant GA304/09/0733).

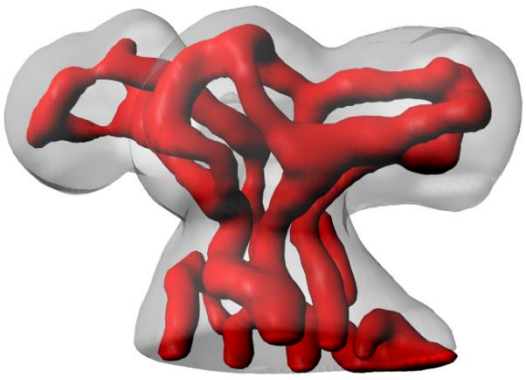


Figure 3. Surface rendering of cpillary bed inside terminal villus of human placenta.

Methods for capillary bed measurement

Capillaries anisotropy in cerebral tissues was studied (Kochová et al., **Journal of Theoretical Biology 286, 2011**). A novel method for evaluating the anisotropy of line systems that involves comparing the observed length densities of lines with the discrete uniform distribution of an isotropic line system with the chi2-test. The rose of

directions, preferential directions and level of anisotropy of linear systems representing the microscopic blood vessels were measured in samples of various regions from human brains (cortex, subcortical gray matter and white matter). The novel method was compared with two other methods used for anisotropy quantification (ellipsoidal and fractional anisotropy). All three methods detected different levels of anisotropy of blood microvessels in human brain. The microvascular bed in the cortex was closer to an isotropic network, while the microvessels supplying the white matter appeared to be an anisotropic and direction-sensitive system.

Survey of principal analytical methods for capillary bed measurement and visualization was published (Janáček et al., **Physiological Research, 63, suppl.1, 2014**). Length, branching, density, tortuosity and orientation of tubular structures in biological samples were mesured. Methods for analysis of large samples by measurement of local differences in geometrical characteristics were introduced. The methods were demonstrated on the structure of the capillary bed in a rat brain

Two photon imaging of yeast colonies

Two photon imaging enabled precise imaging of 3D architecture of structured yeast colonies and helped to reveal mechanisms of the colonies formation. (Váchová et al., **Journal of Cell Biology 194, 5, 2011**). The imaging method was developed by O. Chernyavkiy.

Dose response curves

The department also participated in analysis of dose-response curves. Fitting of general logistic curves by nonlinear minimization enables such analysis even in cases complicated by unavailability of saturation values. The biological problem and the data were provided by department of experimental hypertension (Líšková et al., **BioMed Research International 2014;** Vaněčková et al., **Acta Physiologica 208, 4, 2013;** Behuliak et al., **Journal of Hypertension 31, 10, 2013;** Líšková et al., **European Journal of Pharmacology 667, 1-3, 2011;** Pintérová et al., **Journal of Hypertension 28, 5, 2010).** The method was designed and implemented by P. Karen.

Preprocessing of microscopic images

Methods for preprocessing of microscopic images, namely removal of uneven illumination (vignetting) effect by estimation of structural information (Michálek et al., *Microscopy Research Technique* 74, 9, 2011; Michálek et al., *Microscopy and Microanalysis* 16, Suppl.2, 2010). Another image processing topic was development and validation of original method for registration of images of serial physical sections which can correct minor defects like deformation, fissures or foldings, while current registration algorithms could treat only displacement or smooth deformations. (Michálek and Čapek, *IEEE Transactions on Medical Imaging* 32, 5, 2013; Michálek et al., *Microscopy and Microanalysis* 17, 6, 2011; Michálek et al., *Microscopy and Microanalysis* 16, Suppl.2, 2010). The methods were realized by J. Michálek and M. Čapek in research funded by by ministry of education, youth and sports (grants LC06063 and MEB0810092), and the Czech Science Foundation (grant GA102/08/0691).

Tracking multiple objects in microscopic images

Tracking of multiple objects image sequence was implemented for analysis of movements of microtubules plus-ends in activated mast cells (Hájková et al., *Journal of Immunology* 186, 2, 2011). Activation of mast cells increases the number of growing microtubules in cell periphery. Knock down of STIM1 prevents changes in microtubule dynamics in activated cells. The tracking of glioblastoma cells nuclei was applied to measure relationship between spastin expression and cell migration velocity (Dráberová et al., *Journal of Neuropathology and Experimental Neurology* 70, 9, 2011). Spastin depletion by knock down decreased velocities of the cells. This results suggest that targeting spastin may offer a promising therapeutic strategy directed against glioma cell invasion. The procedure was developed by J. Janáček for laboratory of biology of cytoskeleton of Institute of Molecular Genetics.

Cells growth on surfaces covered by nanodiamonds

Evaluation of cells growth on surfaces covered by nanodiamonds was done in collaboration with department of Biomaterials and Tissue Engineering. Two-photon excitation microscopy and second-harmonic generation imaging was applied to extracellularly distributed collagen and chondrocytes seeded in artificial scaffolds (Filová et al., *Journal of Biomedical Optics,* Vol. 15, 6, 2010 Resulting high-resolution 3-D images enable also to quantitatively analyze the collagen volume and a spatial arrangement of cell-collagen-scaffold systems and to correctly evaluate reaction of seeded chondrocytes to substrate and growing conditions necessary for optimization of the cultivation. Human osteoblast-like cells cultured on nanocrystalline diamond films doped with three distinctively different concentrations of boron increasing diamond conductivity were visualized by fluorescence microscopy (Grausová et al., *PLoS ONE* 6, 6, 2011). Analysis of the adhesion, growth, viability, phenotypic maturation and potential immune activation proved that boron doping is not detrimental for the cells. Some parameters even suggest that boron doping is beneficial for growth. The research was supported by the Czech Science Foundation (grant GAP108/11/0794). Z. Burdíková with L. Kubínová and M. Čapek participated in this project.

Plant anatomy

The department participated in evaluation of anatomical effects of CO2 enrichment on beech and spruce (Lhotáková et al., *Plant Science* 188-189, 2012) supported by the Czech Science Foundation (grant GAP501/10/0340). Necessity of genuine 3D approach for correct cellular organelles counting was demonstrated on data from this project (Kubínová et al., *Journal of Experimental Botany* 65, 2, 2014) in contrast to widely used intuitive 2D methods (e.g. counting of profiles in a thin section). The value is underestimated by 90 per cent when counting chloroplast profiles in 2D and similar disproportion can be expected always when counting small cellular particles. B. Radochová, L. Kubínová, J. Michálek and J. Janáček were involved in this project. The research was done in close collaboration with department of experimental plant biology of faculty of science of Charles University.

Testate amoebae ecology

Application of advanced microscopical techniques on taxonomy and ecology of testate amoebae (Burdíková et al., *Microscopy and Microanalysis* 16, 6, 2010; Burdíková et al., *Microscopy and Microanalysis* 16, Suppl.2, 2010; Burdíková et al., Microbial Ecology 64, 1, 2012; Vohník et al., *Acta Protozoologica* 51, Sp. iss.3, 2012; Vohník et al., *Microbial Ecology* 61, 3, 2011). The research was realized by Z. Burdikova partly in collaboration with Department of mycorrhizal symbioses of Institute of Botany. In microscopic part of the project collaborated also R. Pelc and M. Čapek.

3) **INTERNAL COLLABORATION (within the Institute)** –

Common projects with Department of Biomaterials and Tissue Engineering, Department of Cardiovascular Morphogenesis, Department of Computational Neuroscience and Department of Biochemistry of Membrane Receptors.

Publications with Department of Experimental Hypertension.

4) **DOMESTIC COLLABORATION (within the country)** -

Department of Experimental Plant Biology of Faculty of Science of Charles University – plant anatomy, common publications (Kubínová Z. et al. Journal of Experimental Botany 65, 2010), grant project by Czech Science Foundation.

Laboratory of Biology of Cytoskeleton of Institute of Molecular Genetics – tracking objects in microscopic images, common publications (Hájková et al., *Journal of Immunology* 186, 2, 2011, Dráberová et al., *Journal of Neuropathology and Experimental Neurology* 70, 9, 2011).

Laboratory of Biology of the Cell Nucleus of Institute of Molecular Genetics – spatial statistics in biological images (Philimonenko A. et al. *Journal of Structural Biology*, Vol. 173, 2, 2011; Philimonenko V. et al. *Histochemistry and Cell Biology*, Vol. 134, 3, 2010).

Institute for Clinical and Experimental Medicine, grant Developmental modularity of cerebral tissues in the evolution of avian locomotion using high-

resolution imaging and geometric morphometry **by Czech Science Foundation**.

Institute of Microbiology, common publication (Váchová et al., **Journal of Cell Biology 194, 5, 2011**), grant proposal with L. Vanucci

1st. Faculty of medicine Charles University, publication (Jirkovská et al., *Placenta* 33, 5, 2012), grant by the Czech Science Foundation

5) **INTERNATIONAL COLLABORATION –**

Anatomical institute at University of Ljubljana – travel grant The role of distal nerve stump on remodelling of capillary network and myosin heavy chain isoform transformation in denervated rat skeletal muscle

Loma Linda University (CA, USA) – project Quantitative measurement of vasculature by stereology and 3D image analysis for evaluation of effect of proton or iron ions irradiation on eye and brain blood supply.

6) **KEY METHODOLOGY AND CORE FACILITIES**

Core facility in servis regime with confocal and MP microscope Leica SP2 with laser Coherent Ultra, served by O. Cherniavskyi, Optical tomograph Bioptonics, open SPIM.

Huygens deconvolution and Amira image analysis software.

Development of original procedures and implementations in image analysis in 2D and 3D and interactive methods in 2D and 3D stereology.

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

European Strategy Forum on Research Infrastructures (ESFRI)
Euro-BioImaging, coordinating instition: EMBL, SRN
Coordinating person from CAS for Czech Republic - prof. Pavel Hozák (Institute of Molecular Genetics), for Institute of Physiology – Lucie Kubínová

8) OTHER RELEVANT INFORMATION

2012-2014 joint project with Laboratory Imaging ltd. (author os NIS elements, software for Nikon microscopes) on microscopic image processing funded by Technology Agency of the Czech Republic.

9) SUMMARY AND RESEARCH IMPACT

New original methods for 3D image analysis were developed and applied e.g. in capillary bed studies:

- 3D image analysis methods provided improved measurement of muscle capillarity and other geometrical parameters of the capillary bed **measured from 3D images acquired by confocal microscopy.** The 3D methodology is applied to the anatomical remodelling of capillarity during acute denervation and early reinnervation in and enabled measurement of aging effect on capillary bed.
- Methods for measurement of brain capillary bed in 3D using automatic and interactive stereological methods were improved and tested for precision, applicability and workload: (1) Stereological methods based on a computer generation of isotropic uniform random virtual test probes in 3D, either in the form of spatial grids of virtual “slicer” planes, or spherical probes. (2) Automatic method employing a digital version of the Crofton relations using the Euler characteristic of planar sections of the binary image. (3) Interactive “tracer” method for length measurement based on a manual delineation in 3D of the axes of capillary segments. Interactive methods can be used more widely and sometimes even with low quality images, but are more time consuming.
- Placenta capillary bed branching pattern was described. The quantitative assessment of capillary branching has shown that villous capillaries are more branched in diabetic placentas.
- Toolbox of principal analytical methods for capillary bed measurement and visualization was prepared and tested. Methods for analysis of large samples by measurement of local differences in geometrical characteristics were introduced.

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Two photon imaging enabled precise imaging of 3D architecture of structured yeast colonies and helped to reveal mechanisms of the colonies formation.

Fitting of general logistic curves by nonlinear minimization developed in the department enables dose-response curves analysis even in cases complicated by unavailability of saturation values.

Methods for preprocessing of microscopic images, namely removal of uneven illumination (vignetting) effect by estimation of structural information were developed**.** **D**evelopment and validation of original method for registration of images of serial

physical sections which can correct minor defects like deformation, fissures or foldings was accomplished by the department.

Tracking of multiple objects image sequence was implemented for analysis of movements of microtubules plus-ends in activated mast cells and for tracking of glioblastoma cells nuclei applied to measure relationship between spastin expression and cell migration.

Evaluation of cells growth on surfaces covered by nanodiamonds was done in collaboration with department of Biomaterials and Tissue Engineering and proved beneficial properties of the surfaces for cellscultivation.

Necessity of genuine 3D approach for correct cellular organelles counting was demonstrated on data from this project in contrast to widely used intuitive 2D methods (e.g. counting of profiles in a thin section). The value is strongly underestimated when counting profiles of small cellular particles.

Advanced microscopical techniques were introduced into taxonomy and ecology of testate amoebae.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Biomaterials and Tissue Engineering

1) MAIN RESULTS OBTAINED in 2010-2014:

Although the Institute of Physiology is primarily focused on fundamental research, our department also deals with applied research in collaboration with other research institutes, universities, hospitals and private companies. The main tasks of our laboratory are:

- Improving currently-used synthetic tissue replacements (mainly vascular and bone replacements) by introducing cell and other biological components
- Constructing novel tissue replacements based on materials (synthetic and natural) and cells

To achieve these two main goals, we carry out studies on the molecular mechanisms of cell behavior on their material carriers, such as the adhesion, growth, differentiation, phenotypic maturation, viability, metabolic activity, potential damage and immune activation of cells.

1. Innovation of vascular replacements currently used in clinical practice

Clinically used vascular replacements are made of synthetic polymers, namely polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE). These prostheses are not primarily intended for reconstruction of endothelial cell layer, which is considered as the best prevention of thrombosis, intimal hyperplasia and restenosis of the prosthesis. The material is too hydrophobic, and due to the prosthesis preparation by knitting and weaving technologies, also of inappropriate surface roughness and topography. For the prosthesis endothelialization, it is necessary to modify the inner surface of the prosthesis. In collaboration with the Institute of Macromolecular Chemistry, we have chosen modification with fibrin layers prepared by an in vitro simulation of a part of physiological hemocoagulation process. Fibrin could be isolated in a reasonable quantity in the autologous form, i.e. from the patient´s own blood. The fibrin films have nanofibrous morphology (**Fig. 1A**), and the thickness, length and density of these nanofibers can be controlled by the use of stimulators and inhibitors of fibrin formation from fibrinogen. The adhesion, growth and phenotypic maturation of endothelial cells and the development of a confluent layer by these cells (**Fig. 1B, C**) can be further improved by combination of fibrin with extracellular matrix molecules, such as collagen, fibronectin and laminin (Filová et al., *J Biomed Mater Res A* **102**: 698, 2014). Experiments in dynamic cell culture systems with planar samples (Chlupac et al., *Tissue Eng Part A* **20**: 2253, 2014) and tubular samples (Chlupac et al., *Physiol. Res*. **63**: 167, 2014) revealed that particularly the combinations with fibronectin improved the retention of endothelial cells on the prosthesis under flow and their favorable phenotype. The endothelialized prostheses were also implanted *in vivo* into minipigs, and the obtained results are in preparation for publication.

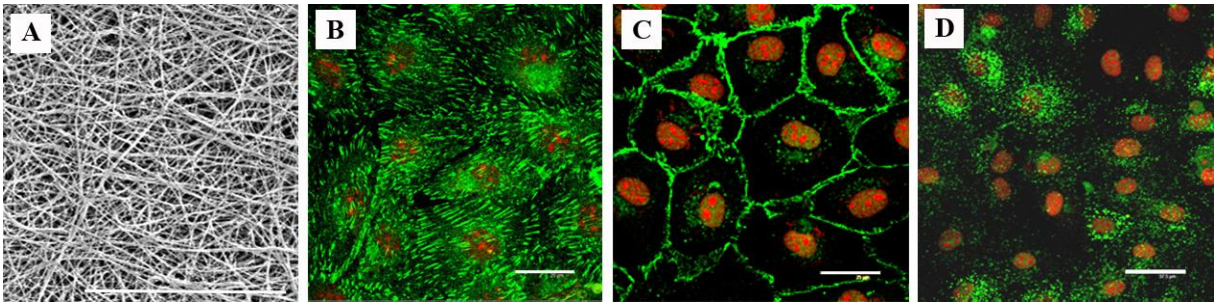


***Fig. 1**. A nanofibrous fibrin layer for inner modification of vascular prostheses (**A**), and immunofluorescence of talin (**B**) VE cadherin (**C**) and von Willebrand factor (**D**) in endothelial CPAE cells in cultures on these layers. Bar 10 μm (A) or 25 μm (B, C ,D).*

2. Construction of novel vascular replacements

Advanced tissue engineering aims at functionalization of biomaterials with short specific ECM-derived oligopeptidic ligands for cell adhesion receptors, which can be prepared synthetically, are not species-specific and thus they are not immunogenic or associated with the disease transmission as the entire protein molecules. In addition, these oligopeptides can be attached to the biomaterial surface in defined types, concentrations, spacing and distribution. Thus, in collaboration with the Institute of Macromolecular Chemistry, RGD-containing oligopeptides, i.e. ligands for integrin adhesion receptors, were attached to a bioinert cell non-adhesive background in defined concentrations. The number of initially adhered vascular endothelial cells and their spreading area then increased with increasing oligopeptide concentration (**Fig. 2**) (Popelka et al., *Eur. Polym. J.* **58**: 11, 2014). In another study, which was submitted to *Biomaterials*, the RGD-containing oligopeptides were combined with a collagen-derived peptide, which markedly increased the endothelial cell retention in a dynamic cultivation system.

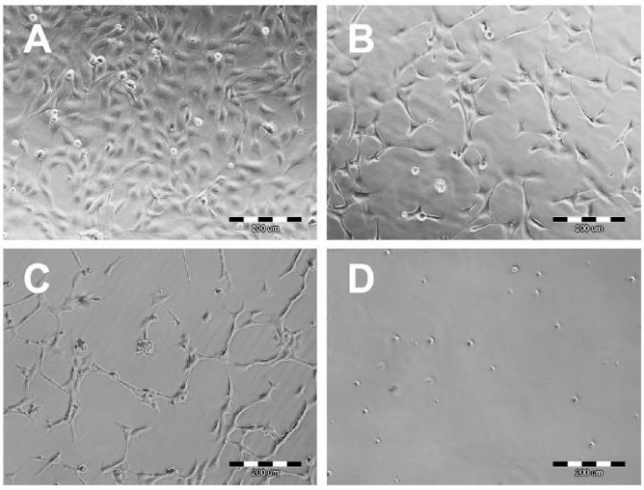


***Fig. 2**. Native vascular endothelial CPAE cells after 24 h of cultivation on PEO/PGMA layer containing RGD ligand at a surface density of 340 (**A**), 200 (**B**), 52 (**C**) and 0 pmol/cm^2 (**D**). Bar = 200 μm.*

The Research on vascular replacements was supported by the Grant Agency of the Czech Republic (GAČR, projects No. P108/10/1106 and P108/11/1857 in collaboration with the Institute of Chemical Technology in Prague and Institute of Macromolecular Chemistry, Acad. Sci CR), and by the Ministry of health of the CR (grant No. NT11270–4/2010 in collaboration with the Institute of Clinical and Experimental Medicine - IKEM). The scientists from our department elaborated the entire part dealing with cell-material interaction, while the other

institutions prepared, characterized or modified the material components of the replacements. IKEM also prepared potential clinical applications of the constructs.

3. Innovation of bone implants currently used in clinical practice

Currently usedbone implants for load-bearing applications, such as bone-integrating parts of big joint replacements, are fabricated of metallic materials. The osseointegration of these implants can be improved by appropriate surface modifications, which can be divided into subtractive modifications (e.g., grinding, polishing, machining, acid etching), or additive modifications (coating with mechanically and chemically resistant bioactive films).

Subtractive technologies, namely combination of electric discharge machining, acid etching and shot peening with ceramic balls were applied in collaboration with the Faculty of Mathematics and Physics, Charles University, Prague, and Beznoska Ltd., Kladno, in order to improve the osseointegration of a hip joint prosthesis made of Ti-6Al-4V alloy. The acid etching played a decisive role in stimulation of the adhesion, spreading, proliferation and mitochondrial activity of human osteoblast-like cells, which was attributed particularly to the increased oxidation and wettability of the material surface. On the other hand, electric discharge machining itself or in combination with shot peening supported osteogenic cell differentiation, manifested by increased expression of collagen I (Havlíková et al., *Mater Sci Eng C Mater Biol Appl.* **39**: 371, 2014). This technology was also patented (Fencl et al., Czech patent, 2014).

An example of additive technologies used in our studies was the development of nanocrystalline diamond films (NCD) for potential coating of bone implants, particularly for the use in stomatology NCD films themselves proved as excellent substrates for the adhesion, growth and osteogenic differentiation of human osteoblast-like cells. These beneficial effects were further enhanced with boron doping of NCD films, which was explained by the electrical conductivity of boron-doped NCD films (Grausová et al., *PLoS One* **6**: e20943, 2011), and by surface termination with oxygen, which increased the surface wettability (**Fig. 3**). By creation of surfaces patterned with oxygen-terminated hydrophilic and hydrogen-terminated hydrophobic microdomains, regionally-selective cell adhesion on O-terminated domains was obtained. This technology was patented (Rezek *et al*., EU patent, 2011).

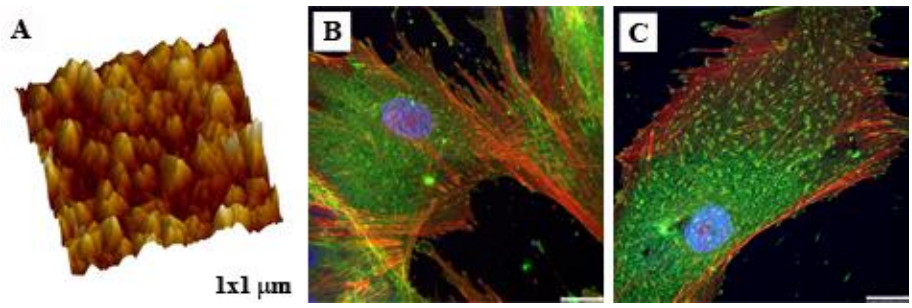


***Fig. 3**. Morphology of nanocrystalline diamond film (**A**) and human osteoblast-like Saos-2 cells in cultures on these films terminated with H (**B**) and O (**C**). AFM microscopy (A), immunofluorescence of talin combined with phalloidine staining of F-actin (B, C). The cells on O-terminated surfaces have better developed talin-containing focal adhesion plaques. Bar=20 μm.*

4. Construction of novel bone tissue replacements

Metallic materials currently used for bone implantation are mechanically strong but they do not allow ingrowth of the bone tissue inside the implant. For these purposes, porous or fibrous polymer scaffolds, reinforced with carbon or ceramic nanoparticles (simulating the inorganic component of the bone matrix) are suitable.

In the first set of studies, we used non-degradable and relatively mechanically strong polymers, such as a terpolymer of polytetrafluoroethylene, polypropylene and polyvinyldifluoride (PTFE/PP/PVDF) or polysulfone (PSU)) reinforced with carbon nanotubes or carbon nanohorns. Both types of carbon nanoparticles improved the mechanical properties of the materials and their colonization with human osteoblast-like cells. The positive effect of the carbon nanoparticles on the cell behavior was more apparent in PTFE/PP/PVDF than in PSU (Staňková et al., *Carbon* **67**: 578, 2014).

In the second set of studies, we concentrated on nanofibrous scaffolds made of degradable polymers, namely poly-(L-lactide) (PLLA) and poly-(L-lactide-co-glycolide) (PLGA), reinforced with carbon or ceramic nanoparticles (**Fig. 4**). PLGA nanofibers loaded with approx. 23 wt.% of nanodiamond particles supported the adhesion and growth of human osteoblast-like MG-63 cells in a similar extent as pure PLGA nanofibers, but accelerated the growth of human bone marrow mesenchymal stem cells (Pařízek et al., *Int. J. Nanomed.* **7**: 1931, 2012; Bačáková et al., *Phys. Status Solidi A,* **211**: 2688, 2014). PLLA nanofibers with 5 wt. % and particularly with 15 wt.% of hydroxyapatite nanoparticles stimulated the growth and osteogenic differentiation of MG-63 cells (Novotná et al., *J. Biomed. Mater. Res. A* **102A**: 3918, 2014).

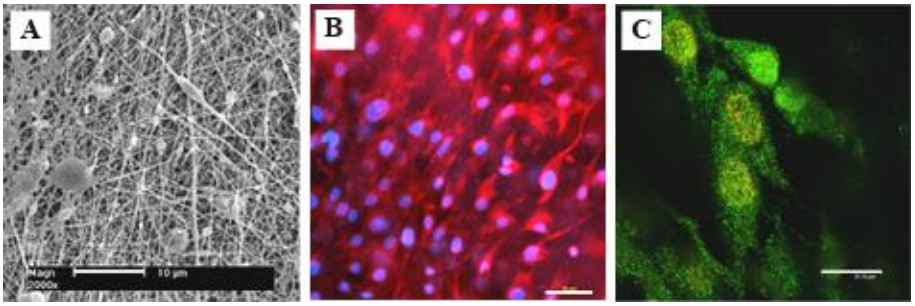


Fig. 4*. Nanofibrous PLGA scaffolds reinforced with 23 wt% of nanodiamond (**A**), human osteoblast-like MG-63 cells in cultures on these scaffolds (**B**) and MG-63 cells in cultures on PLLA scaffolds with 15 wt. % of hydroxyapatite nanoparticles (**C**). Scanning electron microscopy (A), staining with Texas Red-C2-Maleimide and Hoechst #33342 (B), immunofluorescence of osteocalcin (C). Bar 10 μm (A), 50 μm (B) or 20 μm (C).*

The research on bone replacements was supported by the Grant Agency of the Czech Republic (GAČR, grants No P107/12/1025, P108/12/1168, P108/12/G108, 14-04790S) in collaboration with the Institute of Chemical Technology, Prague, Faculty of Mathematics and Physics, Charles University and Institute of Physics, Acad. Sci. CR). Other support was provided by the Technological Grant Agency of the CR (TAČR, grant No. TA0101114. in collaboration with the company Beznoska s.r.o.). The scientists from our department were responsible for the part dealing with the cell-material interaction, the other institutions prepared and charaterized the material.

2) INTERNAL COLLABORATION (within the Institute)

The Dept. of Biomaterials and Tissue Engineering has collaboration with the following Departments in the Inst. Physiol.:

Department of Biomathematics: advanced imaging technologies for studies of cell behavior on artificial materials, e.g. confocal microscopy of cells on porous and fibrous scaffolds or pseudo-3D surfaces with high roughness and complicated topography, SHG technique for visualization of native collagen, quantification of the intensity of fluorescence of various specific molecules in cells (Z. Burdíková). Other fields are statistical evaluation of the obtained results and measurement of mechanical properties of nanofibrous materials (D. Hadraba). We had a common project „Visualization of collagen production in osteogenic cells cultivated on nanocrystalline diamond films", project GAČR, ID P108/11/0794. Principal Investigator: Lucie Kubínová, MSc., PhD, Institute of Physiology, Acad. Sci. CR. Prague. Co-Investigator: Alexander Kromka, MSc. PhD, Institute of Physics, Acad. Sci. CR. Prague, 2011-2013

Department of the Analysis of Biologically Important Compounds: quantification of adsorption of collagen materials with various chemical and physical surface properties, quantification of collagen production by cells on these materials by conventional and capillary electrophoresis and mass spectromectry (A. Eckhardt).

Common publications with these Departments:

Grausová L, Kromka A, Burdíková Z, Eckhardt A, Rezek B, Vacík J, Haenen K, Lisá V, Bačáková L: Enhanced growth and osteogenic differentiation of human osteoblast-like cells on boron-doped nanocrystalline diamond thin films. PLoS One 6(6):e20943, 2011.

Novotna K, Zajdlova M, Suchy T, Hadraba D, Lopot F, Zaloudkova M, Douglas TE, Munzarova M, Juklickova M, Stranska D, Kubies D, Schaubroeck D, Wille S, Balcaen L, Jarosova M, Kozak H, Kromka A, Svindrych Z, Lisa V, Balik K, Bacakova L. Polylactide nanofibers with hydroxyapatite as growth substrates for osteoblast-like cells. J Biomed Mater Res Part A 2014:102A:3918–3930

Department of Cardiovascular Morphogenesis: *in vivo* tests of materials which gave promising results in cell culture systems, e.g. implantation of materials coated with nanocrystalline diamond films into rabbit bones (Prof. D. Sedmera). We have obtained a common project "Bioactive nanostructured surfaces for histocompatible implants" from the Ministry of Health of the Czech Republic, Programme for Supporting the Applied Medical Research and Development (AZV-VES) for years 2015-2019.

3) DOMESTIC COLLABORATION (within the country)

Since the tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function, our Department has a broad collaboration with institutions dealing with material engineering, physics and chemistry, companies producing body implants or materials promising for these implants and hospitals:

Institutes of the Acad. Sci. CR:

Institute of Macromolecular Chemistry: materials functionalized with cell adhesion-mediating proteins, oligopeptidic ligands for cell adhesion receptors, recombinant growth factors

Institute of Physics: nanocrystalline diamond films for biomaterial coating and construction of biosensors and biostimulators, composite polymer-nanodiamond scaffolds for bone tissue engineering, diamond nanoparticles for potential drug delivery and bioimaging

Nuclear Physics Institute: Fullerene and metal-fullerene films for potential biomaterial modification

Heyrovský Institute of Physical Chemistry: surface modification of metallic materials for potential bone implants by thermal oxidation, zeolite-based ceramics for bone tissue engineering

Institute of Inorganic Chemistry: carborane films with golden micro- and nanoparticles for potential surface modification of biomaterials.

Universities:

Institute of Chemical Technology in Prague: synthetic polymeric materials modified with plasma, ion implantation or UV-light irradiation, grafting biomolecules and gold or carbon nanoparticles for vascular, bone and skin tissue engineering (Prof. V. Švorčík). Modification of metallic materials for potential bone implantation with TiO_2 nanotubes (Prof. Joska).

Czech Technical University, Faculty of Mechanical Engineering: metallic materials for construction of bone implants with various surface modifications (deposition of oxides, electroactive films, carbon-based films, controlled surface roughness and topography by grinding and polishing) (Prof. P. Špatenka, Assoc. Prof. V. Starý).

Charles University, Faculty of Mathematics and Physics: hydrocarbon plasma polymer films for surface modification of bone implants (Prof. H. Biederman)

Charles University, 2nd Medical Faculty: modified collagen films for growth regulation of vascular smooth muscle cells (Prof. J. Herget)

J.E. Purkinje University: measurement of physical and chemical properties of biomaterials (Assoc. Prof. Z. Kolská)

Hospitals:

Institute of Clinical and Experimental Medicine (IKEM), Prague: In vivo tests of innovated vascular prostheses after implantation into minipigs (Dr. J. Chlupáč), construction of heart valve replacements (Prof. J. Pirk), development of perivascular drug delivery system (Prof. Pirk).

Faculty Hospital Na Bulovce, Prague, Orthopaedic Clinic: innovation of hip joint replacements, influence of BMP-7 on the growth and osteogenic differentiation of osteoblasts

Faculty Hospital Na Bulovce, Clinic of Plastic Surgery: isolation of adipose stem cells for tissue engineering

General Teaching Hospital, 1st Medical Faculty, Charles University, Prague: surface modifications of stomatological implants

Private Companies:

Beznoska Ltd., Kladno: innovation of big joint replacements by appropriate surface modifications: plasma treatment, sandblasting, polishing, electric discharge machining, deposition of biocompatible films (hydroxyapatite)

Prospon Ltd., Kladno: innovation of big joint replacements by deposition of TiO_2 films

Holzbecher Ltd., Zlíč: development of wound dressings, cellulose-based materials for skin tissue engineering

VUHZ, Joint-Stock Company, Dobrá: modification of metallic bone implants by anodic oxidation

VUP, Joint-Stock Company, Brno: innovation of vascular prostheses
Elmarco Ltd., Liberec: production of nanofibers for tissue engineering
Ceska Vcela Ltd.: production of nanofibers for tissue engineering, contractual research - testing with cells
Nanopharma Ltd.: production of nanofibers for tissue engineering

4) INTERNATIONAL COLLABORATION

University of Pennsylvania, Biophysical Eng'g & NanoBio-Polymers Lab, Philadelphia, PA, USA (Prof. Dennis E. Discher): differentiation of stem cells by the substrate rigidity and deformability
University of Sydney, School of Physics, Sydney, NSW, Australia (Prof. Marcela Bilek): plasma-modified polymers grafted with elastin-derived peptides for vascular tissue engineering
University of Bordeaux, Bioingénierie tissulaire, U1026, Bordeaux, France (Prof. Laurence Bordenave): vascular tissue engineering, laser-assisted bioprinting
University of Ghent, Nano and Biophotonics group, Department of Molecular Biotechnology, Gent, Belgium (Dr. Timothy Douglas): advanced materials for bone tissue engineering: spontaneously-mineralizing hydrogels and nanofibers loaded with alkaline phosphatase
AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Dept. of Biomaterials, Cracow, Poland (Prof. Stanislaw Blazewicz, Prof. Elzbieta Pamula): materials for bone tissue engineering: porous polymer-based scaffolds with ceramic particles or carbon nanotubes, carbon nanotubes with magnetic nanoparticles, hydrogels for controlled drug delivery (e.g., against osteoporosis).
Institute for Technical Physics and Materials Science, Research Centre for Natural Sciences, Thin Film Physics Department, Budapest, Hungary (Dr. Katalin Balazsi): carbides and nitrides for surface modification of bone implants
University of Rome "Tor Vergata", MINIMAlab (Prof. Maria Letizia Terranova): carbon nanotubes and nanodiamonds for bone tissue engineering
University of Sydney, School of Physics, Sydney, N.S.W., Australia (Prof. Marcela Bilek): plasma modiffied polymers functionalized with adhesion oligopeptides for vascular and bone tissue engineering

5) KEY METHODOLOGY AND CORE FACILITIES

For studies on cell-material interaction and creation of tissue engineered constructs, conventional static and also dynamic cell culture systems are used. The dynamic systems include circulatory bioreactors (with flow, tubular and perfusion chambers purchased from Provitro GmbH, Berlin, Germany, and also a lab-made perfusion bioreactor), a rotary bioreactor (Synthecon RCCS-D, Cellon, Luxembourg), a stretching bioreactor (STREX, B-Bridge International, Inc.) and a shaking system (STREX, B-Bridge International, Inc.). Other equipment related to the cell cultivation include laminar flow boxes, cell incubators, centrifuges, autoclaves, hot-air sterilizer, apparatus for preparation of deionized and distilled water, freezers, a cell type isolator, an automated analyser of cell number and viability (Vi-CELL XR, Beckman Coulter), a sensoric xCelligence system (Roche) for real-time monitoring of cell growth. The markers of cell adhesion, growth, differentiation and other cell functions are investigated on mRNA level by real-time PCR (using iQ5 Real-Time PCR Thermal Cycler coupled with Nanodrop and T personal thermocycler), and on protein level using immunofluorescence staining, enzyme-linked immunosorbent assay (ELISA), flow cytometry and immunoblotting. For these purposes, our laboratory is equipped with inverted fluorescence microscopes with digital

cameras and image analysis (Olympus), an ultrasound cell homogenizer, two microplate readers for fluorescence, luminescence and absorbance, a flow cytometer (Accuri), apparatus for protein electrophoresis and Western blotting (Biorad).

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

Projects dealing with cardiovascular tissue engineering:

2010-2014: „The structure and properties of modified polymers for tissue engineering". GAČR, ID P108/10/1106, with the Institute of Chemical Technology, Prague and J.E. Purkinje University, Ústí nad Labem

2010-2013: „Tissue engineering of autologous pericardial heart valve replacements". Ministry of Health of the CR, ID NT1127001, with the Institute of Clinical and Experimental Medicine, Prague

2011-2014: „Polymeric biomaterials for vascular tissue engineering". GAČR, ID P108/11/1857, with the Institute of Macromolecular Chemistry, Acad. Sci. CR

2014-2017: „Small-diameter vascular prostheses seeded with endothelial cells and bone marrow-derived stem cells in a bioreactor". TAČR, ID TA04011345, the National Center of Tissues and Cells, Joint-Stock Company and Czech Technical University, Prague.

Projects dealing with bone tissue engineering:

2009-2012: „Bioinspired Nanocomposite Structures for Bone Tissue Regeneration". GAČR, ID 106/09/1000, with the Institute of Rock Structure and Mechanics, Acad. Sci. CR and Elmarco Ltd., Liberec, CR

2012-2015: „Complex investigation of advanced beta-titanium alloys designed for biomedicine". GAČR, ID P107/12/1025, with the Faculty of Mathematics and Physics, Charles University, Prague.

2012-2016: „Carbon nanolayers, nanostructures and nanoparticles on substrates for potential application in medicine and electronics". GAČR, ID P108/12/1168, with the Institute of Chemical Technology, Prague.

Development of novel bioactive surface modifications of bone implants:

2010-2012: "Stability and Biocompatibility of Surface of Oxidic Layer on a Monophasic TiNb Alloy". GAČR, ID P108/10/1858, with Czech Technical University in Prague and Heyrovský Institute of Physical Chemistry, Acad. Sci. CR, Prague

2011-2013: „Metal-fullerene nanocomposites and their biological applications". GAČR, ID P107/11/1856, with the Nuclear Physics Institute, Acad. Sci. CR, Rez Near Prague.

2011-2013: „Development of implants, tools and fixators with antibacterial coating on the basis of nanostructured surfaces". Ministry of Industry and Trade of the Czech Republic, ID FR-TI3/088, with Prospon Ltd., Kladno, Czech Republic.

2011-2014: „Comprehensive research on joint replacements, with improved functional characteristics, based on beta titanium alloys." TAČR, ID TA01011141, Beznoska Ltd., Kladno.

2012–2015: „Augmentation of biological fixation of titanium implants by biomimetic hydrogel coatings enriched with bioactive compounds: The effect on adhesion and synthetic activity of osteoblasts and cells of immune response. Study in vitro." Project of the Ministry of Health of

the Czech Republic, ID NT13297-4/2012, with the 1st Medical Faculty, Charles University, Prague and Institute of Macromolecular Chemistry, Acad. Sci. CR

2014-2016: „Engineering bulk and surface of diamond nano-objects for biomedicine". GAČR, ID 14-04790S, with theInstitute of Physics, Acad. Sci. CR

Projects dealing with skin tissue engineering

2014-2017: „Matrix systems for healing of skin defects for human and veterinary use". TAČR, ID TA04010065, with Holzbecher Ltd., Zlíč, ČR

Broad aspects of biomaterials and tissue engineering

2012-2018: „Preparation, modification and characterization of materials by radiation". „Center of Excellence", GAČR, ID P108/12/G108, with the Institute of Chemical Technology, Institute of Physics, Acad. Sci. CR and Nuclear Physics Institute, Acad. Sci. CR.

2012-2015: Centre of Biomedical Research (project CZ.1.07/2.3.00/30.0025). This project is cofunded by the European Social Fund and the state budget of the Czech Republic.

''BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University'' (CZ.1.05/1.1.00/02.0109), project from the European Regional Development Fund.

9) SUMMARY AND RESEARCH IMPACT

- We have innovated synthetic polymeric vascular prostheses, currently used in clinical practice, by inner coating with layers based on fibrin nanofibers, which enabled the endothelialization of these prostheses and improved the retention and phenotypic maturation of endothelial cells. This technology was patented and published in renowned interdisciplinary journals (in collaboration with the Institute of Macromolecular Chemistry, Acad. Sci. CR, and the Institute of Clinical and experimental Medicine, Prague). The functionality of these prostheses was verified under *in vivo* conditions after implantation into minipigs.
- We have innovated currently used bone implants, namely femoral stems of hip joint prostheses, by appropriate surface modification which improved the adhesion, growth and osteogenic differentiation of bone cells, and thus they are expected to improve the osteointegration *in vivo*. This technology was patented and published in renowned interdisciplinary journals (in collaboration with the Beznoska Ltd. and the Faculty of Mathematics and Physics, Charles University).
- We have developed tissue engineering constructs based on degradable polymers functionalized with ligands for cell adhesion receptors (for vascular tissue engineering) or reinforced with ceramic or carbon nanoparticles (for bone tissue engineering).
- We also contributed to the fundamental research by studies on the role of cell-matrix communication in controlling the cell adhesion, proliferation and particularly switch between the proliferation and differentiation. As a good model of the matrix, artificial materials were used, as they represent growth substrates with defined physical and chemical properties, such as chemical composition, surface energy, polarity, wettability, roughness and topography, electrical charge and conductivity, pH, zeta potential, rigidity and deformability etc.
- We have established a research group with a new promising research topic, which is not traditional and still not systematically developed in the Czech Republic.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Cellular and Molecular Neuroendocrinology

A) RESEARCH FOCUS

This Laboratory is focused on the research of membrane receptors and secretion in neuroendocrine cells, with a special emphasis on the pituitary and hypothalamus. The Laboratory investigates interactions between plasma membrane electrical events and receptor-controlled pathways at the cellular and molecular levels, determines the manner in which hormones and neurotransmitters utilize calcium and cyclic nucleotides as intracellular messengers, and characterizes channels involved in electrical activity, calcium signaling, and secretion. Specifically, we are addressing how the structural features of channels relate to their functions, and how plasma membrane receptors and the intracellular signaling milieu affect channel activity. To achieve this, we are characterizing both native and recombinant channels that have been cloned from neuroendocrine cells. Our current work is focused on the adenosine-5´-triphosphate (ATP)-gated P2X receptors in pituitary cells. To better understand the complex role of purinergic signaling in various neuroendocrine functions, we investigated also P2X receptors in hypothalamic neurons of rat brain slices.

Main Topics:

(i) Ion channels and electrical excitability of pituitary cells
(ii) Purinergic signaling in hypothalamic suprachiasmatic and supraoptic nuclei
(iii) Relationship between structure and function of purinergic P2X receptor-channels.

B) KEY RESULTS

(i) Ion channels and electrical excitability of pituitary cells and pinealocytes

Background – The pituitary gland controls reproduction, lactation, growth, development, metabolic homeostasis, and the response to stress. It is composed of two embryonically, anatomically, and functionally distinct parts, the neurohypophysis and the adenohypophysis. Neurohypohysis lobe contains the oxytocin and vasopressin secreting axonal terminals of the hypothalamic magnocellular neurons. Anterior lobe of pituitary is composed primarily of five secretory cell types producing six peptide hormones: gonadotrophs produce luteinizing hormone and follicle-stimulating hormone, lactotrophs produce prolactin, somatotrophs produce growth hormone, thyrotrophs produce thyroid gland stimulating hormone thyrotropin and corticotrophs produce adenocorticotropic hormone. The anterior lobe is not directly innervated, but hypothalamic neuropeptides and neurotransmitters act as releasing and inhibitory hormones delivered through the portal vessels. In the past, our work has focused on the gonadotropin-releasing hormone (GnRH) -induced oscillatory calcium release in neonatal pituitary gonadotrophs and the mechanism of melatonin inhibition of these receptors. More recently, in an international collaboration with the Section on Cellular Signalling of NICHD/NIH, Bethesda, USA, we have characterized ligand-gated receptor channels, including the ATP-gated P2X receptors, GABA-A, and nicotinic channels, in several subpopulations of adult pituitary cells. We have also studied the control of membrane potential and spontaneous electrical activity by norepinephrine in the rat pinealocytes, that are still not well characterized electrophysiologically, and investigated several other membrane receptors and ligand-gated receptor channels in pituitary, including TRP channels and multidrug resistance protein channels (Kucka et al., *Mol Pharmacol* **77**: 270, 2010; Kucka et al., *Physiol*

Res **61**: 267, 2012). In collaboration with University of Cambridge we contributed to studies on structure-function relationships of recombinant neuronal nicotinic receptors (Jindrichova et al., *PLoS One* **7**: e32073, 2012; Palczynska et al., *Mol Pharmacol* **82**: 910, 2012). The results on receptor-channels in neuroendocrine cells and their intracellular signaling pathways obtained with major contribution of our laboratory are described in detail bellow.

Characterization of P2X4 receptor in pituitary cells

Previously we characterized the expression and function of P2X2 receptor channels in pituitary gonadotrophs, and in this period we continued in the investigation of the role of P2X receptors in identified subpopulations of anterior pituitary cells (Zemkova et al., *Am J Physiol Endocrinol Metab* **298**: E644, 2010). We showed by quantitative RT-PCR that mRNA transcripts for the P2X4 subunit are the most abundant in rat anterior pituitary tissue and confirmed the P2X4 receptor protein expression by Western blot analysis. Whole-cell patch-clamp recordings showed that extracellular ATP induced an inward desensitizing current in a majority of lactotrophs, resembling the current profile generated by recombinant P2X4 receptor. Activation of these channels led to stimulation of electrical activity and promotion of voltage-gated Ca^{2+} influx. In the presence of ivermectin, a specific allosteric modulator of P2X4 receptor, there was an increase in the maximum amplitude of current, accompanied by an increase in the sensitivity of receptors for ATP. Ivermectin also slowed deactivation of receptors, and enhanced ATP-induced prolactin release. These findings (highlighted by editors in *Physiology* 25: 59, 2010) indicate that lactotrophs express homomeric and/or heteromeric P2X4 receptors, which facilitate Ca^{2+} influx and hormone secretion.

This research was supported by the Grant Agency of the Czech Republic (grant #GA305/07/0681; PI: Hana Zemkova) and Ministry of Education, Youth and Sports of the Czech Republic, the Center for Neuroscience (grant # LC554; PI: Josef Syka) with major involvement of Hana Zemkova who performed electrophysiological experiments, data analysis and contributed to writing of manuscript. This work was done in collaboration with the Section on Cellular Signalling of NICHD/NIH, Bethesda, USA, that performed biochemical experiments and molecular biology analysis and contributed to the writing of the manuscript.

Cholinergic signaling pathway in pituitary cells

Acetylcholine (ACh) has been established as a paracrine factor in the anterior pituitary gland, but the receptors mediating ACh action and the cell types bearing these receptors have not been identified. Our results showed that the expression of the nicotinic subunits mRNAs followed the order $\beta2 > \beta1 = \alpha9 > \alpha4$ in cultured rat pituitary cells. The expression of the subunits in immortalized LβT2 mouse gonadotrophs followed the order $\beta2 > \alpha4 = \alpha1$. M4 > M3 muscarinic receptor mRNA were also identified in pituitary and LβT2 cells. The treatment of cultured pituitary cells with GnRH down-regulated the expression of α9 and α4 mRNAs, without affecting the expression of M3 and M4 receptor mRNAs, and ACh did not alter the expression of GnRH receptor mRNA. We also performed double immunostaining to show the expression of β2-subunit and M4 receptor proteins in gonadotrophs. Functional nicotinic channels capable of generating an inward current, facilitation of electrical activity, and Ca^{2+} influx were identified in single gonadotrophs and LβT2 cells. In both cell types, the M3 receptor-mediated, phospholipase C-dependent Ca^{2+} mobilization activated an outward apamin-sensitive K^+ current and caused hyperpolarization. The activation of M4 receptors by ACh inhibited cAMP production and GnRH-induced LH release in a pertussis toxin-sensitive manner. We concluded that multiple cholinergic receptors are expressed in gonadotrophs and that the main secretory action of ACh is inhibitory through M4 receptor-mediated down-regulation of cAMP production. Our conclusion is that the expression of nicotinic receptors in vitro compensates for the lack of regular GnRH stimulation of gonadotrophs (Zemkova et al., *Endocrinology* **154**: 421, 2013).

This research was supported by Grant Agency of the Czech Academy of Science (grant # IAA500110910; PI: Hana Zemkova) and the Grant Agency of the Czech Republic (grant #GBP304/12/G069; the PROJEKT EXCELENCE in Neuroscience, PI: Ladislav Vyklický) and with major involvement of Hana Zemkova who performed electrophysiological experiments and analysis, and contributed to writing of manuscript. This work was done in collaboration with the Section on Cellular Signaling, PDEG, NICHD, NIH, Bethesda, USA, Section on Neuroendocrinology, PDEG,

NICHD, NIH, Bethesda, USA that performed biochemical experiments and immunostaining, and contributed to the writing of the manuscript.

Phospholipase C signaling pathway in pinealocytes

The pineal gland generates a nocturnal increase in melatonin production, reflecting stimulation by norepinephrine (NE). We used perforated patch clamp recording to study the control of membrane potential and spontaneous electrical activity in the rat pinealocyte by norepinephrine (NE) (Zemkova et al., *Endocrinology* **152**: 3842, 2011). NE did not alter spiking frequency; however, it was found to act through α_{1B}-adrenoreceptors in a concentration-dependent manner to produce a biphasic change in membrane potential. The initial response was a hyperpolarization due to a transient outward K^+ current. This current appears to be triggered by Ca^{2+} released from intracellular stores, based on the observation that it was also seen in cells bathed in Ca^{2+}-deficient medium. In addition, pharmacological studies indicated that this current was dependent on phospholipase C (PLC) activation and was in part mediated by apamin-sensitive Ca^{2+}-controlled K^+ channels. The initial transient hyperpolarization was followed by a sustained depolarization due to an inward current; this response was dependent on PLC-dependent activation of Na^+/Ca^{2+} influx but did not involve nifedipine-sensitive voltage-gated Ca^{2+} channels. Together, these results indicate for the first time that activation of α_{1B}-adrenoreceptors initiates a PLC-dependent biphasic change in pinealocyte membrane potential characterized by an initial transient hyperpolarization mediated by a mixture of Ca^{2+}-activated K^+ channels followed by a sustained depolarization mediated by a Ca^{2+}-conducting non-selective cation channel. These observations indicate that both continuous elevation of intracellular Ca^{2+} and sustained depolarization are associated with and are likely to be required for activation of the pinealocyte.

This research was supported by the Grant Agency of the Czech Republic (grant # GA305/07/0681; PI: Hana Zemkova) with major involvement of Hana Zemkova who performed electrophysiological experiments and analysis, and contributed to writing of manuscript. This work was done in collaboration with the Section on Cellular Signaling, PDEG, NICHD, NIH, Bethesda, USA, Section on Neuroendocrinology, PDEG, NICHD, NIH, Bethesda, USA that contributed to molecular biology and writing of the manuscript.

(ii) Purinergic signaling in hypotalamic suprachiasmatic and supraoptic nuclei

Background – ATP is not only an energy source and important intracellular molecule that controls various intracellular processes, but it is also an extracellular messenger. Extracellular purines and pyrimidines act as autocrine and/or paracrine extracellular messengers via three families of purinergic receptors: seven transmembrane domain P1 receptors and P2Y receptors, and two transmembrane domain P2X receptor-channels. Activation of these receptors controls the accumulation of calcium in the cytoplasm, and activation of numerous signaling molecules both in neurons and endocrine cells (we reviewed in: (Stojilkovic et al., *Mol Cell Endocrinol* **314**: 184, 2010; Stojilkovic et al., *Wiley Interdiscip Rev Membr Transp Signal* **2**: 173, 2013); Vavra et. al., in *Neuroscience - Dealing With Frontiers*, ISBN 978-953-51-0207-6, edited by Carlos M. Contreras) Earlier, we have showed selective expression of the P2X2 receptors in pituitary gonadotrophs. Our recent work was focused on characterization and the roles of other P2X subtypes of anterior pituitary cells and in hypothalamic neurons of slices. In collaborative work with Faculty of Science, Charles University in Prague we also contributed to characterization of glutamatergic signaling in the hypothalamic suprachiasmatic nuclei (Bendova et al., *Synapse* **68**: 85, 2014; Bendova et al., *Neurochem Int* **61**: 43, 2012). The results on our investigation of P2X receptor-channels in neuroendocrine cells and their intracellular signaling pathways obtained with major contribution of our laboratory are described in detail bellow.

Regulation of neurotransmitter release by presynaptic P2X receptors in the supraoptic and suprachiasmatic nuclei of hypothalamus

Expression and function of P2X receptors was investigated in the supraoptic nuclei (SON) (Vavra et al., *Neuroscience* **188**: 1, 2011) and suprachiasmatic nuclei (SCN) (Bhattacharya et al., *J Neurosci* **33**: 8035, 2013) of hypothalamus. SON, the hypothalamic release site of vasopressin and oxytocin, receives a non-glutamatergic, excitatory input from the caudal medulla that uses noradrenaline and ATP as neurotransmitters. SCN, the circadian master clock in mammals, endogenously releases ATP in a rhythm. We found ATP application increased the frequency of action potentials and synaptic potentials

in most neurons of both the SON and SCN. However, ATP induced depolarization of resting membranes in about 80% of SON neurons and only in 7% of SCN neurons. The application of ATP analogue agonists, ATPγS and 2MeSATP, mimicked the effects of ATP, but 2MeSADP, 2MeSAMP and αβmeATP had no effect, both in the SON and SCN. The P2X7 receptor-specific agonist, BzATP, did not induce an inward current, but it increased intracellular calcium in non-neuronal cells in slices. Quantitative real-time PCR showed that P2X2 > P2X7 > P2X4 purinergic receptor mRNAs were expressed in both the SON and SCN tissue. These results showed that SON and SCN neurons express functional presynaptic P2X2 and P2X4 receptors that modulate glutamate and GABA release and control the electrical excitability of neurons. However, there were significant differences between these two hypothalamic regions in that the effect of ATP was higher in the SON, which is involved in endocrine functions of hypothalmus, than in the SCN. These results showed for the first time that extracellular ATP activates presynaptic, not postsynaptic P2X receptors, to modulate synaptic transmission within the SON and SCN.

This research was supported by Grant Agency of the Czech Academy of Science (grant # IAA500110910; PI: Hana Zemkova), the Grant Agency of the Czech Republic (grant #GA305/07/0681; PI: Hana Zemkova, and grant # GD305/08/H037; PI: Hana Zemkova) and Ministry of Education, Youth and Sports of the Czech Republic, the Centrum for Neuroscience (Research Project # LC554; PI: Josef Syka) with major involvement of Vojtech Vavra and Anirban Bhattacharya who performed electrophysiological, biochemical and immunohistochemical experiments, and Hana Zemkova who wrote the manuscripts. This work was done in collaboration with Faculty of Science, Charles University in Prague, Prague, and Department of Biophysics and Cell Biology, Faculty of Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary, that contributed to immunohistochemical studies.

(iii) Relationship between structure and function of P2X receptor-channels

Background – Molecular, physiological, and pharmacological studies have revealed the existence of seven P2X subunits, denoted as P2X1 through P2X7, as well as several spliced forms of these subunits. Each subunit is proposed to have two transmembrane (TM) domains connected by a large extracellular loop, with both N- and C-termini located in the cytoplasm. From the N-termini through the TM2 domain, the cloned subunits exhibit a relatively high level of amino acid sequence homology. In contrast, the C-termini vary in length and show no apparent sequence homology, except for the region nearest to TM2 domain. The functional channels are composed of three subunits, which can form ion permeable pores through homo- and heteropolymerization. P2X receptor subtypes differ with respect to their ligand-selectivity profiles, antagonist sensitivity, and cation selectivity. Their activation leads to an increase in the intracellular Ca^{2+} concentration, with Ca^{2+} influx occurring through the pores of these channels and voltage-gated Ca^{2+} channels, activated following the initial depolarization of cells by P2X receptor-generated Na^{+} currents. Our research is also focused on the structural-functional characterization of recombinant P2X receptors. Positive and negative allosteric modulators of P2X receptors interact with binding sites that are topologically distinct from the orthosteric sites recognized by the receptor endogenous agonist ATP, causing conformational changes that profoundly influence the gating of P2X channels (reviewed in: (Stojilkovic et al., *Cell Mol Neurobiol* **30**: 1251, 2010; Zemkova et al., *Physiol Res* **63 Suppl 1**: S215, 2014). Earlier, we have studied the role of transmembrane domains and residues in P2X receptor gating, we were looking for the binding site of allosteric modulator ivermectin and our work contributed to identification of ATP binding residues of P2X4 receptor in pre-crystalization era. Our ongoing work is focused on detailed analysis of receptor function including pore dilation and identification of binding sites for agonists and allosteric modulators. We are also involved in kinetic modeling of P2X7 receptor function (Khadra et al., *Biophys J* **104**: 2612, 2013) and studies on P2X3 receptor desensitization (Petrenko et al., *Biochemistry* **50**: 8427, 2011). The results on P2X receptors obtained with major contribution of our laboratory are described in detail bellow.

ATP binding site, gating properties and allosteric modulation of P2X4 receptor

The P2X4 receptor subtype is expressed in highest concentration throughout the central and peripheral nervous system, is involved in the learning and memory processes and in the hypersensitivity of nociceptive transmission in injured primary sensory neurons. Crystal structure of P2X4 receptor from

zabrafish revealed that the P2X receptors contain an upper, central, and extracellular vestibule that form a potential pathway for access of into the channel pore. The binding of ATP causes an enlargement of the extracellular vestibule, leading to opening of the channel pore. We studied function of extracellular vestibule in P2X4 receptor using alanine scanning mutagenesis of V47-V61 and F324-N338 sequences, that form lateral portals of the extracellular vestibule. We identified five critical residues that are important for receptor function: V49 is important for receptor trafficking to plasma membrane, and residues Y54, Q55, F324, and G325 are crucial for proper structure, channel gating or vestibule widening. These results indicate multiple roles of the extracellular vestibule (Rokic et al., *PLoS One* **8**: e59411, 2013). Using cysteine scanning mutagenesis of these sequences and cadmium testing of cysteine mutant, we examined the ion accessibility of lateral portals of the P2X4 receptor in the closed and open state. Electrophysiological experiments and analysis of the homology model of the rat P2X4 receptor assumed that there is ion access to extracellular and central vestibules when the channel is closed by interacting predominantly with residues above the TM1, which passes the ion toward the residues located on the upper region of the TM2 upon channel activation. Because the channel vestibules can be charged with ions before opening, they may also have a role as ion reservoirs (Rokic et al., *Front Cell Neurosci* **8**: 3, 2014). Crystal structure of P2X4 receptor revealed that ATP binding site is located extracellularly between subunits, and that a large ATP-binding pocket is formed by two ectodomain non-structuralized segment, the dorsal fin and left flipper. We studied the role of these structures in ATP potency/efficacy using alanine scanning mutagenesis of the R203-L214 (dorsal fin) and the D280-N293 (left flipper) sequences. We identified 15 residues that contribute to the organization of the ATP binding pocket, and are critical for intramolecular signal transduction and receptor desensitization. The results also showed for the first time that the R203 and N204 residues, deeply buried in the protein, may integrate the output signal from these two domains towards the gate (Tvrdonova et al., *PLoS One* **9**: e112902, 2014). Allosteric modulators of ligand-gated receptor channels induce conformational changes of the entire protein that alter potencies and efficacies for orthosteric ligands. We studied the influence of allostery on channel pore dilation using the rat P2X4 receptor and ivermectin, an established specific positive allosteric regulator of this channel. Experiments with vestibular and transmembrane domain receptor mutants showed that ivermectin induces increase in pore conductivity, and that it has distinct effects on opening and dilation of the channel pore, the first accounting for increased peak current amplitude and the latter correlating with changes in the sensitivity and kinetics of receptor deactivation. The corresponding kinetic (Markov state) model indicates that the ivermectin-dependent transition from open to dilated state is coupled to receptor sensitization, which rescues the receptor from desensitization and subsequent internalization. Allosterically induced sensitization of P2X4 receptor thus provides sustained signaling during prolonged and repetitive ATP stimulation (Zemkova et al., *Pflugers Arch* **DOI 10.1007/s00424-014-1546-7**: 2014).

This research was supported by Grant Agency of the Czech Academy of Science (grant # IAA500110910; PI: Hana Zemkova), the Grant Agency of the Czech Republic (grant #GBP304/12/G069, the PROJEKT EXCELENCE in Neuroscience; PI: Ladislav Vyklický) and Ministry of Education, Youth and Sports of the Czech Republic (grant # ED1.1.00/02.0109; PI: Jiří Paleček; and grant # EE2.3.30.0025; PI: Jiří Paleček) with major involvement of Milos Rokic, Vendula Tvrdonova and Hana Zemkova who performed electrophysiological experiments, data analysis and molecular biology. Milos Rokic and Hana Zemkova wrote three of four manuscripts. This work was done in collaboration with the Section on Cellular Signalling, NICHD, National Institutes of Health, Bethesda, MD, USA; Laboratory of Biological Modeling, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA and Department of Physiology, McGill University, Montreal, Quebec, Canada, that performed kinetic modeling and contributed to writing of manuscripts.

Roles of conserved ectodomain cysteines in P2X7 receptor membrane expression

The P2X7 receptor operates as a cytolytic receptor but also controls cell growth and proliferation. To address the question how the same receptor mediates such opposing effects, we have combined electrophysiological, and molecular biology experiments to study its membrane expression (Jindrichova et al., *Purinergic Signal* **8**: 317, 2012). Crucial for the capability of ATP to induce apoptosis via the P2X7 receptor pore-forming mechanism is the ability of cells to express the receptor in the plasma membrane. Mammalian P2X receptors contain 10 conserved cysteine residues in their ectodomains,

which form five disulfide bonds (SS1-5). We analyzed the relevance of these disulfide pairs in rat P2X7 receptor function and trafficking by replacing one or both cysteines with threonine, expressing receptors in HEK293 cells and studying their responsiveness to agonists. We found that all but one single--point mutants and all double-point mutants generated nonfunctional P2X7 receptors and that loss of function was due to decreased cell-surface expression. This study revealed that, in contrast to P2X4 receptor, all disulfide bonds of P2X7 are essential for receptor trafficking. The study on P2X4 receptor revealed that replacing of one or both cysteines with alanine or threonine has much smaller effect on trafficking but affects receptor function (Rokic et al., *Physiol Res* **59**: 927, 2010).

This research was supported by Grant Agency of the Czech Academy of Science (grant # IAA500110910; PI: Hana Zemkova), the Grant Agency of the Czech Republic (grant #GA305/07/0681; PI: Hana Zemkova; grant # GD305/08/H037; PI: Hana Zemkova; grant # GPP304/12/P371; PI: Marie Jindrichova) and Ministry of Education, Youth and Sports of the Czech Republic, the Centrum for Neuroscience (Research Project # LC554; PI: Josef Syka) with major involvement of Marie Jindrichova, Milos Rokic, Vendula Tvrdonova and Hana Zemkova who generated mutants and performed electrophysiological experiments and data analysis. Vojtech Vavra performed confocal microcopy, Pavlo Kuzyk performed western blotting. Hana Zemkova wrote the manuscripts. This work was done in collaboration with the Section on Cellular Signalling of NICHD/NIH, Bethesda, USA, that contributed to molecular biology and writing of manuscripts.

Structural basis of P2X3 receptor desensitization

The P2X3 receptor, highly prone to desensitization, is involved in pain sensation. In this work we examined the role of conserved Tyr37 in the TM1 in receptor function. Tyrosine 37 is highly conserved in ATP-gated P2X receptors suggesting its fundamental role. We tested whether Y37 contributes to the desensitization of P2X3 receptors, which is currently not well understood (Jindrichova et al., *J Neurochem* **119**: 676, 2011). By combining electrophysiological, imaging and modeling approaches, we studied desensitization of various Y37 P2X3 mutants and potential partners of Y37. Unlike the membrane current of the WT receptor, which desensitized in seconds, Y37A mutant current did not fully desensitize even after minutes-long applications of βγ-meATP, αβ-meATP, ATP or 2MeS-ATP. The fractional calcium current was enhanced in the Y37A mutant. Y37F did not rescue the native P2X3 phenotype indicating a role for the hydroxyl group of Y37 for the WT receptor. Homology modeling indicated I318 or I319 in TM2 as potential partners for Y37 in the receptor closed state. We tested this hypothesis by creating a permanent interaction between the two residues via disulfide bond. Whereas single Y37C, I318C and I319C mutants were functional, the double mutants Y37C-I318C and Y37C-I319C were non-functional. Using a cyclic model of receptor operation, we suggest that the conserved tyrosine 37 links TM1 to TM2 of adjacent subunit to stabilize desensitized states and restricts calcium permeability through the ion channel.

This research was supported by Grant Agency of the Czech Academy of Science (grant # IAA500110910; PI: Hana Zemkova) with major involvement of Marie Jindrichova and Hana Zemkova who generated mutants and performed electrophysiological experiments and contributed to writing of manuscript. This work was done in collaboration with Department of Neurobiol., A. I. Virtanen Institute, Kuopio, Finland, that performed imaging studies and molecular modeling and contributed to writing of manuscript.

C) COLLABORATING UNITS:

Internal Collaboration (within Institute of Physiology): Protein Structures, Institute of Physioology CAS (Dr. T. Obšil, 2 joint publications)

Domestic Collaboration (outside Institute of Physiology): Faculty of Science, Charles University in Prague, Prague, Czech Republic (Dr. Z. Bendová, 3 joint publications)

International Collaborations:

Section on Cellular Signaling, Program in Developmental Neurosciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA (Dr. S.S. Stojilkovic, 15 joint publications)

Section on Neuroendocrinology, Program in Developmental Neurosciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA (Dr. D.C. Klein, 1 joint publication)

Department of Neurobiol., A. I. Virtanen Institute, Kuopio, Finland (Dr. R. Giniatullin, 2 joint publications)

Department of Physiology, McGill University, Montreal, Quebec, Canada (Dr. A. Khadra; 2 joint publications)

Department of Neuroscience, Physiology and Pharmacology, University College London, London, United Kingdom (Dr. N.Millar; 2 joint publications)

D) KEY METHODOLOGY AND CORE FACILITIES:

The approach used in the experimental work on main topics is multidisciplinary: electrophysiological (whole-cell patch-clamp recording, perforated-patch recording, analysis of membrane currents and potentials, drug application, waveform modeling), biochemical (western blot analysis of gene expression, quantitative measurements of surface and total P2XR expression, secretory studies, radioimmunoassay of luteinizing hormone secretion, measurement of ATP using a bioluminescent assay kit), fluorimetric (immunohistochemistry, ratiometric imaging of intracellular Ca^{2+} ions), cell and molecular biology-based methods (cDNA cloning and subcloning, PCR amplification, quantitative real-time RT-PCR, sequencing, mutagenesis and heterologous expression of receptors) and structural bioinformatics methods (homology modeling, docking of molecules into receptor structure). The laboratory is equipped for population and single-cell studies in dispersed and intact tissues. These include primary cultures of pituitary cells, pinealocytes and Leydig cells, acute brain slices and organotypic slices, HEK293 and neuroendocrine cell lines (GH3, LβT cells).

E) INVOLVEMENT IN SIGNIFICANT PROJECTS

Hana Zemkova was PI of grant # GD305/08/H037 from the Grant Agency of the Czech Republic, that supported collaboration between 25 students of different subject fields in biomedicine at the level of their doctoral study programs at the Czech Academy of Sciences and Charles University in Prague.

Hana Zemková was involved in grant # LC554 from the Ministry of Education, Youth and Sports of the Czech Republic, the Centrum for Neuroscience (PI: Josef Syka)

Hana Zemková is involved grant # GBP304/12/G069 from the Grant Agency of the Czech Republic, the PROJEKT EXCELENCE in the field of Neuroscience (PI: Ladislav Vyklický)

Hana Zemková is a mentor in grant # CZ.1.07/2.3.00/30.0025 from the Ministry of Education, Youth and Sports of the Czech Republic, supported by the project „Center of biomedical research“ (PI: Jiří Paleček)

Hana Zemková is involved in grant # CZ.1.05/1.1.00/02.0109 from the Ministry of Education, Youth and Sports of the Czech Republic, supported by the project „BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University“, from the European Regional Development Fund.

F) OTHER RELEVANT INFORMATIONS

Postdoctoral stays of students that defended their PhD at the Institute of Physiology CAS:

Aleš Balík (at the Institute of Physiology CAS till 09. 2011) MRC Laboratory of Molecular Biology, Neurobiology Division, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, UK. (Dr. I. Greger).

Milos Rokic (at the Institute of Physiology CAS till 09. 2013) Section on Cellular Signaling, Program in Developmental Neurosciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA . Our collaboration is focused on P2X receptor modeling and physiology in pituitary cells (Dr. S.S. Stojilkovic).

Vojtěch Vávra (at the Institute of Physiology CAS till 09. 2013) Hotchkiss Brain Institute, University of Calgary, Canada. Continues to investigate excitability of hypothalamic neurons (Dr. J. Bains)

Laboratory was also involved in two projects that were primarily aimed to support biomedicine research undirectly:

Ales Balik was PI of grant # M200110971 from the Grant Agency of the Czech Academy of Science, that supported his 2-year training in a top-class laboratory (Laboratory of Molecular Biology (MRC-LMB), Cambridge, UK). His stay resulted in a publication with dedication to this grant (Penn et al., *Neuron* **76**: 503, 2012).

Tomas Radil was PI of grant # GAP407/10/2031 from Grant Agency of the Czech Republic that was aimed to support writing of his bibliography. Tomas Radil is a Czechoslovakian brain- researcher, At the age of thirteen and half he became deported in Birkenau. He was enprisoned there and in the Auschwitz main Camp, till his liberation, on January 27th, 1945. His book represents an analytical micro- history of the Holocaust from the point of view of a teen-age boy. Radil, Tomáš: „Az Auschwitzi fiúk" (The boys of Auschwitz), Bratislava: Kalligram , ISBN 2014978-80-8101-753-7, 632 pages; Rozin P et. al., *J Peace Psychology*. Roč. **20: 412,** 2014.

G) SUMMARY AND RESEARCH IMPACT

Purinergic P2X receptors are ATP-gated ion channels that play an important role in intercellular communication in the brain. Despite the fact that high levels of expression of P2X receptors are observed in many regions of central nervous system, physiological roles are still not well understood. In 2010-2014 we have investigated the function and identified the subtypes of P2X receptors in hypothalamic supraoptic and suprachiasmatic nuclei and in anterior pituitary lactotrophs. Our goal was also to better understand the structure-function relationship of the P2X receptors and their dynamics. We used different electrophysiological approaches and mutagenesis to investigate precise shaping of ATP-binding pocket and signal transduction, and we contributed to the more detailed understanding of gating, allosteric modulation and pore dilation of the P2X4 and P2X7 receptors.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Cellular Neurophysiology

1) **TEAM DESCRIPTION**

i. RESEARCH FOCUS

Our research explores functional and pharmacological properties of ion channels, including those activated during synaptic transmission. For that purpose, we use electrophysiological methods; primarily the patch-clamp technique, combined with molecularbiology, immunohistochemical and microfluorometric methods (Ca^{2+} imaging). Using this approach, we study the properties of excitable cells, such as cultured hippocampal and granular neurons or in vitro preserved neural circuits in hippocampal brain slices, as well as HEK293 and COS cell lines transfected with recombinant N-methyl-D-aspartate receptor (NMDAR) or vanilloid receptors. The goal of our research is to comprehend the relationship between structure and function of ion channels, to characterize the actions of pharmacologically active substances and to explain the molecular mechanism by which they influence ligand-gated ion channels.

For more detailed understanding of physiological mechanisms and pharmacology of ligand-gated ion channels, we use quantitative analytical techniques (including the analysis of single ion channels, measuring of intracellular concentrations of Ca^{2+} and mathematical modeling). Bioinformatics, molecular modeling, molecular dynamics simulations, and docking of small molecules to the ion channel was also used to understand the structure at the atomic/molecular level.

ii. PERSONNEL

Head of the department/team leader:

Ladislav Vyklický, MD, PhD., DrSc. (graduated from medical faculty UK; postdoctoral stay at: 1987 – 1988 Laboratory of Developmental Neurobiology, NIH, Bethesda; 1990 - 1992 Laboratory of Cellular and Molecular Neurophysiology, NIH, Bethesda; 1995 Max Planck Institut für Zellphysiologie, Heidelberg; electrophysiologist, age 59, H-index 24)

Senior scientists:

RNDr. Viktorie Vlachová, PhD., DrSc. (team leader; mathematics, biophysics; electrophysiologist, age 56, H-index 21)

RNDr. Jan Krůšek, Ph.D. (Ph.D. in biophysics; expert in cellular electrophysiology, fluorescent microscopy and spectroscopy; age 53, H-index 10)

Mgr. Martin Horák, Ph.D. (Ph.D. in neuroscience; expert in neurophysiology, molecular biology; trafficking of receptors; 2005-2010 postdoctoral stay at NIDCD, NIH, Bethesda, USA; age 37, H-index 11)

RNDr. Aleš Balík, Ph.D. (Ph.D. in neurophysiology; expert in cellular physiology, neurophysiology, molecular biology; 2008-2011 postdoctoral stay at MRC-LMB, Cambridge, UK; age 37, H-index 9)

Ing. Jiří Černý, Ph.D. (part time; Ph.D. in physical chemistry; expert in structural bioinformatics, molecular modeling, and quantum chemistry; 2006-2007 postdoctoral stay at the Photon Science Institute, University of Manchester, UK; age 36, H-index 16)

Emeritus:

Prof. RNDr. František Vyskočil, DrSc. (Ph.D. in physiology; visiting Professor in the Department of Physiology, University of California, San Francisco, USA and Laboratory of Membrane Biophysics of the Sechenov Institute of Evolutionary Physiology, St. Petersburg; regular professor Kazan State Medical University; age 73, H-index 32)

Research development assistant: Ing. Ivan Dittert, Ph.D.

Junior scientists: Mgr. Miloslav Kořínek, Ph.D., postdoc (Ph.D. in biophysics, chemical and macromolecular physics; 2006 postdoctoral stay at Katholieke Universiteit Leuven, Belgium) • Tereza Smejkalová, Ph.D., postdoc (Ph.D. in neurobiology and physiology, Northwestern University, USA; 2014-2015 postdoctoral stay at Washington University in St. Louis, USA)

Postdoctoral fellows: Mgr. Martina Kaniaková, Ph.D. • Mgr. Lenka Maršáková, Ph.D.

PhD students: Mgr. Jiřina Borovská • Mgr. Anna Hynková • Mgr. Barbora Krausová • Mgr. Marek Ladislav • Mgr. Katarína Lichnerová • Mgr. Lucie Surá-Zímová • Mgr. Filip Touška • RNDr. Vojtěch Vyklický • Bc. Kristýna Skřenková

Undergraduate students: Bc. Štěpán Chvojka • Bc. Pavel Švehla • Bc. Viktor Synytsya • Bc. Jana Vašková

Technicians: Magda Kuntošová • Jiří Kaucký (part time)

Secretary: Miloslava Kuldová

2) KEY RESULTS

A. Structural and functional analysis of ligand gated ion channels

Structural analysis of NMDAR channels. Using a combined approach – involving electrophysiological examination with rapid solution exchange in combination with temperature control, as well as kinetic analysis and modeling, we have elucidated several aspects of NMDAR function and pharmacolgy:

i.) We have characterized in detail the temperature sensitivity of the kinetic transitions between closed, open, and desensitized state of native and recombinant NMDARs, as well as the temperature sensitivity of the amplitude and deactivation kinetics of the NMDAR component of the excitatory postsynaptic currents in hippocampal neurons.

ii.) We have shown that the sensitivity of NMDAR to the potentiating effect of pregnenolone sulfate is phosphorylation dependent.

iii.) We have found that cholesterol, which naturally occurs in plasma membranes, strongly modulates the function of NMDARs. Higher levels of membrane cholesterol enhance NMDAR activity whereas decreased levels diminish activity. NMDAR desensitization is also affected by changes of membrane cholesterol concentration. Surprisingly, the function of other glutamate receptors (AMPA and kainate) is independent of membrane cholesterol.

iv.) We have identified the molecular basis of the use-dependent and voltage-independent inhibitory effect of neurosteroids on NMDAR responses. The site of action is located at the extracellular vestibule of the receptor's ion channel pore and is accessible after receptor activation. Mutations in the extracellular vestibule in the SYTANLAAF motif disrupt the inhibitory effect of negatively charged steroids. In contrast, positively charged steroids inhibit mutated NMDAR responses in a voltage-dependent manner. These results, in combination with molecular modeling, characterize the structure details of the open configuration of the NMDAR channel. Molecular dynamic simulations together with experimental data on steroid permeability through the ion channel suggest a wide open extracellular vestibule of the pore with the dimensions of the narrowest region in the ring of threonines.

Papers: • Korinek et al., *Neuroscience* **165**: 736, (2010) • Adamusova et al., *Physiological research* **62**: 731, (2013) • Korinek et al., *Journal of Physiology* (accepted 2015: http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1469-7793/accepted; see also Perspectives article written by Lonnie Wollmuth - "Is cholesterol good or bad for your brain? - NMDARs have a say") • Vyklicky et al., *Scientific reports* (2015).

Team's contribution: Team – Laboratory of Cellular Neurophysiology (LCN – Institute of Physiology CAS): Major contribution. Performed in vitro and in vivo experiments; prepared samples of tissue for kinetic experiments; based on electrophysiological data (patch-clamp recording) suggested possible mechanism of action; structural and molecular dynamic modeling. Team at UOCHB (HC): Synthetized the different steroid compounds.

Support: Czech Science Foundation (GACR) 309/07/0271, EC FP6 PHOTOLYSIS LSHM-CT-2007-037765, 1M0002375201, LC554, P304/12/G069, 303/12/1464, P303/110075, TE01020028, BIOCEV CZ.1.05/1.1.100/02.0109, P303/11/P391, 14-02219S, TE01020028.

Understanding the fundamental principles of multimodal gating of nociceptor-specific thermosensitive transient receptor potential (TRP) channels. The results from the project demonstrate that:

i.) The carboxyl terminus of the ankyrin TRPA1 channel contains several specific positively charged residues that are important determinants of voltage- and chemical-induced activation. Some of the identified residues are predicted to interact with membrane lipids or contribute to permeation.

ii.) The conserved acidic motif in the C-terminus of human ankyrin TRPA1 channel is actively involved in channel regulation by intracellular calcium. This proposed mechanism represents an important self-modulating feedback loop that first augments and then inhibits the initial activation of TRPA1.

iii.) The fourth transmembrane domain (S4) and the S4–S5 linker contribute to voltage sensing in TRPV1 channel. Despite their highly conserved nature, these regions regulate the temperature and chemical gating in the various TRPV channels, specifically TRPV2 and TRPV3, in different ways, surprisingly indicating that not all structural determinants, although conserved in primary sequence, play functionally conserved roles across the TRP channel family.

iv.) A small contact surface between S1 and the pore-helix is required for TRPV1 channel functioning. Analogous to Kv channels, the S1-pore interface might be important for the energetic coupling between S1-S4 sensor activation and gate opening, serving to stabilize conformations associated with TRPV1 channel gating.

v.) The molecular mechanism by which the TRPV1 channel is activated by camphor is separable from other TRPV1 activation mechanisms and depends on the short helical segment within the permeation pore. An important finding is that camphor induces changes in the spatial distribution of important lipids on the inner leaflet of the plasma membrane, which provides clues about the effects of camphor on other TRP proteins.

vi.) In the framework of the long-term collaboration with the University in Erlangen, Germany, we contributed to studies aimed at understanding the role of thermosensitive TRP channels and their functional synergism in sensory neurons. Particularly, the results confirmed an important role for TRPA1 in the pathogenesis of cold allodynia and characterized the functional synergism between M-currents and TRPM8-mediated currents in cutaneous cold nociceptor nerve endings.

Papers: Zima et al., *Neuropharmacology*, 10.1016/j.neuropharm.2015.02.018, (2015) • Witschas et al., *BBA Biomembranes* **1848:** 1147, (2015) • Boukalova et al., *BBA Mol Cell Research* **1833:** 520, (2013) • Vetter et al., *J Neurosci* **33:** 16627, (2013) • Sura et al., *J Biol Chem* **287:** 18067, (2012) • Marsakova et al., *Anesthesiology* **116:** 903, (2012) • Vetter et al., *EMBO J* **31:** 3795, (2012) • Samad et al., *Biochem J* **433:** 197, (2011) • Boukalova et al., *J Biol Chem* **285:** 41455, (2010) • Benedikt et al., *BBA Mol Cell Research* **1793:** 1279, (2009)

Reviews: Boukalova et al., *Physiol Res* **63 Suppl 1:** 205, (2014) • Touska et al., *Curr Pharm Biotechnol* **12**: 122, (2011)

Editorial: V. Vlachova was the Guest Editor of a special "Hot Topic" issue of Current Pharmaceutical Biotechnology, 2011, Vol. 12, No. 1, focusing on TRP ion channels, contributed by 14 highly-valued experts in the field.

Team's contribution: Team – LCN - IP CAS: Designed research; performed and analyzed electrophysiological experiments (patch-clamp recording); prepared recombinant DNAs; suggested possible mechanisms and structural interpretations; performed molecular docking simulation. Team LCN - IP CAS: Contributed to design; performed research and analyzed data in the framework of the long-term collaboration with the University in Erlangen (Vetter et al., 2012, 2013). Team- Protein Structures - IP CAS: Prepared some of the DNA clones and contributed to experimental design. Team from the Institute of Systems Biology and Ecology, Nove Hrady: For the study Samad et al. (2011) contributed to structural explanation of the data. Team from the Faculty of Mathematics and Physics, Prague: The homology model of the C-terminus of TRPA1.

Support: Czech Science Foundation (GACR) 305/06/0319, 305/09/0081, 303/07/0915, 301/10/1159, 202/09/0806, 305/08/H037 and P304/12/G069, , IAA 600110701, EC FP6 PHOTOLYSIS LSHM-CT-2007-037765, and by the Ministry of Education, Youth and Sports of the Czech Republic 1M0517, LC06010, MSM6007665808, MSM0021620835, SVV-2010-261 304, CZ.1.07/2.3.00/30.0025 and LC554.

B. Pharmacology of ligand gated ion channels

***Steroid modulation of the NMDAR channel function*.** We have analyzed the effect of pregnanolone sulfate (PA-S) analogues at NMDARs. The results of our study show that:

i.) There is a requirement for the steroid molecule to be charged (either negatively or positively) to preserve its inhibitory activity at NMDARs.

ii.) Structural modifications at the A and/or D ring can increase the steroid inhibitory efficacy by more than 100-fold (to 10-100 nM range).

iii.) Pregnanolone sulfate (PA-S), a steroid with a voltage-independent action, has a higher potency to inhibit tonically activated NMDARs than those synaptically activated. Using a reaction scheme for the NMDAR, simulations of various types of pharmacological modulation indicate that this difference is due to the use-dependent onset and use-independent offset of steroid inhibition. Analysis of newly synthetized steroids indicate that for certain steroid analogues is their potency more than 10-fold higher to inhibit tonically than phasically activated (synaptic) receptors. This is considerably better than the ratio for memantine (NMDAR inhibitor approved for the treatment of Alzheimer disease).

iv.) Neurosteroids with inhibitory activity at NMDARs have neuroprotective effect on neurodegeneration induced in rats by ischemia or by pharmacologically induced excitotoxicity. Surprisingly behavioral tests shows no signs of psychomimetic-like effects typical for other classes of NMDAR inhibitors.

Papers: Borovska et al., *British journal of pharmacology* **166:** 1069, (2012) • Vidrna et al., *Steroids* **76:** 1043, (2011) • Stastna et al., *Steroids* **74:** 256, (2009) • Kapras et al., *Steroids* **77:** 282, (2012) • Cerny et al., *Steroids* **77**: 1233, (2012) • Rambousek et al., *Neuropharmacology* **61**: 61, (2011) • Vales et al., *Behavioral brain research* **235**: 82, (2012)

Reviews: Korinek et al., *Steroids* **76:** 1409, (2011) • Vyklicky et al., *Physiological Research* **63**: S191, (2014)

Book Chapter: Vyklicky et al., Analysis of whole-cell NMDAR currents, *Springer Business + Science Media Series*, editor: Wolfgang Walz; *Ionotropic Glutamate Receptor Technologies,* editor: Gabriela K Popescu

Patents: (1) Czech Patent (UPV) No.: 303037: Pregnanolone derivatives substituted in position 3alpha, process for their preparation and use (Chodounska et al., CZ patent No. 303037, 2012); (2) Czech Patent (UPV) No.: 303443: Pregnanolone derivatives substituted in position 3alpha with cationic group, process of their preparation, their use and composition containing them

(Chodounska et al., CZ patent No. 303443, 2012); (3) US patent No.: US8575376: Steroid anionic compounds, method of their production, usage and pharmaceutical preparation involving them (Chodounska et al., US patent No. US8575376, 2014); (4) PCT Patent Application WO/2010/136000: Steroid anionic compounds, method of their production, usage and pharmaceutical preparation involving them.

Team's contribution: Team – LCN - IP CAS: Performed in vitro and in vivo experiments; prepared samples of tissue for kinetic experiments; designed some molecules of the inhibitory steroids; based on electrophysiological data (patch-clamp recording) suggested a possible mechanism of action and a neuroprotective effect. Team at the Institute of Organic Chemistry and Biochemistry CAS (mainly HC and EK): synthetized the steroids. Team - Neurophysiology of memory (IP CAS): performed the behavioral experiments.

Supported: Czech Science Foundation (GACR) 309/07/0271, 203/08/1498, 309/08/H079, P303/11/0075, P303/12/1464, ED0007/01/01, 1 M0517, LC554, Z4 055 0506, Z5 011 0509, LC06077, NS10365, P304/12/G069, TE01020028, NR 9180-3, 309/09/0286, EC FP6 PHOTOLYSIS LSHM-CT-2007-037765.

Pharmacological modulation of the nicotinic type of acetylcholine receptor. We studied mechanisms by which lobeline interacts with neuronal nicotinic receptors α4β2, α3β4 and muscle receptor using the patch-clamp technique. We found that despite some differences, the common molecular mechanism of action in all nicotinic receptors is connected to the interaction with agonist/competitive antagonist binding site. The result of the interaction is complicated by partial agonistic activity of lobeline and its slow on and off rate of binding. The result of the interaction is either inhibition or potentiation depending on the concentrations of the agonist and lobeline and on the time schedule of drug application. In the α3β4 receptor this mechanism is combined with voltage-dependent channel block.

Papers: Kaniakova et. al., *Eur J Pharmacol* **658**: 108, (2011) • Kaniakova et. al., *Eur J Pharmacol* **738**: 352, (2014)

Supported: Czech Science Foundation (GACR) 202/09/0806, Grant Agency of the Academy of Sciences IAA 500110905

***Team's contribution*:** Team – LCN - IP CAS: designed and performed experiments and data analysis including the mathematical analysis of receptor activation and inhibition, wrote the paper. First Faculty of Medicine: contributed medical aspects of lobeline physiological action.

C. iGluR posttranscriptional and posttranslational regulations and trafficking

Activity-driven regulation of AMPAR RNA processing. Initially Ales Balik's postdoctoral project (MRC-LMB, Cambridge, UK, 2008-2011) exceeded the duration of his stay in the foreign laboratory and was completed in the subsequent years back in Prague. Moreover, the results from the original study have initiated a new collaboration and the study with Prof. M. Jantsch (MFPL, Vienna).

In summary, the activity-dependent control of AMPAR RNA processing was revealed in this project. The major results are:

(i) Changes in neuronal activity (in vitro TTX/BIC treatment) modulate AMPAR RNA processing in the CA1 hippocampal subfield.

(ii) The activity-driven changes affect both processes – AtoI editing and alternative splicing – bidirectionally and in a reversible fashion.

(iii) The observed RNA changes are subsequently transformed into the new AMPAR assemblies delivered to the cell membrane impacting on the quality of excitatory signal transduction. We proposed a new AMPAR-mediated short-term homeostatic mechanism.

(iv) The expression levels of newly identified ADAR2 (editing enzyme) regulation factors (in the group of M. Jantsch) also respond to the altered neuronal activity.

Papers: Balik et. al., *Nucleic Acid Research* **41**: 1124, (2013) • Tariq et. al*., Nucleic Acid Research* **41**: 2581 (2013)

Review: Penn et. al., *Frontiers in Neuroscience* **7**: 61, (2013)

Team's contribution: AB co-designed and performed majority of the experiments (organotypic brain slices, RNA isolation, PCR and sequencing-based analysis) and also co-wrote the article (Balik et. al., 2013). Second, he provided RNA samples from CA1 hippocampus and performed part of the RNA expression analysis (Tariq et. al., 2013). Finally, he co-wrote the review article on the current knowledge about neuronal activity-driven RNA editing (Penn et. al., 2013).

Supported: Czech Science Foundation (GACR) P304/12/G069

Structural determinants of NMDARs important for receptor trafficking*.* We have identified several structural determinants of the GluN subunits which play a critical role in the early processing of the NMDARs. The results of our studies show that:

i.) The M3 domains of both GluN1 and GluN2 subunits contain key amino acid residues that contribute to the regulation of the number of functional surface NMDARs.

ii.) A single amino acid residue within the fourth membrane domain (M4) of GluN1 subunit (L830) regulates the surface number of NMDARs.

iii.) Surface delivery of GluN2C-containing receptors is reduced compared to GluN2A- and GluN2B-containing receptors.

iv.) Three distinct regions within the N-terminus, M3 transmembrane domain, and C-terminus of GluN2C subunits are required for proper intracellular processing and surface delivery of NMDARs.

Papers: Lichnerova et al., *Front Cell Neurosci* **8:** 375, (2014). • Kaniakova et al., *Journal of Neurochemistry* **123**: 385-95, (2012). • Kaniakova et al., *Journal of Biological Chemistry* **287:** 26423-34, (2012)

Review: Horak et al., *Front Cell Neurosci.* **8:** 394, (2014)

Book Chapter: Horak et al., Trafficking of Glutamate Receptors and Associated Proteins in Synaptic Plasticity, *The Synapse: Structure and Function*, pp. 221-279, Neuroscience-Net Master Reference Book Series, Elsevier, managing editor: John E. Johnson Jr. (2014); Horak et al**.,** Counting NMDARs at the cell surface, *Springer Business + Science Media Series*, editor: Wolfgang Walz; *Ionotropic Glutamate Receptor Technologies*, editor: Gabriela K Popescu

Team's contribution: Team – LCN - IP CAS: Performed all described experiments (molecular biology, microscopy, biochemistry, electrophysiology).

Supported: Czech Science Foundation (GACR) P303/11/P0075, 14-02219S, 14-09220P and Marie Curie IRG PIRG-GA-2010-276827.

3) INTERNAL COLLABORATION (within the Institute)

L. Vyklický – cooperation with Dr. K. Valeš - Neurophysiology of memory (IP CAS). The project involves behavioral experiments concerning effects of steroids, supported by joint grants.

4) DOMESTIC COLLABORATION (within the country)

L. Vyklický - cooperation with Dr. H. Chodounská and E. Kudová - Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague and M. Nekardova – structural modeling

L. Vyklický/A. Balík - cooperation with prof. J. Horáček - National Institute of Mental Health, Prague, Klecany

H. Krůšek/F. Vyskočil/ M. Horák - cooperation with prof. K. Kuča, Dr. O. Soukup, Mgr. V. Šepsová - Department of Toxicology and Department of Public Health, Faculty of Military Health Sciences, University of Defense, Hradec Králové (Sepsova et al., *Physiol Res* **63**: 771, (2014); Soukup et al., *Curr Alzheimer Res* **10**: 893, (2013); Soukup et al., *Physiol Res* **60**: 679, (2011))

H. Krůšek/F. Vyskočil - cooperation with prof. S. Ďaďo - Czech Technical University in Prague, Prague. The impedance of cell layer (J. Krusek et al., *Physiol Res* **63**: 705, (2014))

H. Krůšek/F. Vyskočil - cooperation with prof. P. Šebo - Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology, CAS (Osickova et al., *Mol Microbiol* **75**: 1550, (2010); Sebo et al., US, European and international patents: US 08017132; EP 2233569-A1; US 2012071393-A1; EP 2233569-B1; ES 2505324-T3; US 2010239550-A1; WO 2010136231-A1; US 8017132-B2; CA 2755694-A1; EP 2411513-A1; CN 102439146-A ; JP 2012521207-W; HK 1166644-A0; RU 2011138706-A; IN 201107232-P1)

H. Krůšek/F. Vyskočil - cooperation with prof. S. Adámek - 3. Surgical Department, First Faculty of Medicine, Charles University, Prague (Adamek et al., *Brain Res* **1370**: 215, (2011))

V. Vlachová - cooperation with the group of doc. I. Barvík - Institute of Physics, Faculty of Mathematics and Physics, Charles University, Prague. Expertise on homology modeling.

5) INTERNATIONAL COLLABORATION

L. Vyklický – cooperation within groups involved in SIXTH FRAMEWORK PROGRAMME - PRIORITY 1 - LifeSciHealth - Life sciences, genomics and biotechnology for health (*LSHM-CT-2007-037765). PHOTOLYSIS* - Specific Targeted Research Project - Development of flash photolysis for deep uncaging in vivo and high throughput characterization of neurotransmitter-gated ion channels in drug discovery.

L. Vyklický – cooperation with M. Petrovic, MD, PhD - School of Pharmacy and Biomedical Sciences, Maudland Building, MB140; University of Central Lancashire, Preston, Lancashire, PR1 2HE, UK.

T. Smejkalová - cooperation with S. Mennerick, PhD - Washington University in St. Louis, Division of Biology and Biomedical Sciences, Department of Psychiatry, 660 S. Euclid Ave., St. Louis, MO 63110, USA. Our collaboration is focused on the modulation of excitatory and inhibitory synaptic transmission by steroids.

M. Horák - cooperation with Y. H. Suh, Ph.D. – Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, South Korea. Our collaboration is focused on the regulation of glutamate receptor surface stability and synaptic localization by *N*-glycosylation.

A. Balík - cooperation with dr. I. Greger - MRC Laboratory of Molecular Biology, Neurobiology Division, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, UK. Our collaboration is focused on AMPAR biogenesis in neuronal circuits.

A. Balík - cooperation with prof. M. Jantsch - Department of Chromosome Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria. Our collaboration is focused on molecular mechanisms involved in AMPAR mRNA processing.

H. Krůšek/F. Vyskočil - bilateral experimental cooperation with prof. E. E. Nikolsky and prof. E. A. Bukharaeva - Kazan branch of Russian Academy of Sciences and Kazan Medical University. Our collaboration was focused on the properties and regulation of quantal and nonquantal neurotransmitter release and synaptic transmission at neuromuscular synapses. At the rat neuromuscular synapse, the quantal and non-quantal Ach release is controlled by glutamatergic and purinergic systems, voltage sensitive P/Q type of calcium channels and ryanodine sensitive Ca-dependent /Ca-release (Khuzakhmetova et al., *Int J Dev Neurosci* **34**: 9, (2014); Lindovsky et al., *Eur J Pharmacol* **688**: 22, (2012); Malomouzh et al., *Neurosci Res* **71**: 219, (2011); Nurullin et al., *Physiol Res* **60**: 5, (2011); Malomouzh et al., *Physiol Res* **60**: 185, (2011); Petrov et al., *Br J Pharmacol* **163**: 732, (2011); Volkov et al., *Comp Biochem Physiol A* **158**: 520, (2011)).

V. Vlachová - long-term cooperation with prof. P.W. Reeh and doc. K. Zimmermann - Institute of Physiology and Experimental Pathophysiology, University of Erlangen. Several papers have been published in direct collaboration and a unique method for the application, heating and cooling of solutions was developed.

6) KEY METHODOLOGY AND CORE FACILITIES

Electrophysiology

patch-clamp recording
whole-cell and isolated-patch recording
macroscopic and single-channel analysis
drug application and subsequent non-stationary analysis
solution application at defined temperature 15-40°C
modeling

In vitro cellular models

preparation of primary hippocampal neurons and cerebellar granule cells
neuronal micro-island cultures
acute brain slices
organotypic brain slices
heterologous expression of receptors in HEK293 and COS cell lines
transfection of neurons
virus assisted DNA transfection/infection

Molecular biology

complex DNA/RNA analysis
PCR based technology
PCR analysis of glutamate receptor genes in patients suffering from schizophrenia
in situ hybridization

Bioinformatics

molecular modeling and structural bioinformatics
protein/protein and protein/small molecule docking
homology modeling
molecular dynamics
quantum chemical calculations
genome/RNA expression data analysis

Optical methods

methods of ratiometric imaging of intracellular Ca^{2+} ions
FRET method using CellR imaging system for quantification of protein interaction
immunofluorescence widefield and confocal microscopy

Biochemistry

western blot analysis of gene expression
quantitative measurements of surface and total NMDAR expression – colorimetric assay
lectin binding assay and deglycosylation assay

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

List of international and domestic grants in which several institutes from The Czech Academy of Sciences and/or Charles University participate(d):

Project of excellence in the field of neuroscience - GBP304/12/G069; Czech Science Foundation; coordinator L. Vyklický, (2012-2018) - (four institutions, 8 laboratories in the IP CAS)

Neuroscience Centre - LC554; Ministry of Education, Youth and Sport; co-investigator L. Vyklický, (2005 -2011) - Institute of Experimental medicine CAS (two institutes, 8 laboratories in the IPCAS)

Center of Neuropsychiatric Studies (Neurobiology in Clinical Application) - 1M0517; Ministry of Education, Youth and Sports; co-investigator L. Vyklický, (2005-2011) – National Institute of Mental Health (two institutes, 8 laboratories in the IP CAS)

Elucidating mechanisms that regulate early trafficking of NMDA receptors (PIMarie Curie IRG (FP7) - PIRG-GA-2010-27682; EU; M. Horák, (2011-2015)

Center of Biomedical Research - CZ.1.07/2.3.00/30.0025; Ministry of Education, Youth and Sport; L.Vyklický, V. Vlachová, (2012-2015)

Center for Development of Original Drugs - TE01020028; Technology Agency of the Czech Republic; co-investigator L. Vyklický, (2012 – 2019) - Institute of Organic Chemistry and Biochemistry CAS

SIXTH FRAMEWORK PROGRAMME - PRIORITY 1 – LifeSciHealth - LSHM-CT-2007-037765; Life sciences, genomics and biotechnology for health; PHOTOLYSIS - Development of flash photolysis for deep uncaging in vivo and high throughput characterization of neurotransmitter gated ion channels in drug discovery; L. Vyklický, (2008-2011)

8) OTHER RELEVANT INFORMATION

Postdoctoral stays (at least a year) of students that defended their PhD at the IP CAS

Lucie Svobodová (at the IP CAS till 2012) - Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, Sant Joan d'Alacant, 03550-Alicante, Spain

Ondřej Cais, PhD. (at the IP CAS till 2012) - MRC Laboratory of Molecular Biology, Neurobiology Division, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge CB2 0QH, UK

Miloslav Sedláček, PhD. (at the IP CAS till 2011) - NIDCD, 31 Center Drive, MSC 2320, Bethesda, MD USA

Miloš Petrovic, MD, PhD. (at the IP CAS 2014) - School of Pharmacy and Biomedical Sciences, Maudland Building, MB140; University of Central Lancashire, Preston, Lancashire, PR1 2HE, UK

Katja Witschas, PhD. (Dr. rer. nat.), Postdoctoral Research Assistant - Department of Pharmacology, University of Oxford, UK

Jan Benedikt, PhD. (Assistant Professor) - Universidad Central del Caribe; Puerto Rico, USA

RNDr. Jiří Lindovský, PhD. (at the IP CAS till 2010) – Institut de Neurobiologie Alfred Fessard CNRS, Gif-sur-Yvette, France

9) SUMMARY AND RESEARCH IMPACT

Ion channels are membrane proteins that conduct ionic currents across cellular membranes and contribute to numerous physiological processes such as synaptic transmission, cell excitability and sensory transduction. They are implicated in the pathogenesis of numerous human diseases and represent one of the most important drug targets. Detailed information about ion channel structure and function is prerequisite for efficient drug design. The main focus of our research are ligand-gated ion channels in particular - specifically glutamate and transient receptor potential receptors. We use different biophysical and biochemical techniques, molecular biology, DNA mutagenesis and electrophysiology with the aim of combining the quantitative data to unifying structural or kinetic models. Our goal is to contribute to a better understanding of ligand-gated ion channel structure and function and to propose new therapeutic interventions that may be beneficial for patients suffering from neurological and psychiatric diseases.

This Report was also aimed to show that the described research team is solving relevant experimental questions attractive to young students and that the education and skills they learn allow them to gain competitive positions in respected foreign laboratories.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Epithelial Physiology

A. TEAM DESCRIPTION

Research focus

Experimental work of the team is focused on two research domains:

- biology, physiology and pathophysiology of the intestine
- cellular and molecular mechanisms of corticosteroid regulations

In these domains, our team focused during the years 2010-2014 on the following topics:

Topic 1: *Inflammatory bowel disease and colorectal cancer*

The aim of our research was to determine the expression of pro-proliferative and anti-apoptotic genes in colitis-associated neoplasia and to identify changes of local metabolism of glucocorticoids. The topic was based on several lines of evidence, which implicate inflammatory bowel disease as a stimulator of epithelial-derived tumours in the intestine and glucocorticoids as anti-proliferative and pro-apototic factors.

Topic 2. *Peripheral circadian clock in the intestine*

The goal was to identify whether genes encoding transporters of intestinal absorption and secretion are regulated by circadian clock and whether the rhythmicity of circadian clock is changed during tumourigenesis because epidemiologic studies indicate that abnormal light/dark regimens (e.g. shift work) lead to increased incidence of tumours.

Topic 3. *Local metabolism of glucocorticoids and neuroendocrine regulatory pathways during stress and inflammation*

The main goal was (i) to characterize the effect of stress on the local metabolism of glucocorticoids both in brain and peripheral tissues and (ii) to determine whether changes in glucocorticoid metabolism are encompassed in the feedback regulation of

hypothalamus-pituitary-adrenal (HPA) axis at the level of limbic structures, PVN, pituitary and adrenal gland. The approach taken in this topic combined behavioural, neuroendocrine and biochemical approaches to identify whether various stressors (homotypic, heterotypic, psychosocial, inflammatory) are able to modulate local glucocorticoid signalling in secondary lymphoid organs and in HPA axis. The topic was based on widely recognized role of stress in health problems of humans and animals and on findings that dysfunction in the limbic system and dysregulation of HPA axis are involved in the pathophysiology of a range of diseases.

To achieve these aims, our group utilized a quite diverse set of experimental tools including biochemical analysis (i.e. determination of enzyme activities), genomic and proteomic techniques, microanatomy (laser microdissection of microscopic samples of brain and colon structures and neoplastic tissue), genetic models of weak and strong HPA axis activity (Fisher 344 and Lewis rats) and animal models of colitis, arthritis and colitis-associated colorectal cancer. Due to collaboration with the University Hospital we could work also with human samples of colorectal cancer and pre-malignant colorectal polyps.

1.2. *Personnel*

The team members are an interdisciplinary group with complementary background including physiologists and biochemists that have also competencies for working with laboratory animals. Actually our team consists of 1 senior scientist Jiří Pácha, Ph.D., D.Sc. (team leader, h-index 17) and 2 junior scientists (Jana Bryndová, Ph.D., h-index 8; Peter Ergang, Ph.D., h-index 6), 1 potdoctoral fellow (Karla Mazancová-Vagnerová, Ph.D., h-index 9; since September 2013 after returning from maternity leave), 1 PhD student (Martin Vodička, M.Sc, since October 2011), 2 laboratory assistants and 2 diploma students. In addition, 3 pregraduate and 6 graduate students were actively involved in our research projects during the evaluated period, 3 of them were awarded Master's degree and 4 Doctoral degree: M. Hock (Ph.D. in August 2011, now in business), P. Klusoňová (Ph.D. in February 2011, then postdoctoral fellow in our team and since February 2013 in the laboratory of Prof. A. Odermatt, Pharmacenter, Univ. Basel, Switzerland,), M. Soták (Ph.D. in October 2014, now postdoctoral fellow in AstraZeneca R&D Center in Mölndal, Sweden), J. Švec, M.D., (Ph.D. in January 2012, now a clinician in the University Hospital).

B. MOST IMPORTANT RESULTS

The following report outlines the selection of the most important results and the publications obtained in the frame of the research projects during the period 2010-2014.

Topic 1 and 2.

It is well known that cell proliferation in rapidly renewing tissues such as intestinal epithelium is a temporally regulated process showing circadian rhythmicity and that glucocorticoids are one of the major circadian oscillators whose rhythmic secretion is able to shift the phase of peripheral clock and to modulate proliferation, differentiation and apoptosis. In addition, the local concentration of glucocorticoids *in situ* does not depend only on plasma concentration of the hormone but also on local metabolism of glucocorticoids catalyzed by enzymes 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11HSD1, 11HSD2). 11HSD1 increases the local concentration of cortisol (corticosterone in rat and mice) due to the reduction of cortisone (or 11-dehydrocorticosterone) whereas 11HSD2 decreases this concentration due to the oxidation of cortisol (or corticosterone). It means that the former enzyme is able to amplify local glucocorticoid signals whereas the latter reduces these signals. The accumulating evidence indicates that disruption of circadian homeostasis is linked to cancer development and progression and we have shown in previous papers the existence of peripheral circadian clock in the colon (Sládek et al. *Gastroenterology* 133:1240,2007) and downregulation of 11HSD2 expression and activity in colorectal adenocarcinoma, (Žbánková et al. *Cancer Lett* 210:95,2004). Therefore we focused on the relationship between intestinal circadian clock and intestinal transporters, cell cycle and corticosteroid metabolism in healthy and neoplastic intestine.

In collaboration with the pathologists of the Third Faculty of Medicine, Charles University, Prague, and physicians of the University Hospital we studied the quantitative changes of a panel of pro-proliferative, anti-apoptotic and pro-inflammatory genes and their encoding proteins in the early stages of transition from ulcerative colitis to epithelial neoplasia and compared them with the changes observed in sporadic colorectal cancer using mucosal biopsies of human patients (Švec et al. *Inflamm Bowel Dis* 16:1127, 2010). We demonstrated quantitative differences of the studied genes between neoplastic and non-neoplastic mucosa and showed that some of these genes are upregulated already at the early stages of neoplasia in patients with chronic inflammation. The study illuminated the potential relevance of these markers at precancerous stages of ulcerative colitis. Our team (J. Švec, P. Ergang and J.Pácha, corresponding author) performed all molecular biological measurements (quantification of gene

transcripts) and prepared the manuscript. Histopathological and immunohistochemical analyses were done in the Institute of Pathology, Third Faculty of Medicine (T. Jirásek, V. Mandys) and samples were collected in the University Hospital (M. Kment). Detailed analysis of 11HSD1 and 11HSD2 expression in premalignant colorectal polyps and colorectal adenocarcinoma (Moravec et al. *Histol Histopathol* 29:489, 2014) proved that 11HSD2 is downregulated not only in adenocarcinoma but also in the early stages of neoplastic transformation (adenoma with low-grade dysplasia). These findings indicate the role of glucocorticoid signalling changes during transformation of colorectal epithelium from the early stage of tumour development. Histopathological and imunohistochemical analysis was done in the Institute of Pathology, Third Faculty of Medicine (V. Mandys), samples were collected in the University Hospital (Z. Zádorová, J. Hajer, M. Kment), the analysis of 11HSD1 and 11HSD2 expression and preparation of the manuscript was done by our team (M. Moravec, J Švec, P. Ergang, L. Řeháková and J. Pácha, corresponding author).

To study colorectal cancer in more detail we adopted the mouse models of sporadic (A/J mice) and colitis-associated cancer (ICR mice) and compare the expression of pro-proliferative, pro-inflammatory and anti-apoptotic genes in mouse and human tumours (Švec et al. *Int J Exp Pathol* 91:44,2010). This study proved that genetic background and/or mechanism of tumourigenesis associated with genotoxic insult and colonic inflammation influence the gene expression in colon epithelial neoplasia. Induction of tumour, quantification of gene expression in microsamples of neoplastic tissues isolated by laser microdissection and preparation of the manuscript were done by our team (J. Švec, P. Ergang and J. Pácha, corresponding author) whereas histopathological analysis of neoplastic changes was done by the colleagues from the Third Faculty of Medicine (V. Mandys, M. Kment). The mouse model of colitis-associated colorectal cancer was further used for studying the colonic circadian clock during neoplastic transformation and for analyzing the daily profiles of the clock-controlled genes regulating cell cycle (Soták et al. *Int J Cancer* 132:1032,2012). We showed that the circadian clock in primary tumours is severely disrupted and is not able to drive rhythmically the expression of clock-controlled genes including Wee1, a kinase that regulates the transition from interphase to mitosis. This is the first study demonstrating the relationship between the circadian clock and intestinal carcinogenesis “under physiological conditions”; previous studies used only genetically-modified animals. The study arose in collaboration of our team (M. Soták, P. Ergang and J. Pácha, corresponding author) with the Department of Neurohumoral Regulations of our Institute (L. Polidarová, A. Sumová). The design of the experiment was conceived jointly by both teams, the paper was prepared by J. Pácha and A. Sumová (expert in chronobiology).

Based on these results, we were asked by the Editor of the journal *Annals of Medicine* to prepare a review on topic „circadian clock, cell cycle and cancer“ (Soták et al. *Ann Med* 46:221,2014), where A. Sumová prepared the chapter about circadian clock and J. Pácha (corresponding author) together with M. Soták the chapters about molecular organization of the cell cycle, its circadian rhythmicity and interplay between cell cycle, circadian clock and tumourigenesis including meta-analysis of rhythmicity of putative genes associated with cell cycle progression, proliferation and apoptosis based on 39 published microarray data sets.

Circadian clock controls not only genes regulating cell cycle, proliferation and apoptosis but also many other genes that play important role in various biological and physiological processes. In 2011, we have identified the role of colonic circadian clock in colonic electrolyte absorption (Soták et al. *Am J Physiol Gastrointest Liver Physiol* 301:G1066,2011). We showed circadian rhythmicity in the expression of genes encoding transporters and channels operating in colonic NaCl absorption and showed that these genes are in phase with colonic clock genes under normal and restricted feeding conditions. In addition, we showed the relationship between regulation of sodium absorption and expression of clock genes via aldosterone, which stimulated not only sodium absorption but also expression of clock gene *Per1*. Our findings show that circadian system may facilitate the colon to anticipate changes in food and chyme entry. In addition, our findings are of potential importance to further studies of gastrointestinal physiology and nutrition science, in particular gastrointestinal transport, as a majority of studies in rats is done during daytime, i.e. in the period of day when transport activity of noctural rats is significantly decreased. This study was done in collaboration with the team of A. Sumová (L. Polidarová, A. Sumová) who contributed the chronobiological expertise and participated in planning the experiments and writing the paper. The experimental work was done predominantly by our team (M. Soták, J. Musílková, M. Hock and J. Pácha, corresponding author). Based on these findings we were asked by the editor of *Acta Physiologica* to write a review about circadian regulation of intestinal epithelium (Pácha, Sumová *Acta Physiol* 208:11,2013). A. Sumová prepared the chapters about the mammalian circadian system and its molecular mechanisms whereas J. Pácha (corresponding author) the chapters devoted to circadian regulation of epithelial proliferation, digestion, absorption and secretion.

Topic 3.

11HSD1, an enzyme which regulates local glucocorticoid activity, is considered to play an important role in various diseases. In previous studies we demonstrated upregulation of 11HSD1 and downregulation of 11HSD2 in experimental TNBS- and DSS-colitis of rats and

mice and in patients with ulcerative colitis (Bryndová et al. *Scand J Gastroenterol* 39:549,2004; Vagnerová et al. *J Physiol* 191:497,2007; Žbánková et al. *J Gastroenterol Hepatol* 22:1019,2007). Based on these findings we studied the effect of inflammation on immune cells and secondary lymphoid organs in the period 2010-2014 (Ergang et al. *J Steroid Biochem Mol Biol* 126:19,2011). We showed that colitis is characterized by increased bioavailability of endogenous glucocorticoids in lymphoid organs and cells and that pro-inflammatory cytokines play an important role in this process. This study was one of the first detailed identification of changes of local metabolism of glucocorticoids in cells and organs of mucosal immune system during colitis and was entirely done by our team. The positive regulatory role of pro-inflammatory cytokines on 11HSD1 during inflammation and its physiological meaning we supported in further experiments using rats with adjuvant arthritis (Ergang et al. *Mol Cell Endocrinol* 323:155, 2010). We showed that inflammation upregulated synovial 11HSD1 together with cyclooxygenase 2 (COX2) and pro-inflammatory cytokines TNFα and IL-1β. In addition, treatment with 11HSD1 inhibitor in vivo increased the expression of cytokines whereas cytokine antagonists reduced expression of 11HSD1. The results suggest that inflammatory stress controls local 11HSD1 by pro-inflammatory cytokines and that the increased supply of local biologically active glucocorticoids might contribute to feedback regulation of inflammation. The study was designed and performed by our team (P. Ergang, P. Leden, K. Vagnerová, P. Klusoňová and J. Pácha, corresponding author), I. Mikšík from other team of the Institute of Physiology performed HPLC analysis of corticosteroids and the colleagues from the Third Faculty of Medicine and University Hospital (J. Jurčovičová, M. Kment) provided arthritic rats and their clinical evaluation.

Social stress has profound influence on immune and inflammatory responses and therefore we also studied the effect of social stress on activity of 11HSD1 in specific structures of HPA axis and in secondary lymphoid organs (Vodička et al. *Plos One* 9:e89421, 2014).Similar to inflammatory stress, chronic social stress upregulated 11HSD1 in secondary lymphoid organs but not in canonical components of HPA axis (paraventricular nucleus of hypothalamus, pituitary and adrenal gland). However, social stress selectively upregulated 11HSD1 in brain structures associated with indirect regulation of HPA axis, specifically in prefrontal cortex, amygdala and some regions of hippocampus. These findings support the hypothesis of indirect modulation of glucocorticoid feedback of the HPA axis by 11HSD1. The study was designated and performed predominantly by our team (M. Vodička, P. Ergang, L. Řeháková, P. Klusoňová, J. Makal, M. Soták, J. Musílková and J. Pácha, a corresponding author), A. Mikulecká from other team of the Institute of Physiology designed the social stress

paradigm and performed ethological analysis and P. Zach (Third Faculty of Medicine) contributed the expertise in neuroanatomy. In very recent paper published electronically in December 2014 (Ergang et al. *Psychoneuroendocrinology* 53:49,2015) we showed that the above mentioned effect of stress on 11HSD1 does not depend on quality of stress and duration of stress exposition. Similar effects on 11HSD1 were induced by short-term variable stress combining both physical and emotional stress. This study was designated and performed by our team (P. Ergang, M. Vodička, M. Soták, P. Klusoňová, L. Řeháková and J. Pácha, a corresponding author), only M. Behuliak (Department of Experimental Hypertension) performed the analysis of catecholamine biosynthetic pathway and P. Zach (Third Faculty of Medicine) contributed the expertise in neuroanatomy.

C. INTERNAL COLLABORATION (WITHIN THE INSTITUTE)

Our team established a fruitful partnership with several other teams of the Institute of Physiology. In particular, we have a long-lasting cooperation with the teams of I. Mikšík (Dpt. of Analysis of Biologically Active Compounds, DABAC), A. Sumová (Dpt. of Neurohumoral Regulations, DNR) and J. Zicha (Dpt of Experimental Hypertension, DEH). Other less intensive collaboration represented in the evaluated period the collaboration with the Department of Developmental Epileptology, in particular with A. Mikulecká, who participated in our study published last year (Vodička et al. *Plos One* 9:e89421, 2014).

3.1. *Collaboration with DABAC*

Mikšík's team of analytical chemists is indispensable in projects in which it is necessary to analyze and quantify corticosteroids using high pressure liquid chromatography and mass spectrometry (identification and quantification of steroids) and in implementation of proteomic methods, which we plan to use more frequently in the upcoming period 2015-2019 (our first collaborative proteomic paper was published in November 2014 in *Analytical and Bioanalytical Chemistry*). The collaboration with the team of I. Mikšík carried out during the period 2010-2014 has yielded 5 international publications in peer-reviewed journals (Ergang et al. *Mol Cell Endocrinol* 323:155,2010; Vagnerová et al. *Steroids* 76:577,2011; Ergang et al. *J Steroid Biochem Mol Biol* 126:19,2011; Klusoňová et al. *Steroids* 76:1252,2011; Mikšík et al. *Anal Bioanal Chem* 406:7633,2014).

3.2. *Collaboration with DNR*

Similarly, our team also has a very fruitful and long-lasting cooperation with the team of A. Sumová, whose scientific program focuses on chronobiology. Collaboration between our teams led to development of a new project (supported by Czech Science Foundation) devoted to the role of circadian clock in physiology and pathophysiology of gastrointestinal tract. In addition to the above mentioned 4 papers corresponded by our team (Soták et al. *Am J Physiol Gastrointest Liver Physiol* 301:G1066,2011; Soták et al. *Int J Cancer* 132:1032,2012; Pácha, Sumová *Acta Physiol* 208:11,2013; Soták et al. *Ann Med* 46:221,2014) we collaborated in 2 other papers (Polidarová et al. *Chronobiol Int* 28:204,2011; Polidarová et al. *Am J Physiol Gastrointest Liver Physiol* 306:G346,2014) corresponded by DNR. These two papers provided details (i) about the relationship between the central circadian clock of brain (the suprachiasmatic nucleus) and the peripheral clock in specific intestinal segments and (ii) about the ontogeny of intestinal circadian clock.

3.3. *Collaboration with DEH*

In collaboration with the team of J. Zicha we studied regulation of local metabolism of glucocorticoids in vascular smooth muscle cells (Vagnerová et al. *Steroids* 76:577,2011) and in Prague hereditary hypertriglyceridemic rat (HHTg), a unique non-obese model of metabolic syndrome (Klusoňová et al. *Steroids* 76:1252,2011). In the former study, we showed that in vascular smooth muscle cells 11HSD1 is regulated by ligands of peroxisome proliferator-activated receptor-γ via transcription factors C/EBPα and C/EBPζ. In the latter study we tested in a model of non-obese metabolic syndrome the local metabolism of glucocorticoids in liver, adipose tissue and skeletal muscles because the data from genetically modified mice suggested that 11HSD1-dependent glucocorticoid amplification may contribute to insulin resistance, hypertension and dyslipidemia.

D. DOMESTIC COLLABORATION (WITHIN THE COUNTRY)

Our team has long-lasting collaboration with the Third Faculty of Medicine. This collaboration reflects the limits of our professional expertise in research projects that require histopathological and neuroanatomical analyses. In addition, this collaboration enables our team to analyse some pathophysiological problems of tumourigenesis and intestinal bowel disease not only at the level of animal models but also at the level of real disease of the patients. During the period 2010-2014 the collaboration was supported by grants from Czech Research

Council and from Ministry of Health and the colleagues from the Medical faculty participated in 6 papers, which were corresponded by our team (see above).

One member of our team (J. Pácha) is a teacher at the Faculty of Natural Science, Charles University, in the Bachelor, Master and Ph.D. programs in Biology and Physiology. This activity at the faculty facilitates participation of the pregradual and gradual students in research of our team.

E. KEY METHODOLOGY AND CORE FACILITIES

The team uses a broad spectrum of genomics related techniques such as quantitative RT-PCR, techniques for protein separation, radiometric methods for measurement steroid enzyme activities, separation and analysis of steroids, laser microdissection and electrophysiological analysis of the epithelium (Ussing chamber, voltage clamp). As regard the infrastructure, the team has all necessary equipment suitable for biochemical and molecular biological measurements (electrophoresis, PCR cyclers, immunoblotting, MagnaLyser homogenizer, spectrophotometer, facilities for cell culture experiments and laser microdissection (Leica LMD 6000)) and for electrophysiological analysis (voltage clamp) and the facility for housing animals. Some facilities for genomic, proteomic and metabolomic analyses are shared with other teams of the Institute of Physiology (mass spectrometers, HPLC systems, phospho/fluoro-imager, ABI PRISM 7000 Sequence Detection System, etc.) or with other institutes of the Biomedical Campus (Cytometry service). Our team developed technique of laser microdissection of specific areas of histological microscopic preparations followed by genomic analysis of small tissue samples and trained some colleagues from other teams to use this very precise method for analysis of relationship between gene expression and local structure.

F. INVOLVEMENT IN SIGNIFICANT PROJECTS

During the evaluated period our team was involved in three projects supported by the Czech Science Foundation and Ministry of Health corresponding to grants *Local metabolism of glucocorticoids and neuroendocrine regulatory pathways during stress* (PI: J. Pácha), *Role of internal time-keeping system in pathogenesis of colorectal cancer* (PI: J. Pácha) and *Predictive molecular markers of colorectal cancer development* (Co-PI: J. Pácha, collaborative research grant of our team and the colleagues from the Third Faculty of Medicine, Charles Univ., Prague)

G. SUMMARY AND RESEARCH IMPACT

During the period under the review 2010-2014, we have been working on 3 projects and

achieved results mentioned above. As specific scientific outcomes of the team we can underline: (1) unequivocal demonstration that many physiological functions in colon epithelium are controlled by circadian clock, a fact that has been usually ignored in gastrointestinal research, (2) demonstration of disruption of circadian homeostasis in colorectal cancer and (3) ability of inflammatory and psychosocial stress to modulate local metabolism of glucocorticoids, which indicates the possibility for pharmacological intervention in various diseases. Last, but not least, several PhD students worked in our team and the original results of their research activity were included in their PhD theses.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Functional Morphology

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

The main research interest of our department is to study **mechanisms of pain** and to explore new possibilities of pain treatment, especially in chronic states. Our experimental work is concentrated on the modulation of nociceptive information at the spinal cord level that is the relay center between the periphery and the higher brain areas. Our goal is to study these modulatory mechanisms in order to improve therapy for pain conditions, such as allodynia, hyperalgesia, neuropathic and cancer related pain. In our research we use mainly electrophysiological, immunohistochemical and behavioral methods.

Another project is directed towards the role of thyroid hormones (THs) and omega-3-polyunsaturated fatty acid (n-3 PUFA) in the **physiology of cardiac and skeletal muscles**. Models of hyper- and hypothyroid status are used to analyze potential influence of n-3 PUFA on the induced pathological changes. Muscle grafting model is also used to analyze effect of THs on regeneration of slow and fast skeletal muscles.

ii. PERSONNEL

- Senior scientist(s)

Jiří Paleček MD, PhD, department head, H index 20, citations >1520
Expertise in mechanisms of pain

Tomáš Soukup, RNDr, PhD, H index 20, Citations>1460
Expertise in muscle development, regeneration and muscle fiber transformation

- Junior scientist(s):

Diana Špicarová, MSc, PhD, H index 4, Citations>55
Expertise in mechanisms of pain,
Received her PhD degree in 2010, maternal leave in 2011

- Postdoctoral fellow(s):

Mickael Diallo PhD, H index 5, Citations>58
Expertise in mechanisms of pain
Works at the department since 2012

- PhD/MSc/BSc students (7/4/4), laboratory assistants (2)

PhD students

David Sojka PhD, Diana Špicarová PhD, Petr Novák PhD, Petra Mrózková MSc, Vladimír Nerandžič MSc, Natalia Kalynovská MSc, Pavel Adámek MSc

2) KEY RESULTS

i. Pain Mechanisms

Physiological pain is an important biological mechanism designed to protect organism from potentially harmful stimuli affecting both somatic and visceral organs. Pathological states characterized by increased sensitivity to innocuous and noxious stimuli (allodynia, hyperlagesia) and spontaneous chronic pain can become debilitating diseases, often recalcitrant to the best available treatment efforts. Millions of chronic pain patients suffer and unrelieved chronic pain problems often result in an inability to work and to lower quality of life. There are also significant costs associated with pain and chronic pain treatment by the society every year. The cellular and physiological mechanisms underlying the development of chronic pain and associated phenomena are still not fully understood. This makes it difficult to design new and more effective therapeutic alternatives especially for the chronic pain patients. The mechanisms of allodynia and hyperalgesia are investigated by numerous laboratories and it is now clear that both peripheral and central processes may play a role in their development. In our work, we have concentrated on identifying some of the cellular mechanisms responsible for modulation of nociceptive synaptic transmission at the spinal cord level, as an underlying mechanism of hyperalgesia and allodynia. **Our main focus is on the role of spinal cord transient receptor potential vanilloid-1 (TRPV1) receptors in nociceptive transmission and modulation of their function during pathological pain states** (reviewed in Spicarova et al. Physiol. Res.,2014, 63, 1, 225-236).

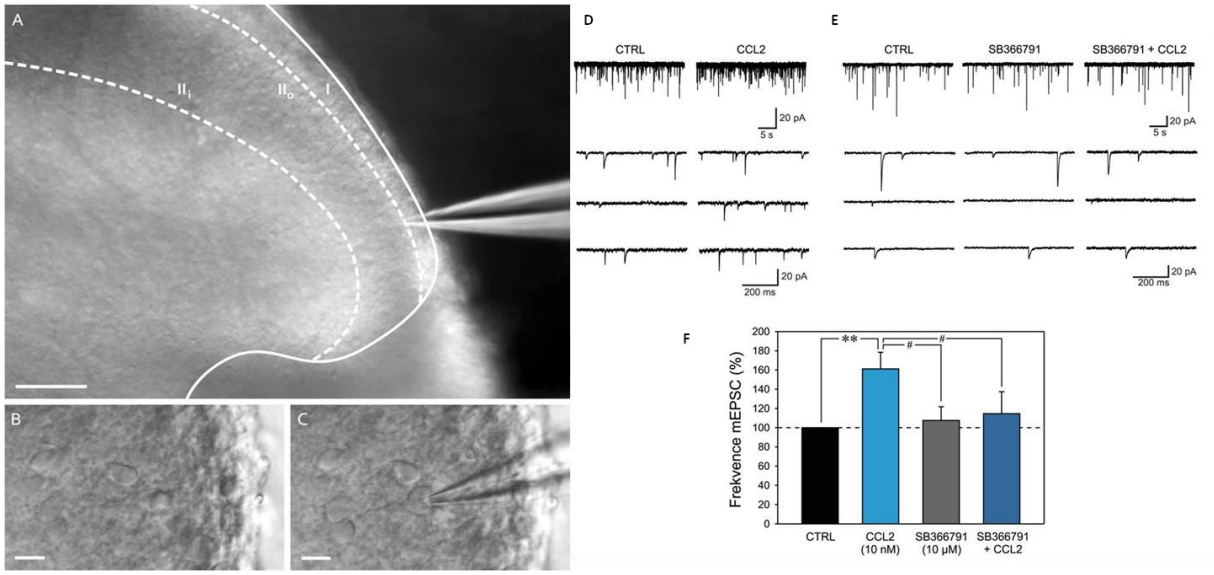
Modulation of synaptic transmission in the spinal cord dorsal horn is thought to be involved in the development and maintenance of different pathological pain states. Transient receptor potential vanilloid 1 (TRPV1) receptors are known as molecular integrators of nociceptive stimuli in the periphery, but their role on the presynaptic spinal endings of nociceptive DRG neurons is unclear. In our previous experiments we have shown that after phosphorylation and during peripheral inflammation, the sensitivity of spinal TRPV1 receptors is increased and application of a very low concentration of endogenous TRPV1 receptor agonist N-oleoyldopamine (OLDA) may lead to their activation (Spicarova and Palecek, J Neurophysiol 2009, 102, 234–243). In the experiments described here, we have evaluated the influence of neuroinflammatory cytokines and chemokines in the process, especially under the conditions of peripheral neuropathy.

The proinflammatory cytokine, tumor necrosis factor α (TNFα), is an established pain modulator in both the peripheral and the central nervous system. Up-regulation of TNFα and its receptors (TNFR) in dorsal root ganglion (DRG) cells and in the spinal cord has been shown to play an important role in neuropathic and inflammatory pain conditions. In our experiments the possible influence of TNFα on presynaptic spinal cord TRPV1 receptor function was investigated. Using patch-clamp technique, we have showed that a population of dorsal horn neurons with capsaicin sensitive primary afferent input had significantly higher basal mEPSC frequency after the TNFα pretreatment when compared to the controls. The incubation with TNFα also induced robust sensitivity to endogenous TRPV1 agonists. Our results indicated that TNFα has a significant impact on nociceptive signaling at the spinal cord level that could be

mediated by increased responsiveness of presynaptic TRPV1 receptors to endogenous agonists. This could be of major importance, especially during pathological conditions, when increased levels of TNFα and TNFR are present in the spinal cord. Špicarová and Paleček, Journal of Neuroinflammation. 2010, 7, 49. *This work was done entirely at the department with the following grant support (GACR 305/09/1228, MSMT LC554).*

To further investigate the role of TNFα and its receptors in pain modulation under conditions of peripheral neuropathy, we have extended our experiments and used a model of peripheral neuropathy. Recordings from superficial dorsal horn neurons in spinal cord slices after axotomy showed robust, significant increase in the spontaneous EPSC frequency after TNFα incubation (7.9 ± 2.2 Hz) when compared to the neurons without incubation (2.8 ± 0.8 Hz). The effect of TNFα treatment was much less pronounced in the slices from the control animals. Tetrodotoxin (TTX) application in slices from axotomized animals and after TNFα incubation decreased the mEPSC frequency to only 37.4 ± 6.9% of the sEPSC frequency. This decrease was significantly higher than in the slices without the TNFα treatment (64.4 ± 6.4%). The nerve injury also induced significant increase in sensitivity to endougenous agonists of presynaptic TRPV1 receptors. Our results indicated that TNFα may enhance spontaneous transmitter release from primary afferent fibres in the spinal cord DH by modulation of TTX-sensitive sodium channels following sciatic nerve injury. Modulation of TRPV1 receptors function on primary sensory terminals by TNFα may play an important role in neuropathic pain development. Špicarová, Nerandžic and Palecek Journal of Neuroinflammation. 2011, 8, 177. *This work was done entirely at the department with the following grant support (GACR 305/09/1228, MSMT LC554, GAUK 309211, P303/12/P510).*

In the next set of experiments we have investigated the role of the chemokine CCL2 (C-C motif ligand 2) in the development of neuropathic pain after peripheral nerve injury. In our experiments we have studied the effect of CCL2 application and TRPV1 receptor activation on nociceptive signaling and the modulation of synaptic transmission. Intrathecal drug application in behavioral experiments and patch-clamp recordings of spontaneous, miniature and dorsal root stimulation-evoked excitatory postsynaptic currents (sEPSCs, mEPSCs, eEPSCs) from superficial dorsal horn neurons in acute rat spinal cord slices were used. The intrathecal application of CCL2 induced thermal hyperalgesia and mechanical allodynia, while pretreatment with the TRPV1 receptor antagonist SB366791 diminished the thermal but not the mechanical hypersensitivity. Patchclamp experiments showed an increase of sEPSC and mEPSC frequency and eEPSCs amplitude in dorsal horn neurons after acute CCL2 application. Immunohistochemical analysis showed that CCL2 induced increase of phosphorylated extracellular signal regulated kinase (pERK) in the dorsal horn that was dependent on the TRPV1 receptors activation. Our results demonstrated that activation of spinal TRPV1 receptors plays an important role in the modulation of nociceptive signaling induced by the neuroinflammatory chemokine CCL2. The mechanisms of cooperation between the CCL2 activated receptors and TRPV1 receptors on the central branches of primary afferent fibers may be especially important during different pathological pain states. Špicarová, Adámek, Kalynovska, Mrózková and Palecek Neuropharmacology. 2014, 81, 75-84. *This work was done entirely at the department with the following grant support (GACR 305/09/1228, GACR P303/12/P510, GACR P304/12/G069, MSMT LH12058, RVO:67985823, GAUK 253154).*



The role of TRPV1 receptors in modulation of nociceptive information at the spinal cord level. In acute spinal cord slices (A), synaptic activity of individual neurons (B) is recorded with patch clamp technique (C). Inflammatory chemokine CCL2 application, released during neuropathic states, leads to increased mESPC activity of spinal dorsal horn neurons (D). This increase is dependent on TRPV1 receptors activation and is prevented by their antagonist SB366791 (E, F).

Acute postoperative pain is one of the frequent reasons for pain treatment. However, the exact mechanisms of its development are still not completely clear. In continuation of our previous experiments with local capsaicin application (Pospisilova and Palecek, Pain, 125, 233–243, 2006) we have investigated the contribution of TRPV1 receptors expressed on cutaneous peripheral nociceptive fibers and on the presynaptic endings of primary afferents in the spinal cord, to the development and maintenance of hypersensitivity to thermal and mechanical stimuli following surgical incision. A rat plantar incision model was used to test paw withdrawal responses to thermal and mechanical stimuli. Plantar incision in a rat model induced mechanical allodynia, hyperalgesia and thermal hyperalgesia. A single intrathecal administration of TRPV1 receptor antagonist significantly reduced postincisional thermal hyperalgesia and also attenuated mechanical allodynia, while mechanical hyperalgesia remained unaffected. Local intradermal TRPV1 antagonist treatment reduced thermal hyperalgesia and mechanical allodynia without affecting mechanical hyperalgesia. Our experiments confirmed that both peripheral and spinal cord TRPV1 receptors are involved in increased cutaneous sensitivity following surgical incision. The strong analgesic effect of the intrathecal TRPV1 receptor antagonist application was especially evident in the reduction of thermal hyperalgesia. This work further supports our view of a key role of spinal TRPV1 receptors in modulation of nociceptive activity and pain. Uchytilová, Špicarová and Palecek Molecular Pain. 2014, 10, 67. *This work was done entirely at the department with the following grant support (P304/12/G069, LH12058,P303/12/P510, CBV CZ.1.07/2.3.00/30.0025 European Social Fund and the state budget of the Czech Republic, BIOCEV CZ.1.05/1.1.00/02.0109 European Regional Development Fund, RVO67985823).*

Glutamate AMPA receptors are critical for sensory transmission at the spinal cord dorsal horn. Plasma membrane AMPA receptor endocytosis that can be induced by insulin may underlie long term modulation of synaptic transmission. Insulin receptors are known to be expressed on spinal cord DH neurons, but their possible role in sensory transmission has not been studied. In this work we tested the hypothesis that insulin application would induce decrease of AMPA mediated excitatory postsynaptic currents recorded in spinal cord dorsal horn neurons, evoked by dorsal root stimulation, possibly due to AMPA receptors internalization. Our experiments showed that in 75% of the neurons the size of the AMPA eEPSCs was significantly reduced when insulin was applied. This eEPSC reduction was dependent on tyrosine kinase activation as application of its inhibitor, lavendustin A, prevented the insulin induced AMPA eEPSCs depression. These findings suggested a new possible role of the insulin pathway in modulation of sensory and nociceptive synaptic transmission in the spinal cord. Špicarová and Palecek, Neuroscience 2010, 166, 1, 305-311. *This work was done entirely at the department with the following grant support (GACR 305/06/1115, GACR 305/09/1228, MSMT CR LC554, AV0Z 50110509).*

ii. Physiology of cardiac and skeletal muscles

Research on Omega 3 FA supplementation in rats with altered thyroid status

Alterations of thyroid hormones (THs) level play a significant role in human health and they become an important problem of contemporary society. THs exert unequivocally positive roles in healthy organism, however, alterations of their level (excess or deficiency) can result in the deterioration of many physiological functions including lipid level alterations, metabolic changes, membrane functions and severe diseases such as cardiac arrhythmias (reviewed in Tribulova et al., Vascul Pharmacol 52: 102-112, 2010) or even psychiatric disorders (Říčný et al. 2011). On the other hand, omega-3 polyunsaturated fatty acids (n-3 PUFA) have been suggested in many clinical trials and in animal models to possess multiple positive effects, like reduction of lipid levels, metabolic effects, direct interactions with cytosolic or membrane bound proteins, alteration of membrane fluidity or cardiac tissue remodeling and cell-to-cell communications. Our preliminary experiments suggested that n-3 PUFA applied as dietary supplementation (Vesteralens, Norway) can partially ameliorate some of these pathological changes (Říčný et al., WCBR, Prague, 2011, Radosinska et al., J Hypertension 31: 1876-1884, 2013; Bacova et al., Exp Clin Cardiol 18 (Suppl A): 41A-46A, 2013; Radosinska et al., In: Adaptation Biology and Medicine: New Challenges. Eds. L.M.Popescu, A.R.Hargens and P.K.Singal. Narosa Publishing House, New Delhi, Vol. 7, pp. 19-34, 2014; Radošinská et al., Cardiology letters 23: 10-16, 2014 and this topic was reviewed by T.Soukup (Soukup T, Physiol Res 63,Suppl 1: S119-S131, 2014). On the other hand, n-3 PUFA effect on serum lipids as revealed in our experiments was not significant (Rauchová et al., Horm Metab Res 43: 43–47, 2011; Pavelka et al., Industrial Toxicology 2012, pp. 139-143; Pavelka S and Soukup T, Industrial Toxicology 2012, pp. 231-235; Rauchová et al., Horm Metab Res 45: 507-512, 2013; Rauchová et al., Industrial Toxicology, pp. 140-145, 2013; Pavelka et al., Industrial Toxicology, 109-115, 2014).

We have used hyperthyroid and hypothyroid rats as models of pathological state and analyzed whether and how n-3 PUFA applied as the OMACOR (the only preparation approved for clinical use) ameliorated pathological changes caused by

alteration of THs levels in adult Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) of both sexes. We have also tried to evaluate the influence of genetic background given by different rat strains. It was shown previously that many pathophysiological changes are strengthened by high blood pressure and high lipid levels, as well as by sex differences, as thyroid gland dysfunction in humans affects about 5-7% of the population, but the ratio of women and men among these patients is about 6:1. As n-3 PUFAs possess multiple sites of potential actions rather than a specific mode of action, we believe that our complex research will contribute to better understanding of n-3 PUFA actions and eventually to better treatment options of severe life style diseases with great socio-economic impact in humans. As one of the main results, we found that n-3 PUFA intake suppressed incidence of ventricular fibrillation and facilitated sinus rhythm restoration in SHR rats at early and late stage of hypertension. We have attributed the antiarrhythmic effect to the attenuation of abnormal distribution, expression and phosphorylation of myocardial Cx43, to positive modulation of PKCε and δ signaling and to normalization of MyHC profiles. These findings support the n-3 PUFA prophylactic use to minimize risk of lethal arrhythmias in hypertensive individuals. Radosinska et al., J Hypertension 31: 1876-1884, 2013. *Experiments were performed by the laboratory staff (Kopecká, Pytlová, Zachařová, Soukup) and in collaborations with other Departments of the Institute (Dr. Rauchová, Department of Experimental Hypertension, lipid evaluation, Dr. Pavelka, Department of Radiometry, TH determination), with Dr. Říčný, National Institute of Mental Health, Klecany, membrane fluidity and Dr. Žurmanová, Laboratory of muscle physiology and bioenergetics, Faculty of Science, Charles University, MyHC and other protein RT-PCR., as well as with Dr. Narcisa Tribulová, Institute for Heart Research, Slovak Academy of Sciences, WB and electrophysiology of the heart. All experiments were supported by following grants: MYORES LSH-CT-2004-511978; Czech Science Foundation GACR 304/08/0256; Ministry of Education and Sports of the Czech Republic 7AMB12SK158 and 7AMB14SK123 PI: Tomáš Soukup; 303/09/0570, 304/12/0259 PI: Hana Rauchová; GACR 304/12/0252, GACR 305/09/1228, GACR P303/10/P227 PI: Jiří Paleček; and Research Project RVO: 67985823, AV0Z 50110509. Some of the papers prepared in collaboration were also supported by grants of participating co-authors: GAAVIAA601110908, MSM0021620858 Jitka Žurmanová, GAUK628412 Petra Arnoštová, VEGA 2/0046/12, 2/0049/09, MZ0PCP2005, APVV SK-CZ-2013-2560027-11, APVV 51-059505, 51-017905 Narcisa Tribulová).*

Research on muscle fiber types transformation

It is known for decades that the differentiation of muscle fiber types depends on intrinsic factors, i.e. genetic factors given by the muscle cell lineage and on extrinsic ones such as innervation, contractile activity, muscle stretch and thyroid hormones (TH) level. Mammalian skeletal muscles consist of heterogeneous population of physiologically and biochemically diverse fibers and myosin heavy chain (MyHC) isoforms, each encoded by a specific MyHC gene, and THs are known to be one of the main determinants of fiber phenotype. It was shown that reduced neuromuscular activity, hyperthyroidism or mechanical unloading stimulate slow to fast fiber type transitions, while increased neuromuscular activity, hypothyroidism and higher mechanical loading result in fast to slow fiber type transitions.

THs are undoubtedly one of the extrinsic factors whose influence on striated and cardiac muscles has been studied intensively and they are powerful regulators of many muscle-specific genes including MyHC isoforms. Actually, TH are important

pathophysiological factor leading to MyHC transitions in skeletal as well as in cardiac muscles (Arnoštová et al. J Biomed Biotechnol ,e634253, 2011; Říčný and Soukup, Physiol Res, 60, 899-904, 2011; Soukup et al., Physiol Res 61, 575-586, 2012; Žurmanová J and Soukup T, Physiol Res, 62, 445-453, 2013).

Although small changes in MyHC (fiber type) composition can have pathophysiological consequences even in adult animals, changes of THs during development may have smashing effect. As regenerating muscle fibers repeat in some way muscle development and adapt more rapidly than surviving adult fibers, we have introduced a model of a so-called heterochronous isotransplantation (Jirmanová I and Soukup T, Anat Embryol 192: 283-291, 1995), whereby slow soleus (SOL) or fast extensor digitorum longus (EDL) muscles from young rats are intramuscularly transplanted into the EDL or SOL host muscle of inbred adult recipients of the same strain with altered TH status. This approach makes it possible to analyze the contribution of genetic factors given by the fast or slow muscle cell lineage, the fast or slow impulse frequency of the host re-innervating axons and of the experimentally altered levels of TH in a single experimental model. It also enables to compare the response of differentiated adult and regenerating grafted muscles to altered TH status (since 1995 we have published 27 papers related to this topic). Besides analyzing changes in skeletal and heart calsequestrin (CSQ1 and 2) expression, we have focused on the fiber type transitions in muscle grafts of rats with altered thyroid status. Recently we have published paper on effect of regeneration, altered innervation and thyroid hormone alteration on fiber type transitions in the slow SOL grafted (GRAFT) into host EDLh muscles of euthyroid (EU), hyperthyroid (HT) and hypothyroid (HY) 2-month-old female Lewis strain rats (Kopecka et al., Histochem Cell Biol 142, 677-684, 2014). We found that after an average regeneration period of 6 to7 months and after re-innervation by the “fast” peroneal nerve of EDLh muscle, originally slow GRAFT was transformed into a fast muscle. However, the extent of GRAFT transformation varied with different thyroid hormone status. In the EU rats, GRAFT contained about 95% of fast fibers, among which type 2X and 2B fibers predominated (about 75%). The transition towards fast muscle phenotype was more pronounced in HT status, where the fastest type 2B fibers predominated. On the contrary, in HY status, the slow to fast transformation was less pronounced, as GRAFT contained less type 2B and 2X but more type 2A and 1 fibers. These results confirm that the type of innervation is the crucial factor for the slow to fast fiber type transitions in GRAFT, but the extent of muscle transformation is further modulated by the altered TH levels.

Regarding changes in CSQ, we found that the protein and mRNA levels for CSQ1 were highest in the EDL muscle, the relative CSQ1 protein levels in the soleus muscle were two times lower and the transcript levels were more than 5 times lower compared to the EDL. In the left heart ventricle, protein isoform and CSQ1 transcript were also present, although at protein level, CSQ1 was hardly detectable. TH status increased and HY status decreased the expression of CSQ1 in the EDL, but its relative levels in the soleus and in the heart did not change. The regenerated soleus transplanted into EDL, as well as EDL transplanted into soleus exhibited protein and mRNA levels of CSQ1 corresponding to the host muscle and not to the graft source. TH status increased the percentages of the fastest 2X/D and 2B fibers at the expense of slow type 1 and fast 2A fibers in the EDL and that of fast 2A fibers in the soleus at the expense of slow type 1 fibers. HY status led to converse fiber type changes. We have suggested that the observed changes in CSQ1 levels in TH and HY compared to EU rats can be related to fiber type changes caused by alteration of the thyroid status rather than to the direct effect of TH on CSQ1 gene expression. These results are in

agreement with idea that the type of innervation is the crucial factor also for expression of calcium handling proteins as shown in GRAFT, but that the extent of CSQ1 expression can be further modulated by the altered TH status (Novak et al., Physiol Res 59: 783-801, 2010; Novák P, Soukup T, Physiol Res 60: 439-452, 2011). Our transplantation research was summarized in the invited review Soukup T, Smerdu V, Histochem Cell Biol 143: 123-130, 2015.

Experiments on CSQ were performed by the laboratory staff (P.Novak, K. Kopecká, G. Zachařová, T. Soukup) in collaboration with V.Sulimenko a V.Marková (Laboratory of Biology of Cytoskeleton, Institute of Molecular Genetics of the ASCR, v.v.i.) who contributed to the WB and PCR. All grafting experiments were performed by the laboratory staff, Dr Smerdu in last 2 papers contributed to single fiber co-expression analysis. Some experiments were performed also in collaboration with Dr.Říčný (ELISA) and with another PhD student P. Arnoštová and Dr. Žurmanová (SDS-PAGE and PCR of MyHC isoforms). The experiments were supported by the following grants MYORES LSH-CT-2004-511978, GACR 304/08/0256 and by The Czech-Slovenian Intergovernmental S&T Cooperation Program grant, Slovenian Research Agency P3-0043, PI:Tomáš Soukup, GACR 305/09/1228, GACR 304/12/0259, GACR P304/12/G069, MSMT LH12058, MSMT LC554 PI: Jiří Paleček, and by Research Project RVO: 67985823, AV0Z 50110509. Papers prepared in collaboration were also supported by grants of the participating co-authors GAAV IAA 601110908, MSM0021620858, IAAX01110901PI:Jitka Žurmanová, MZ0PCP2005, APVV SK-CZ-0027-11 PI: Narcisa Tribulová.

3) **INTERNAL COLLABORATION**

There is an ongoing collaboration with several other departments and scientists at the institute. While most of it is on informal level, we have also team work projects within the workframe of several grants and centers (LC554, Neuroscience Centre, 2005-2011; GBP304/12/G069, Project of excellence in the field of neuroscience, 2012-2018 and EE2.3.30.0025, MŠMT Center of Biomedical Research, 2012-2015), where several other departments take part. Dr. Soukup has also long lasting productive collaboration with Dr. Rauchova, Department of Experimental Hypertension and Dr. Pavelka, Department of Radiometry reflected in 7 joint papers since 2010.

4) **DOMESTIC COLLABORATION**

Our department is part of the new project BIOCEV (Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University) a European Centre of Excellence in biomedicine and biotechnology where new collaborations are established. We have also intensive collaboration with Dr. Žurmanová at the Faculty of Science, Charles University based on the grant of the 6th FP Network of Excellence Multi-organismic Approach to Study Normal and Aberrant Muscle Development, Function and Repair (5 papers since 2010) and with Dr Říčný at the National Institute of Mental Health, Klecany. In cooperation with the Institute of Molecular Genetics (Dr. Beck, Dr. Schuster) we work on a transgenic mice model.

5) **INTERNATIONAL COLLABORATION**

Very important and productive is collaboration with Dr. Tribulová, Institute for Heart Research of the Slovak Academy of Sciences, Bratislava focusing on effect of thyroid hormones and/or n-3 PUFA on heart structure and function (8 papers after 2010). Long-lasting collaboration with Dr. Vika Smerdu, Institute of Anatomy, Medical Faculty, Ljubljana, Slovenia, started by the Czech-Slovenian Intergovernmental S&T

Cooperation Program grant concentrated on muscle transitions after isotransplantation. Last results of this collaboration were published recently (2014, 2015). We have also started collaboration with Prof. Dougherty at The University of Texas MD Anderson Cancer Center, Houston, TX under the Czech-American collaborative project (LH12058, Mechanisms of Neuropathic Pain States) with coauthored results submitted for publication. We have also collaboration with prof. Pohl, INSERM, Paris on the role of neuroinflammation in the spinal cord modulatory mechanisms. Long-lasting interactions with Prof. Lars-EricThornell, University of Umea, Sweden and Prof. Gerhard Asmussen, University of Leipzig, Leipzig, Germany, resulted in many papers in the past and are continuing at present on the topic of human muscle spindles development and function.

6) KEY METHODOLOGY AND CORE FACILITIES

In our research we are using mostly combination of electrophysiological, immunohistochemical, molecular genetic and behavioral methods. The key methodological approach is recording of electrical activity from superficial dorsal horn neurons with patch clamp technique in acute spinal cord slices. This technique provides us with the possibility to study modulation of synaptic transmission at the spinal cord level under different experimental conditions. We have also well equipped immunohistochemical laboratory. All the crucial equipment needed for our work is available within our department. We are also using some of the core institutional facilities such as confocal microscope, RT-PCR, HPLC and others.

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

Our department was part of the 6th FP Network of Excellence Multi-organismic Approach to Study Normal and Aberrant Muscle Development, Function and Repair (MYORES LSH-CT-2004-511978). Under this project we have participated on several experimental topics and we have also organized a meeting of the network in Prague. More recently we are part of a center of excellence for neuroscience that was supported by two grant projects (LC554 2005-2011 and GBP304/12/G069 2012-2018). Dr. Palecek is also a leader of the project supporting several groups/departments at the institute to educate new postdoctoral fellows, supports their international training and provides specialty courses for out of Prague universities students (EE2.3.30.0025, Center of Biomedical Research, 2012-2015). Our group is also part of a European Centre of Excellence in Biomedicine and Biotechnology BIOCEV, an EU funded infrastructure built by the Czech Academy of Sciences and the Charles University. Participation in this project will provide us with a unique opportunity to use the new core facilities at the center and collaborate more closely with other groups.

8) OTHER RELEVANT INFORMATION

During the evaluated period the members of the department have coauthored altogether 19 peer reviewed publications in journals with impact factor.

While the results for the evaluation are based only on the already published results during the period, number of other results were also obtained that were published in 2015 or are currently under review in journals.

Soukup T, Smerdu V. Effect of altered innervation and thyroid hormones on myosin heavy chain expression and fiber type transitions. Histochem Cell Biol. 2015 Feb;143(2):123-30.

Soukup T, Diallo M. Proportions of myosin heavy chain mRNAs, protein isoforms and fiber types in the slow and fast skeletal muscles are maintained after alterations of thyroid status in rats. Physiol Res. 2015 Mar 3;64(1):111-8.

Uchytilova E, Spicarova D, Palecek J. Single high-concentration capsaicin application prevents c-Fos expression in spinothalamic and postsynaptic dorsal column neurons after surgical incision. Eur J Pain. 2015 Feb 25.

Nerandzic V and Palecek J, Modulation of spinal cord synaptic activity by N-acyl phosphatidylethanolamine (NAPE) in a model of peripheral inflammation. Submitted.

Diallo M, Kalynovska N and Palecek J, The effect of angiotensin II receptor 1 antagonist losartan on development of neuropathic pain in rats. Manuscript in preparation.

9) SUMMARY AND RESEARCH IMPACT

Our department is working in two major areas of interest: pain mechanisms and physiology of skeletal and cardiac muscles.

Using electrophysiological, immunohistochemical and behavioral techniques we have demonstrated the significance of presynaptic TRPV1 receptors at the central branches of primary afferents in the spinal cord for the modulation of nociceptive synaptic transmission and pain. Our results showed that under pathological conditions, these TRPV1 receptors become more sensitive to endogenous agonists and may enhance nociceptive signalling. Experiments in models of neuropathic pain proved the involvement of spinal TRPV1 receptors in the neuroinflammatory mediators induced changes in pain modulation. Our results strongly suggest that TRPV1 receptors in the spinal cord dorsal horn serve as molecular integrators of numerous different modulatory inputs/molecules and have significant impact on pain transmission and modulation. Our results may lead to development of new analgesic drugs.

In the area of muscle physiology we have demonstrated a positive effect of omega-3 polyunsaturated fatty acids (n-3 PUFA) on cardiac muscle function under different experimental conditions including hypertension and altered thyroid status. These results support the existing evidence that n-3 PUFA treatment may help ameliorate some of the pathological changes. Our extensive results studying the role of thyroid hormone levels in muscle fiber types transformation brought new evidence in this area and further confirmed the importance of innervation in muscle fiber type differentiation and determination.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Genetics of Model Diseases

Introduction

Metabolic syndrome is a cluster of several risk factors for type 2 diabetes and cardiovascular disease, including obesity, hypertension, insulin resistance, and dyslipidemia. These pathological conditions are determined multifactorially by many genes and their interactions with environmental effects. Genome wide association studies (GWAS) in humans identified only a minor proportion of the total heritability of complex traits so far. In addition, associations of practically all significant variants with complex traits are based only on statistical evidence and most likely do not represent causal alleles, especially when they are often located within noncoding regions. Studies in animal models of human complex diseases can provide a useful alternative and provide functional tests for variants identified in GWAS.

The spontaneously hypertensive rat (SHR) is the most widely used animal model of human essential hypertension and left ventricular hypertrophy and under special environmental conditions also develops disturbances of lipid and glucose metabolism that are typical for metabolic syndrome. Similar to humans, these hemodynamic and metabolic disturbances in the SHR are also determined multifactorially. To identify genetic determinants of such complex traits, we used a combination of linkage and correlation analyses with intermediary phenotypes in the BXH/HXB recombinant inbred (RI) strains and follow-up *in vivo* functional testing in SHR congenic and SHR transgenic or knockout lines.

The BXH/HXB recombinant inbred (RI) strains were derived from reciprocal crosses between BN-*Lx* and SHR progenitors by Pravenec and Křen. For genetic dissection of complex pathophysiological traits in RI strains, it is possible to take the advantage of accumulated genotypes and intermediary phenotypes. Intermediary phenotypes have simpler genetic architectures and can be used for connecting variabilty at the DNA level with complex pathophysiological traits. For instance, abundance of mRNA in tissues is a highly heritable trait and represents very useful intermediary phenotype since it is possible to identify *cis*- and *trans*-regulated expression quantitative trait loci (eQTL) as candidate genes for complex traits. The avalability of genome sequences of both progenitor strains, the SHR and BN-*Lx*, enables rapid screening for functional variants of such candidate genes (Attanur, …Pravenec et al. *Genome Res* **20**: 791, 2010; Simonis, …Pravenec et al. *Genome Biol* **13**: r31, 2012). We used this approach to identify QTL at the molecular level in the SHR, including mutant *Endog* (endonuclease G) gene (McDermott-Roe, …Pravenec et al. *Nature* **478**: 114, 2011) that predispose to left ventricular hypertrophy, *Ebi2* (Epstein-Barr virus induced gene 2) gene that is associated with an inflammatory gene network and its human ortholog with predisposition to Type 1 diabetes (Heinig, …Pravenec et al. *Nature* **467**: 460, 2010). Using similar approach, additional candidate genes have been identified, including genes associated with cardiac microvascular remodeling (Mancini, …Pravenec et al. *Basic Res Cardiol* **108**: 316, 2013) or catecholamine sythesis and

secretion (Jirout, …Pravenec et al. *Hum Mol Genet* **19**:2567, 2010; Friese, …Pravenec et al. *Hum Mol Genet* **22**: 3624, 2013). In addition to *cis*-regulated eQTL, systems-level approaches combining radiotelemetry blood pressures and transcriptome data revealed conservation of *trans*-regulated genes in the rat and genetic determinants of blood pressure in humans (Langley, …Pravenec et al. *Cardiovasc Res* **97**: 653, 2013). Epigenetic marks such as histone modification or cytosine methylation are important determinants of cellular and whole-body phenotypes. However, the extent of, and reasons for inter-individual differences in these epigenetic marks and their association with phenotypic variation are poorly characterised.

We annotated the rat genome with histone modification maps, identified differences in histone trimethyl-lysine levels among strains, and described their underlying genetic basis at the genome-wide scale using ChIP-seq in heart and liver tissues in a panel of rat BXH/HXB recombinant inbred and their progenitor strains. We identified extensive variation of histone methylation levels among individuals and mapped hundreds of underlying *cis*- and *trans*-acting loci throughout the genome that regulate histone methylation levels in an allele-specific manner. Our data suggest that genetic variation has a widespread impact on histone trimethylation marks that may help to uncover novel genotype-phenotype relationships (Rintisch, …Pravenec et al. *Genome Res* **24**: 942, 2014).

We used whole-genome bisulfite sequencing in the SHR and BN progenitor strains to define the genetic architecture of cytosine methylation in the mammalian heart and to test for association between methylation and pathophysiological phenotypes. In the BXH/HXB RI strain panel, we found significant correlation of CpG methylation and levels of serum chromogranin B (CgB), a proposed biomarker of heart failure, which is evidence for a link between germline DNA sequence variation, CpG methylation differences and pathophysiological phenotypes in the SHR strain. Together, these results provide a starting point for understanding the relationship between the genetic control of CpG methylation and disease phenotypes (Johnson, …Pravenec et al. *PLoS Genet* **10**: e1004813, 2014).

The above mentioned results were obtained in an international collaborative effort within the European Community's Seventh Framework Programme under grant agreement # HEALTH-F4-2010-241504 (EURATRANS). Some of these results and additional results obtained mainly in domestic collaborations are described in detail below.

Genetic regulation of catecholamine synthesis, storage and secretion in the spontaneously hypertensive rat

Jirout ML, Friese RS, Mahapatra NR, Mahata M, Taupenot L, Mahata SK, Křen V, Zídek V, Fischer J, Maatz H, Ziegler MG, Pravenec M, Hubner N, Aitman TJ, Schork NJ, O'Connor DT. Genetic regulation of catecholamine synthesis, storage and secretion in the spontaneously hypertensive rat. Hum Mol Genet 19: 2567-2580, 2010; Friese RS, Altshuler AE, Zhang K, Miramontes-Gonzalez JP, Hightower CM, Jirout ML, Salem RM, Gayen JR, Mahapatra NR, Biswas N, Cale M, Vaingankar SM, Kim HS, Courel M, Taupenot L, Ziegler MG, Schork NJ, Pravenec M, Mahata SK, Schmid-Schönbein GW, O'Connor DT. MicroRNA-22 and promoter motif polymorphisms at the Chga locus in genetic hypertension: functional and therapeutic implications for gene expression and the pathogenesis of hypertension. *Hum Mol Genet* **22**: 3624-3640, 2013; Michal Pravenec is the corresponding author)

Understanding catecholamine metabolism is crucial for elucidating the pathogenesis of hereditary hypertension. Here we integrated transcriptional and biochemical profiling

with physiologic quantitative trait locus (eQTL and pQTL) mapping in adrenal glands of the BXH/HXB recombinant inbred (RI) strains, derived from the spontaneously hypertensive rat (SHR) and normotensive Brown Norway (BN-Lx). We found simultaneous down-regulation of five heritable transcripts in the catecholaminergic pathway in young (6 weeks) SHRs. We identified *cis*-acting eQTLs for *Dbh*, *Pnmt* (catecholamine biosynthesis) and *Vamp1* (catecholamine secretion); enzymatic activities of *Dbh* and *Pnmt* paralleled transcripts, with pQTLs for activities mirroring eQTLs. We also detected *trans*-regulated expression of *Vmat1* and *Chga* (both involved in catecholamine storage), with co-localisation of these *trans*-eQTLs to the *Pnmt* locus. *Pnmt* re-sequencing revealed promoter polymorphisms that result in decreased response of the transfected SHR promoter to glucocorticoid, compared with BN-Lx. Of physiological pertinence, *Dbh* activity negatively correlated with systolic blood pressure in RI strains, whereas *Pnmt* activity was negatively correlated with heart rate. The finding of such *cis*- and *trans*-QTLs at an age before the onset of frank hypertension suggests that these heritable changes in biosynthetic enzyme expression represent primary genetic mechanisms for regulation of catecholamine action and blood pressure control in this widely studied model of hypertension.

This research was supported by Grant Agency of the Czech Academy of Sciences (grant # IAA500110604, PI: Michal Pravenec) with major involvement of Michal Pravenec and Václav Zídek who contributed to study design, data analysis, manuscript preparation and provided tissues and physiological data from RI strains. Michal Pravenec was an International Research Scholar of the Howard Hughes Medical Institute (grant # 55005624). This work was done in collaboration with Department of Medicine, University of California, San Diego, La Jolla, that contributed to biochemical and statistical analyses.

Effects of human C-reactive protein on pathogenesis of features of the metabolic syndrome

(Pravenec M, Kajiya T, Zídek V, Landa V, Mlejnek P, Šimáková M, Šilhavý J, Malínská H, Oliyarnyk O, Kazdová L, Fan J, Wang J, Kurtz TW. Effects of human C-reactive protein on pathogenesis of features of the metabolic syndrome. Hypertension 57: 731-737, 2011)

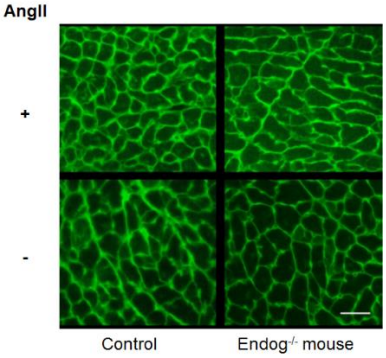
(This is the Top Paper published in *Hypertension* in 2011 in Basic Science Category)

Major controversy exists as to whether increased C-reactive protein (CRP) contributes to individual components of the metabolic syndrome or is just a secondary response to inflammatory disease processes. We measured blood pressure and metabolic phenotypes in spontaneously hypertensive rats (SHRs) in which we transgenically expressed human CRP in the liver under control of the apolipoprotein E promoter. In transgenic SHR-CRP rats, serum levels of human CRP approximated the endogenous levels of CRP normally found in the rat. Systolic and diastolic blood pressures measured by telemetry were 10 to 15 mm Hg greater in transgenic SHR-CRP expressing human CRP than in SHR controls ($P<0.01$). During oral glucose tolerance testing, transgenic SHR-CRP exhibited significant hyperinsulinemia compared with controls. Transgenic SHR-CRP also exhibited significant resistance to insulin stimulated glycogenesis in skeletal muscle, hypertriglyceridemia, reduced serum adiponectin, and microalbuminuria. Transgenic SHR-CRP had evidence of inflammation and oxidative tissue damage with significantly increased serum levels of interleukin 6 and increased hepatic and renal thiobarbituric acid reactive substances, suggesting that oxidative stress may be mediating adverse effects of increased human CRP. These findings are consistent with the hypothesis that increased CRP is more

than just a marker of inflammation and can directly promote multiple features of the metabolic syndrome.
This research was supported by Czech Science Foundation (grant # 301/10/0290, PI: Michal Pravenec) with major involvement of all workers from Department of Model Diseases who contributed to study design, derivation of transgenic rats, data analysis, and manuscript preparation. Michal Pravenec was an International Research Scholar of the Howard Hughes Medical Institute (grant # 55005624). This research was done in collaboration with the Institute for Clinical and Experimental Medicine that contributed to biochemical analyses and with University of California, San Francisco that contributed to study design and manuscript editing.
We also demonstrated that the SHR-CRP transgenic strain represents a useful model for testing anti-inflammatory effects of drugs such as statins (Šilhavý et al. *Cardiovasc Ther* **32**: 59, 2014) or fumaric acid esters (Šilhavý et al. *PLoS One* **9**: e101906, 2014)

Endonuclease G is a novel determinant of cardiac hypertrophy
(McDermott-Roe C, Ye J, Ahmed R, Sun XM, Serafín A, Ware J, Bottolo L, Muckett P, Cañas X, Zhang J, Rowe GC, Buchan R, Lu H, Braithwaite A, Mancini M, Hauton D, Martí R, García-Arumí E, Hubner N, Jacob H, Serikawa T, Zídek V, Papousek F, Kolar F, Cardona M, Ruiz-Meana M, García-Dorado D, Comella JX, Felkin LE, Barton PJ, Arany Z, Pravenec M, Petretto E, Sanchis D, Cook SA. Endonuclease G is a novel determinant of cardiac hypertrophy and mitochondrial function. *Nature* **478**: 114-118, 2011)
Left ventricular mass (LVM) is a highly heritable trait and an independent risk factor for all-cause mortality. So far, genome-wide association studies (GWAS) have not identified the genetic factors that underlie LVM variation, and the regulatory mechanisms for blood-pressure-independent cardiac hypertrophy remain poorly understood. Unbiased systems genetics approaches in the rat now provide a powerful complementary tool to GWAS, and we applied integrative genomics to dissect a highly replicated, blood-pressure-independent LVM locus on rat chromosome 3p. Here we identified endonuclease G (*Endog*), which previously was implicated in apoptosis but not hypertrophy, as the gene at the locus, and we found a loss-of-function mutation in *Endog* that is associated with increased LVM and impaired cardiac function. Inhibition of *Endog* in cultured cardiomyocytes resulted in an increase in cell size and hypertrophic biomarkers in the absence of pro-hypertrophic stimulation. Genome-wide network analysis unexpectedly implicated ENDOG in fundamental mitochondrial processes that are unrelated to apoptosis. We showed direct regulation of ENDOG by ERR-α and PGC1α (which are master regulators of mitochondrial and cardiac function), interaction of ENDOG with the mitochondrial genome and ENDOG-mediated regulation of mitochondrial mass. At baseline, the *Endog*-deleted mouse heart had depleted mitochondria, mitochondrial dysfunction and elevated levels of reactive oxygen species, which were associated with enlarged and steatotic cardiomyocytes. Our study has further established the link between mitochondrial dysfunction, reactive oxygen species and heart disease and has uncovered a role for *Endog* in maladaptive cardiac hypertrophy.

Figure 1 Endog$^{-/-}$ mice have increased cardiomyocyte cross-sectional area at baseline and following angiotensin II (AngII) stimulation. Representative fluorescence photomicrographs of left ventricular sections from Endog$^{-/-}$ and wildtype (WT) mice at baseline (-) and following AngII-induced hypertrophic stimulation (+).



This research was supported by Czech Science Foundation (grant # 301/08/0166, PI: Michal Pravenec), Fondation Leducq (grant # 06 CVD 03, Co-Investigator: Michal Pravenec), and the European Community's Seventh Framework Programme under grant agreement # HEALTH-F4-2010-241504 (EURATRANS) (Co-Investigator: Michal Pravenec) with major contribution by Michal Pravenec and Václav Zídek who provided tissues from RI strains and derived SHR.BN-*Endog* congenic strain and analyzed the data. This research was done in a large international collaboration.

Effects of mtDNA in SHR conplastic strains on reduced OXPHOS enzyme levels, insulin resistance, cardiac hypertrophy, and systolic dysfunction (Houštek J, Hejzlarová K, Vrbacký M, Drahota Z, Landa V, Zídek V, Mlejnek P, Šimáková M, Šilhavy J, Mikšík I, Kazdová L, Oliyarnyk O, Kurtz T, Pravenec M. Nonsynonymous variants in mt-Nd2, mt-Nd4, and mt-Nd5 are linked to effects on oxidative phosphorylation and insulin sensitivity in rat conplastic strains. *Physiol Genomics* **44**: 487-494, 2012; Houštěk J, Vrbacký M, Hejzlarová K, Zídek V, Landa V, Šilhavý J, Šimáková M, Mlejnek P, Kazdová L, Mikšík I, Neckář J, Papoušek F, Kolář F, Kurtz TW, Pravenec M. Effects of mtDNA in SHR-mtF344 versus SHR conplastic strains on reduced OXPHOS enzyme levels, insulin resistance, cardiac hypertrophy, and systolic dysfunction. *Physiol Genomics* **46**: 671, 2014) (Michal Pravenec is a corresponding author of both papers)

Common inbred strains of the laboratory rat can be divided into four major mitochondrial DNA (mtDNA) haplotype groups represented by the BN, F344, LEW, and SHR strains. We investigated the metabolic and hemodynamic effects of the SHR vs. LEW mitochondrial genomes by comparing the SHR to a new SHR conplastic strain, SHR-mt^{LEW}; these strains are genetically identical except for their mitochondrial genomes. Complete mitochondrial DNA (mtDNA) sequence analysis comparing the SHR and LEW strains revealed gene variants encoding amino acid substitutions limited to a single mitochondrial enzyme complex, NADH dehydrogenase (complex I), affecting subunits 2, 4, and 5. Two of the variants in the *mt-Nd4* subunit gene are located close to variants known to be associated with exercise intolerance and diabetes mellitus in humans. No variants were found in tRNA or rRNA genes. These variants in *mt-Nd2*, *mt-Nd4*, and *mt-Nd5* in the SHR-mt^{LEW} conplastic strain were linked to reductions in oxidative and nonoxidative glucose metabolism in skeletal muscle. In addition, SHR-mt^{LEW} conplastic rats showed increased serum nonesterified fatty acid levels and resistance to insulin stimulated incorporation of glucose into adipose tissue lipids. These results provide evidence that inherited variation in mitochondrial genes

encoding respiratory chain complex I subunits, in the absence of variation in the nuclear genome and other confounding factors, can influence glucose and lipid metabolism when expressed on the nuclear genetic background of the SHR strain.
We also investigated the metabolic and hemodynamic effects of the SHR vs. F344 mtDNA by comparing the SHR vs. SHR-mt^{F344} conplastic strains that are genetically identical except for their mitochondrial genomes. Altogether 13 amino acid substitutions in protein coding genes, seven single nucleotide polymorphisms in tRNA genes, and 12 single nucleotide changes in rRNA genes were detected in F344 mtDNA compared with SHR mtDNA. Analysis of oxidative phosphorylation system (OXPHOS) in heart left ventricles (LV), muscle, and liver revealed reduced activity and content of several respiratory chain complexes in SHR-mt^{F344} conplastic rats compared with the SHR strain. Lower function of OXPHOS in LV of conplastic rats was associated with significantly increased relative ventricular mass and reduced fractional shortening that was independent of blood pressure. In addition, conplastic rats exhibited reduced sensitivity of skeletal muscles to insulin action and impaired glucose tolerance. These results provide evidence that inherited alterations in mitochondrial genome, in the absence of variation in the nuclear genome and other confounding factors, predispose to insulin resistance, cardiac hypertrophy and systolic dysfunction.
This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grant # LL1204 within the ERC CZ program, PI: Michal Pravenec) and the European Community's Seventh Framework Programme under grant agreement # HEALTH-F4-2010-241504 (EURATRANS) (Co-Investigator: Michal Pravenec) with major involvement of research workers from department Genetic of Model Diseases in study design, derivation of conplastic strain, data analysis and manuscript preparation. This research was done in collaboration with departments of Bioenergetics and Developmental Cardiology, Institute of Physiology, Czech Academy of Sciences. This work was done in collaboration with the Institute for Clinical and Experimental Medicine that contributed by measuring parameters of lipid and glucose metabolism.

Plzf as a candidate gene predisposing the spontaneously hypertensive rat to hypertension, left ventricular hypertrophy, and interstitial fibrosis.

(Liška F, Mancini M, Krupková M, Chylíková B, Křenová D, Šeda O, Šilhavý J, Mlejnek P, Landa V, Zídek V, d' Amati G, Pravenec M, Křen V. Plzf as a candidate gene predisposing the spontaneously hypertensive rat to hypertension, left ventricular hypertrophy, and interstitial fibrosis. *Am J Hypertens* **27**: 99-106, 2014)
Recently, a quantitative trait locus (QTL) that influences heart interstitial fibrosis was mapped to chromosome 8. Our aim was to dissect the genetic basis of this QTL(s) predisposing SHR to hypertension, LVH, and interstitial fibrosis. Hemodynamic and histomorphometric analyses were performed in genetically defined SHR.PD-chr.8 minimal congenic strain (PD5 subline) rats. The differential segment, genetically isolated within the PD5 subline, spans 788kb and contains 7 genes, including the promyelocytic leukemia zinc finger (Plzf) gene that has been implicated in hypertrophy and cardiac fibrosis. Mutant Plzf allele contains a 2,964-bp deletion in intron 2. The PD5 congenic strain, when compared with the SHR, showed significantly reduced systolic blood pressure, amelioration of LVH, and reduced interstitial fibrosis. Cardiac expression of Plzf was significantly reduced in prehypertensive (8 and 21 days) congenic animals compared with controls. These results provide compelling evidence of a significant role for genetic factors in regulating blood pressure, LVH, and cardiac fibrosis and identify mutant Plzf as a prominent candidate gene.

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grant # LL1204 within the ERC CZ program, PI: Michal Pravenec), Czech Science Foundation (grant # P301/12/0696, PI: Michal Pravenec) and the European Community's Seventh Framework Programme under grant agreement # HEALTH-F4-2010-241504 (EURATRANS) (Co-Investigator: Michal Pravenec) with major involvement of research workers from department Genetic of Model Diseases in study design, measurement of hemodynamic parameters, data analysis and manuscript preparation. This research was done in collaboration with the Institute of Biology and medical Genetics, Charles University, Prague and Sapienza, University of Rome, Italy.

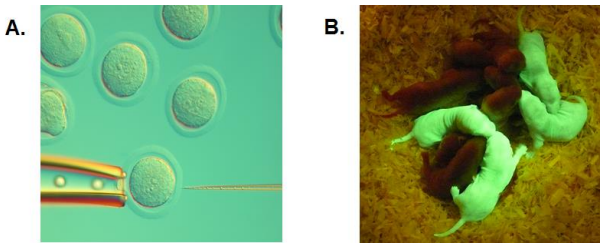
Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons.

(Ivics Z, Mátés L, Yau TY, Landa V, Zídek V, Bashir S, Hoffmann OI, Hiripi L, Garrels W, Kues WA, Bösze Z, Geurts A, Pravenec M, Rülicke T, Izsvák Z. Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons. *Nat Protocol* **9**: 773, 2014; Michal Pravenec is a corresponding author)

We describe a protocol for high-efficiency germline transgenesis and sustained transgene expression in two important biomedical models, the mouse and the rat, by using the Sleeping Beauty transposon system. The procedure is based on co-injection of synthetic mRNA encoding the SB100X hyperactive transposase, together with circular plasmid DNA carrying a transgene construct flanked by binding sites for the transposase, into the pronuclei of fertilized oocytes. Upon translation of the transposase mRNA, enzyme-mediated excision of the transgene cassettes from the injected plasmids followed by permanent genomic insertion produces stable transgenic animals. Generation of a germline-transgenic founder animal by using this protocol takes ~3 months. Transposon-mediated transgenesis compares favorably in terms of both efficiency and reliable transgene expression with classic pronuclear microinjection, and it offers comparable efficacies to lentiviral approaches without limitations on vector design, issues of transgene silencing, and the toxicity and biosafety concerns of working with viral vectors.

This work was supported by Ministry of Education, Youth and Sports of the Czech Republic (grants # LH12061 and # LL1204 (within the ERC CZ program) and by Technological Agency of the Czech Republic (grant # TA02010013, Co-Investigator: Michal Pravenec) with major contribution of Vladimír Landa, Václav Zídek and Michal Pravenec in development of Sleeping Beauty transgenesis in the rat.

Figure 2 Derivation of transgenic rats with a SB transposon vector encoding the Venus fluorescent protein. (A) Pronuclear microinjection of an ovum with the help of holding (left) and injection (right) capillaries; ova already injected are shown in the upper part. (B) Two-day-old transgenic rats and their nontransgenic siblings.



INTERNAL COLLABORATION (within the Institute)

We have a productive collaboration with Department of Bioenergetics when we collaborate on grant # LL1204 (within the ERC CZ program) from the Ministry of Education, Youth and Sports of the Czech Republic (PI: Michal Pravenec) and together with Department of the Biology of adipose tissue, we also collaborate on grant 14-36804G (MITOCENTER) from Czech Science Foundation. We also collaborate with Department of Developmental Cardiology (grant # 13-10267S from Czech Science Foundation, PI: Jan Neckář), with Department of Neurophysiology of Memory, and with department of Experimental Hypertension. All these collaborations resulted in coauthorship of many papers.

DOMESTIC COLLABORATION (within the country)

We have a long-term productive collaboration with Ludmila Kazdová from the Institute for Clinical and Experimental Medicine, with Vladimír Křen and František Liška from the Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, with Viktor Kožich and Stanislav Kmoch from Institute of Inherited Metabolic Diseases, 1st Medical Faculty, Charles University.

INTERNATIONAL COLLABORATION – zahraniční spolupráce

We have a long-term, very productive collaboration with the following scientists:
Theodore W. Kurtz (University of California, San Francisco) – 74 joint papers
Timothy J. Aitman (Imperial College, London) – 23 joint papers
Norbert Hübner (MDC Molecular medicine, Berlin) – 18 joint papers
Stuart A. Cook (Duke-NUS, Singapore) – 12 joint papers
Enrico Petretto (Duke-NUS, Singapore) – 14 joint papers
Edwin Cuppen (Hubrecht Institute KNAW, Utrecht) – 6 papers
Zsuzsanna Izsvák (MDC Molecular medicine, Berlin) – 5 joint papers
Dominique Gauguier (The Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford – 4 joint papers

KEY METHODOLOGY AND CORE FACILITIES

Our department can use modern animal facility for breeding of RI strains, congenic and conplastic lines, transgenic and knockout strains of rats.
We have equipment for derivation of transgenic rats using the Sleeping Beauty constructs and mRNA of SB100X transposase as well as production of knockout rats using ZFN from Sigma or TALEN techniques.
We have equipment for radiotelemetry measurements of blood pressure and heart rates and for molecular biological studies.

INVOLVEMENT IN SIGNIFICANT PROJECTS

Michal Pravenec was an international research scholar of the Howard Hughes Medical Institute in 2006-2011 (grant # 55005624).
Michal Pravenec was a co-investigator of grant # 06 CVD 03 of Fondation Leducq in 2007-2012

Michal Pravenec was a co-investigator of the European Community's Sixth Framework Programme under grant agreement # LSHG-CT-2005-019015 (EURATools) in 2006-2010
Michal Pravenec was a co-investigator of the European Community's Seventh Framework Programme under grant agreement # HEALTH-F4-2010-241504 (EURATRANS) in 2011-2015
Michal Pravenec is PI of the grant #LL1204 (within the ERC CZ program) from the Ministry of Education, Youth and Sports of the Czech Republic in 7/2012-6/2017 (the ERC CZ program is for ERC proposals submited to EU that passed the evaluation thresholds, thereby confirming it as a fundable proposal, however, under the limitations of the overall budget available for the call, the proposal was not retained for funding).
Michal Pravenec was the work package leader of Center for Applied Genetics grant # 1M6837805002 from the Ministry of Education, Youth and Sports of the Czech Republic
Michal Pravenec is the work package leader of Mitocenter grant # 14-36804G from the Czech Science Foundation.

SUMMARY AND RESEARCH IMPACT

During 2010-2014, Department Genetics of model diseases contributed significantly to genetic analysis of metabolic syndrome in the spontaneously hypertensive rat (SHR). Specifically, several QTLs were identified at the molecular level as quantitative trait genes (QTG) including mutant *Endog* gene predisposing to left ventricular hypertophy, *Ebi2* gene associated with inflammatory gene network or *Plzf* gene predisposing to left ventricular hypertrophy, cardiac fibrosis and metabolic disturbances. Altogether, 40 papers were published during the evaluation period, including 2 in *Nature*, 3 in *Nature Protocols*, 2 in *Genome Research* and other prestigious journals.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Cardiovascular Morphogenesis

RESEARCH FOCUS

The main focus of the department is on cardiovascular development. In particular, we study the morphology and physiology of the cardiac conduction system. We rely two complementary approaches, which have proved successful in the past. The first one is the morphological examination of various developmental stages, allowing us to trace the structures of interest in time and to interpret the experimentally-induced changes in developmental context. The range of techniques employed is from macroscopy to electron microscopy. The second approach relies upon optical techniques to study functional aspects of the heart. Here we use video recordings, ultrasound biomicroscopy, and optical mapping of voltage and calcium transients. Together, we strive to derive new knowledge about pathogenesis of cardiovascular diseases and uncover new potential therapeutic interventions using the developmental approach to disease.

KEY RESULTS

The work (and publication activity) of the lab in the past 5 years could be divided into five **main thematic circuits:** 1) mechanisms of conduction system development, 2) environmental effects on myocardial morphogenesis, 3) reviews and book chapters, 4) collaborations with the Institute of Clinical and Experimental Medicine on adult disease models,, and 5) collaboration with the Veterinary and Pharmaceutical University in Kosice on toxicity of agrochemicals using the embryonic chick model.

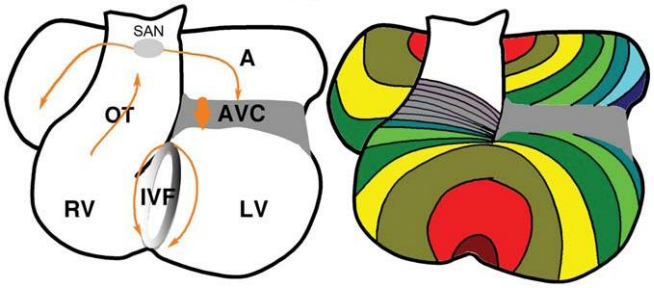


Figure 1*. Scheme of conduction through the embryonic mouse heart.*

1. The **first series** revolves around a 5-year project of the Grant Agency of the Czech Republic (304/09/0615, Molecular mechanisms of conduction system formation). The first and most significant paper in this series was the characterization of functional and morphological development of cardiac conduction system in the mouse (Sankova et al., *Cardiovasc Res* **95**: 469, 2012; ***Figure 1***). Soon afterward followed another paper on mechanisms governing induction of the pacemeker of the heart through the Pitx2 gene dosage (Ammirabile et al., *Cardiovasc Res* **93**: 291, 2012). The third publication in this series was the study on dysmorphogenesis of the ventricular conduction system due to overexpression of mutated ion channel SCN5, mimicking the clinical scenario of the long QT syndrome (de la Rosa et al., *Cardiovasc Res* **98**: 504, 2013). The next recent paper in this series was the work on the mysterious *Lethal8* mutant mouse, a product of the ENU mutagenesis screen, which displayed abnormalities in atrial conduction due to a mutation in the ErbB2 gene (Tenin et al., *PLoS One* **9**: e107041, 2014). The last one so far was the final work of Dr. Benes, who graduated from the PhD programme in 2014, on the effects of connexin40 dosage in conduction through the embryonic atria (Benes et al., *FEBS Lett* **588**: 1465, 2014). For all these papers, our laboratory contributed both experimentally (optical mapping, microscopy) as well as by manuscript writing and revising. In addition, we accommodated the visiting scientists from the collaborating labs (Dr. Ammirabile from the University of Padova, Dr. de la Rosa from the University of Jaen, and Dr. Tennin from the University of Manchester), taught them the principles of optical mapping, and guided them in data analysis and interpretation.

2. The second main topic of the laboratory is the morphogenesis of the myocardium. In the past few years we focused on the effects of the epigenetic factors on this process, and our efforts are supported by the grant P302/11/1308 from the Grant Agency of the Czech Republic. The first papers were devoted to the effects of altered hemodynamics on morphogenesis of cardiac valves and cardiac conduction system, respectively (Sedmera, *Progress in Pediatric Cardiology* **29**: 11, 2010, Sankova et al., *Am J Physiol Heart Circ Physiol* **298**: H1571, 2010). Molecular impact of altered hemodynamic loading was put into developmental context using microarray analysis (Krejci et al., *Physiol Res* **61 Suppl 1**: S137, 2012). One paper showing the importance of realistic morphological data for mathematical modelling of strains in the embryonic

heart was published in collaboration with Bucknell University (Buffinton et al., *Biomech Model Mechanobiol* **12**: 1037, 2013).

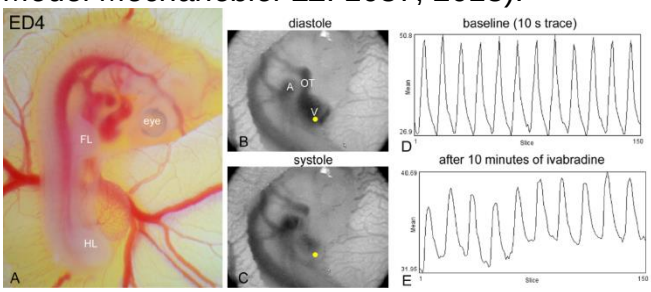


***Figure 2**. In ovo video monitoring of cardiac function in the embryonic chick model.*

Series of two papers on the effects of prenatal administration of beta blockers on the developing heart were produced by our graduate student, Radka Kockova, MD (Kockova et al., *Am J Physiol Heart Circ Physiol* **304**: H895, 2013, Hejnova et al., *Biomed Res Int* **2014**: 463123, 2014; **Figure 2**). These results showed that any drug causing bradycardia in the developing heart leads also to decreased cardiac output and carries an embryolethal potential. Finally, our collaboration with the group of Dr. Pavlinkova from the Institute of Biotechnology on the mammalian model of gestation diabetes as a factor influencing cardiovascular morphogenesis resulted in two papers in respectable journals (Bohuslavova et al., *J Mol Cell Cardiol* **60C**: 129, 2013, Bohuslavova et al., *BMC Endocr Disord* **14**: 11, 2014). This series is characterized also by major research contibution by our department members, providing both the experimental data (microscopy, functional studies, embryonic samples for biochemical analysis) as well as their interpretation and presentation.

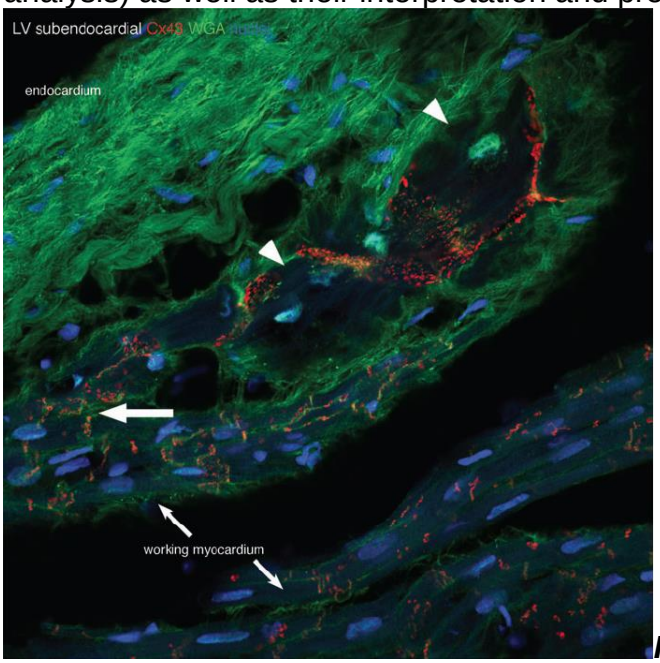


***Figure 3**. Purkinje fibers in the sheep are distinguished by high density of Connxin43 expression (red).*

3. Publication activities of the department were not limited only to primary papers, but several well-cited **reviews** resulted from the data mentioned above as well. Topics reviewed included the role of myocyte proliferation in cardiac growth, remodeling and regeneration (Sedmera and Thompson, *Dev Dyn* **240**: 1322, 2011), relationship between form and function in the cardiovascular system (Sedmera, *Cardiovasc Res* **91**: 252, 2011), and edited book on Ontogeny and Phylogeny of the Vertebrate Heart (Sedmera and Wang, 2012) including the chapter on myocardial morphogenesis (Sedmera et al., in *Ontogeny and Phylogeny of the Vertebrate Heart* 147, 2012), and

a book chapter on prenatal cardiac adaptations in a book edited by Prof. Ostadal (Pesevski et al., in *Cardiac Adaptations* 41, 2013). In collaboration with the Department of Developmental Cardiology (Prof. Ostadal), we published a review on developmental determinants of cardiac sensitivity to low oxygen tension (Ostadal et al., *Can J Physiol Pharmacol* **92**: 566, 2014). Together with Prof. Robert Gourdie (Virginia Tech Carilion Research Institute) we published a review on morphology of Purkinje fibers in different species (**Figure 3**, Sedmera and Gourdie, *Physiol Res* **63 Suppl 1**: S9, 2014). We also collated our experience with optical approaches to study cardiac function (Vostarek et al., *Prog Biophys Mol Biol* **115**: 261, 2014). On all these reviews, our department members are either lead or corresponding authors.

4. There was a productive **collaboration with** several departments of the Institute of Clinical and Experimental Medicine (**IKEM**). Together with the adult experimental cardiologists, we helped with characterization of the obturator device in a sheep model (Sochman et al., *Physiol Res* **63**: 157, 2014). Together with Dr. Melenovsky we provided morphological characterization of the volume overload heart failure model in the rat (Petrak et al., *Proteome Sci* **9**: 69, 2011, Melenovsky et al., *Mol Cell Biochem* **354**: 83, 2011, Benes et al., *Anat Rec (Hoboken)* **294**: 102, 2011, Benes et al., *Clin Sci (Lond)* **121**: 29, 2011). This data formed also a part of the PhD thesis work of Jiri Benes Jr, MD (defended successfully in 2014). For these studies, we provided morphological evaluation of the normal and experimental hearts or tissues, and actively participated in manuscript preparation (Drs. Sedmera and Benes). Finally, one of the graduate students in the lab, Dr. Kockova, comes primarily from this institution, and our laboratory was instrumental for the experimental part of her PhD thesis, scheduled for defence in April 2015.

5. Last part of our **collaboration** was with the Veterinary and Pharmaceutical school in Kosice, **Slovakia**. Dr. Eva Petrovova did most of her thesis work in our lab, graduating in 2009, and maintains since this time close ties with our lab. These efforts resulted in joint publications using the chick embryonic model for the purposes of monitoring potentially toxic substances in agriculture (Petrovova et al., *J Environ Sci Health A Tox Hazard Subst Environ Eng* **47**: 1312, 2012, Petrovova et al., *Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment* 469, 2011).

INTERNAL COLLABORATION (within the Institute)

Our department has an extensive collaboration with the Department of Biomathematics (Dr. Janacek, Dr. Capek) on the topics of imaging, ranging from confocal to microCT. Due to the closeness of the investigated themes as well as spatial proximity, we maintain a productive collaboration with the Department of Developmental Cardiology (Profs. Ostadal, Kolar, Dr. Neckar). We share our expertise in morphology, providing precise histological and ultrastructural characterization of their models, while we draw on their experience with physiological setups and share some critical instrumentation (laminar flow hood, fluorescence microscope, ECG recorders). For the purposes of analysis of our samples for collagen contents, we coordinate with the Department of Analysis of Biologically Important Compounds (Dr. Eckhardt) who provides quantitative MS analysis of heart extracts for collagens. Lipidomics profiling of the embryonic chick heart is performed in collaboration with Dr. Kuda from the Department of Adipose Tissue Biology. The most recent collaborative project was started with the Department of Bioenergetics (Dr. Vrbacky, Dr. Houstek) for the purposes of phenotypic analysis of the TMEM70 null mice.

DOMESTIC COLLABORATION (within the country)

Within the Czech Republic, our main collaborating partner is the Institute of Anatomy at the Charles University in Prague, First Faculty of Medicine. With its Laboratory of Molecular Embryology we have close personal ties, and most of our publications stem from this joint effort. We share the expertise as well as the equipment, drawing on strengths of both institutions.

We have also ongoing collaboration (2 recent papers) with the group of Dr. Pavlinkova at the Institue of Biotechnology of the Czech Academy of Sciences (Bohuslavova et al., *J Mol Cell Cardiol* **60C**: 129, 2013, Bohuslavova et al., *BMC Endocr Disord* **14**: 11, 2014).

On the clinical front, our main collaborating partner (7 joint papers in the past five years) is the Institute of Clinical and Experimental Medicine (IKEM). Here the most productive was the work performed with the group of Dr. Melenovsky on volume overload heart failure in the rat model (Petrak et al., *Proteome Sci* **9**: 69, 2011, Melenovsky et al., *Mol Cell Biochem* **354**: 83, 2011, Benes et al., *Anat Rec (Hoboken)* **294**: 102, 2011, Benes et al., *Clin Sci (Lond)* **121**: 29, 2011).

INTERNATIONAL COLLABORATION

Geographically closest collaborating lab is located at the Veterinary and Pharmaceutical University in Kosice, Slovakia with Dr. Petrovova we work on aspects of developmental toxicity of agrochemicals Petrovova et al., *J Environ Sci Health A Tox Hazard Subst Environ Eng* **47**: 1312, 2012, Petrovova et al., *Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment* 469, 2011). Our collaborators at the Universite de Aix-Marseille, France (Drs. Miquerol, Thevanieu-Ruissy, Kelly) provided us wtih the Connexin40:GFP transgenic mouse line (Sankova et al., *Cardiovasc Res* **95**: 469, 2012), and sent in a student, Sabrina Breyer, for physiological analysis of the *Tbx1* null phenotype. Dr. Marina Campione at the University of Padova, Italy we collaborated on the model of partial Pitx2 deletion (Ammirabile et al., *Cardiovasc Res* **93**: 291, 2012) and analysed the functionality of the cardiac pacemaker; we plan to extend this to optogenetics, which is a developing theme in her lab at the present. Dr. Hentges at the University of Manchester, UK sent in two mutant mouse lines and a postdoctoral fellow, Dr. Tenin, for their electrophysiological analysis (Tenin et al., *PLoS One* **9**: e107041, 2014). Drs. Diego Franco and Amelia Aranega (University of Jaen, Spain) sent in a PhD student, Angel Jose de la Rosa Sanchez for analysis of conduction defects of their mouse model of long QT syndrome (de la Rosa et al., *Cardiovasc Res* **98**: 504, 2013). We also maintain collaboration with the researches at the Medical University of South Carolina, USA (e.g. Sedmera and Thompson, *Dev Dyn* **240**: 1322, 2011), where Dr. Sedmera stayed between 1998 and 2005.

KEY METHODOLOGY AND CORE FACILITIES

The main forte of our lab is our experience with manipulations of the avian embryos. This includes embryonic and foetal surgery, both in the egg (*in ovo*) or in the explanted setup (e*x ovo* culture). For this purposes we have an extensive set of fine surgical instruments, some of them custom made, high-end ergonomical operating microscope (Leica) and sterile conditions for post-incubation of the operated embryos. For monitoring of the function of the developing cardiovascular system we rely during the early stages on videomicroscopy, for later embryos that are not accessible visually we use ultrasound biomicroscopy (Visual Sonics VeVo, housed at the animal facility at the Institute of Biotechnology, since it is also used for mouse work). For studies of the

explanted hearts, our workhorse is the technique of Optical Mapping, which is performed on tissues stained with voltage-sensitive dyes (di-4-ANEPPS) or calcium probes (Rhod-2, fluo-4). Changes in fluorescence are monitored using an EM-CCD camera from Andor, capable of maximum resolution of 512x512 pixels and speed over 1,000 fps in the binning crop mode. For confocal microscopy and 3D imaging we use the core facility of the Department of Biomathematics, which houses two confocal microscopes, two OPT instruments and recently constructed OpenSPIM setup.

INVOLVEMENT IN SIGNIFICANT PROJECTS

The philosophy of our lab is that of informal, "goal-oriented" collaborations with adequately funded laboratories. We have tried in past without success both individual and joint applications for EU funds and concluded that our mode of operation is productive enough. Similar results were obtained with our attempt for the Centre of Excellence in cardiovascular diseases (this programme of the Grant Agency of the Czech Republic is currently on hold). This is not to say we are not going to try in future for larger-scale collaborative grants, just that we do not wish to rely on this type of funding.

OTHER RELEVANT INFORMATION

Not applicable.

SUMMARY AND RESEARCH IMPACT

We pride ourselves in advancement of current state of knowledge mainly along the lines outlined in the section KEY RESULTS, specifically in the field of cardiac conduction system development and effects of hemodynamics on myocardial morphogenesis and functioning. We have provided the first quantitative characterization of function of the developing murine conduction system (Sankova et al., *Cardiovasc Res* **95**: 469, 2012), which will serve as a baseline for interpretation of any future data in transgenic mice. We have proved the value of this work by analysis of several mouse mutants with altered conduction both in the ventricles (Sankova et al., *Cardiovasc Res* **95**: 469, 2012, de la Rosa et al., *Cardiovasc Res* **98**: 504, 2013) and in the atria (Ammirabile et al., *Cardiovasc Res* **93**: 291, 2012, Tenin et al., *PLoS One* **9**: e107041, 2014, Benes et al., *FEBS Lett* **588**: 1465, 2014). On the second front, we provided valuable data for construction of finite element models of the embryonic left ventricle and showed that ignoring of realistic microanatomy results in errors of up to three orders of magnitude in overly simplistic models (Buffinton et al., *Biomech Model Mechanobiol* **12**: 1037, 2013). We have shown that mechanical loading is an important epigenetic factor especially during the early stages of conduction system development (Sankova et al., *Am J Physiol Heart Circ Physiol* **298**: H1571, 2010). The studies comparing developmental toxicity of commonly used cardiac drugs highlighted the types for which the embryo is more sensitive than the adult, and reassured about the relative safety of specific beta blockers (Kockova et al., *Am J Physiol Heart Circ Physiol* **304**: H895, 2013). Showing that drug-induced embryonic bradycardia results in decreased cardiac output and embryo demise, we could generalize that any drugs that significantly slow down the embryonic heart rate have a significant potential for serious adverse effects.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Membrane Transport

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

- Structure, function and regulation of **cell transport systems** at protein molecule level**.**
- Role of transporters in **specific cell properties** and in **diseases**.
- Role of transporters in ***Candida* virulence and pathogenicity.**
- Development of new techniques to estimate **cell physiological parameters**.

Research topics in 2010-2014

1) Transporters involved in pH and cation homeostases – isolation of corresponding genes; biochemical, physiological and molecular characterization of their products; employment of yeast as a model to study transport systems in mammalian and plant cells; heterologous expression of transporters from higher eukaryotes in yeast
2) Molecular basis of high osmotolerance in non-conventional yeasts – identification of genes encoding transporters responsible for extremely high osmotolerance, their functional expression in *S. cerevisiae* strains
3) Development of tools for genetic engineering of non-conventional yeasts
4) Identification and characterisation of transporters involved in yeast virulence and pathogenicity
5) Yeast as a tool for recombinant protein production.

ii. PERSONNEL

There were many changes in personnel during the years 2010-2014, mainly due to the retirement of senior scientists (A. Kotyk (0.1 FTE) retired in 2014, J. Kolínská (0.3 FTE) retired in 2013, J. Horák (0.5 FTE) retired in 2013), maternal/parental leaves of students and post-docs (H. Elicharová, Z. Antošová, L. Kraidlová) or due to finishing their post-docs or PhD studies (e.g. L. Marešová, S. Petrezselyová, J. Zahrádka, M.J. Leandro).

Situation at the end of 2014:

- Senior scientist(s)
  - Hana Sychrová PhD DSc (team leader), biochemist, age 55, H-index 19
- Junior scientist(s):

- Olga Zimmermannová (Kinclová) PhD, biochemist, age 40, H-index 9

- Postdoctoral fellow(s) (total FTE 2.05):
  - Marie Kodedová, PhD, biophysicist, age 31, H-index 2
  - Klára Papoušková, PhD, biologist, age 35, H-index 3 (came back in autumn 2012 after 6 years of parental leave)
  - Zuzana Antošová, PhD, biochemist, age 31, currently on parental leave
- PhD/MSc/BSc students (6/0/2) (one of the PhD students is currently on parental leave)
- Laboratory assistants (1) and research assistants (2, total FTE 1.35)

2) **KEY RESULTS**

The key research results of the team can be divided into three main categories reflecting the topics mentioned above. One category of results mainly covers basic research in the area of **potassium and pH homeostases** in yeast cells, the other involves the results of our studies aiming to characterize the **transporters of pathogenic *Candida* species** that may serve as targets for the development of new antifungal drugs, and the last consists of results obtained with **non-conventional osmotolerant yeast species**, which may be useful for applied research in biotechnology and the food industry. In total, in 2010-2014, we have published 35 papers in international journals with IF, 2 papers in new journals (without an IF at the time of publication), 4 manuscripts are in press, and 3 have been submitted (situation in March 2015).

As described in section 7, we were involved in two large follow-up ERA-NET projects Translucent I and Translucent II. The Translucent projects were developed within the context of the SysMo (System Biology of Microorganisms) initiative and focused on the study of cation homeostasis using the well-known yeast *Saccharomyces cerevisiae* as the model. The aim of the projects was to develop a model of the intracellular homeostasis of alkali metal cations and verify it by a series of experimental approaches. The maintenance of monovalent cation homeostasis (mainly K^+ and Na^+) is vital for cell survival, and cation toxicity is at the basis of a myriad of relevant phenomena, such as salt stress in crops and diverse human diseases. Full understanding of the importance of monovalent cations in cell biology can only be achieved from a systemic perspective. Within the projects, the combination of biochemical, genetic, genomic and computational approaches boosted our knowledge in the field and provided the basis for a more comprehensive and coherent vision of the role of monovalent cations in the biology of the cell. The role of our team was to construct a series of mutant strains lacking various combinations of all known genes encoding potassium and sodium transporters (both plasma-membrane and organellar) and some genes whose products were supposed to participate in the regulation of cation homeostasis. Our mutant strains were mandatory used by the whole consortium. To achieve this, we first had to choose a suitable parental strain and optimize some of the techniques (Maresova et al., *Yeast* **27**: 317, 2010; Petrezselyova

et al., *Fungal Biology* **114**: 144, 2010). We performed detailed phenotypic studies (e.g. growth, salt-stress tolerance, drug tolerance) on the series of constructed knock-outs (altogether more than 35 strains ranging from single to quintuple deletions) and estimated their physiological parameters (e.g. relative membrane potential, intracellular pH, potassium and sodium content) and the changes in these parameters upon salt stress, potassium starvation, extreme external pH etc. In collaboration with the other partners, we performed detailed studies on the role of Trk proteins (the main potassium uptake systems in *S. cerevisiae*), and found that the lack of Trk transporters not only affects the potassium content in cells, but also physiological parameters and the cell proteome both in potassium-sufficient and limiting conditions (Curto et al., *J Proteomics* **73**: 2316, 2010; Herrera et al., *Biochim Biophys Acta* **1838**: 127, 2014; Navarette et al., *FEMS Yeast Research* **10**: 508, 2010). In this part of the work we elucidated the so-far unknown roles of the minor Trk2 potassium uptake system (Borovikova et al., *FEMS Microbiol Lett* **350**: 28, 2014; Petrezselyova et al., *Folia Microbiol* **56**: 23, 2011), and discovered a new type of cation-chloride cotransporter in yeast vacuoles and elucidated its role in vacuole morphology and cation homeostasis (Petrezselyova et al., *Biochim Biophys Acta* **1828**: 623, 2013). Besides characterizing the properties and roles of cation transporters, we also focused our work on identifying key non-transporting players in the osmotolerance and maintenance of cation homeostasis. We participated in a whole-genome screen performed within the consortium which identified several genes whose role in the potassium homeostasis was not known (Barreto et al., *Eukaryot Cell* **10**: 1241, 2011), and within our team, we identified several other genes whose products participated in salt tolerance, cation and pH homeostases (Maresova et al., *FEMS Yeast Res* **10**: 802, 2010; Maresova et al., *FEMS Yeast Res* **12**: 332, 2012; Papouskova et al., *FEMS Yeast Res* **in press** doi: 10.1093/femsyr/fov007 2015), and membrane potential as an important regulator of the activity of cation-efflux systems, Ena1 ATPase and the Nha1 cation/proton antiporter (Zahradka et al., *FEMS Yeast Res* **12**: 439, 2012). For the first time, we described the role of 14-3-3 proteins in yeast salt tolerance, and identified them as regulators of the activity of the Nha1antiporter, both via genetic interaction and directly on the level of protein—protein interaction (Zahradka et al., *Biochim Biophys Acta* **1820**: 849, 2012).

Many years of work on yeast cation transporters resulted in the invitation from two prestigious journals to summarize our current knowledge in a review article (Arino et al., *Microbiology and Molecular Biology Reviews* **74**: 95, 2010) and to summarize the published and non-published results of the Translucent consortium (Arino et al., *Adv Microb Physiol* **64**: 1, 2014). Last but not least, our experience in the characterization of cation transporters, together with our series of mutant strains and know-how on heterologous expression led to our involvement in a joint research effort with groups aiming to characterize cation transporters in higher eukaryotes. Two putative mammalian cation transporters, the NHA2 antiporter from osteoclasts and NKCC2 cation-chloride cotransporter, were expressed in our strains and characterized using our methods (Huang et al., *Biochim Biophys Acta* **1800**: 1241, 2010; Petrezselyova et al., *Yeast* **30**: 395, 2013). Similarly, by heterologous expression in

yeast, we contributed to the discovery that *Os*HKT1;3 is a sodium (and not potassium as predicted from the protein structure) transporter which needs the „cornichon" cargo receptor (Rosas-Santiago et al., *J Exp Bot* **in press**: doi:10.1093/jxb/erv069, 2015).

Our second topic was related to opportunistic *Candida* species. As these pathogenic yeast species live in host organisms, they must secrete specific host-tissue degrading enzymes and express specific transporters with high affinities and capacities to ensure efficient uptake of nutrient sources that are common to the host and pathogen (e.g. amino acids, sugars, potassium, phosphate etc.). Simultaneously, these yeasts must efficiently maintain their cation and pH homeostases to survive in very different niches within the host organism (e.g. urinary vs. digestive tracts). In the last few decades, *Candida* infection has become a significant threat, mainly for immunocompromised patients, and this threat is becoming even greater with rapidly emerging drug-resistant strains. The characterization of *Candida* transporters with a mechanism of activity and protein structure different from those of the host may help to identify new targets for the development of novel antifungal drugs. In connection with our work with the model yeast species *S. cerevisiae*, we first compared the salt tolerance of various pathogenic *Candida* species (Krauke et al., *Curr Microbiol* **61**: 335, 2010), characterized the main transporters ensuring their salt tolerance (Krauke et al., *Folia Microbiol (Praha)* **55**: 435, 2010; Krauke et al., *FEMS Yeast Res* **11**: 29, 2011) and found a surprising synergism between fluconazole (the most commonly used antifungal drug to which many clinical isolates are resistant, and which inhibits one of the ergosterol-biosynthesis steps) and salt treatment. Detailed studies elucidated that the changes in plasma-membrane lipid composition caused by fluconazole treatment result in a hyperpolarization of the plasma membrane, which in turn escalates the non-specific influx of toxic cations (e.g. Na^+, Li^+, hygromycin B, TMA etc.) and affects cation homeostasis (Elicharova et al., *Med Mycol* **51**: 785, 2013; Elicharova et al., *Microbiology* **160**: 1705, 2014). The other group of *Candida* transporters, in which we were interested, were the amino-acid permeases. The model yeast *S. cerevisiae* has almost 20 specific amino-acid permeases for one or a small group of structurally related amino acids, and only one general amino acid permease (Gap1) which is able to recognize and transport all amino acids. To our surprise, we found six genes homologous to *S. cerevisiae GAP1* in the genome of *C. albicans.* The expression of these six genes in *S. cerevisiae* lacking its own amino-acid permeases showed that the products of all six *C. albicans* genes differ in their transport capacities and substrate specificities. Moreover, for the first time, we showed that three of the *C. albicans* Gap transporters also function as transceptors, i.e. sensors that are involved in the intracellular signal-transduction pathways (Kraidlova et al., *Eukaryot Cell* **10**: 1219, 2011). We also contributed (by heterologous expression of corresponding genes in *S. cerevisiae*) to the characterization of two enzymes (secreted proteases and fatty-acid desaturases) which, via ensuring the special composition of the plasma-membrane lipids and transporters in these species, contribute to *Candida*'s virulence and pathogenicity (Bucek et al., *PLoS One* **9**: e93322, 2014; Vinterova et al., *J Microbiol* **51**: 336, 2013).

As for our study of transporters in non-conventional yeast species, we have focused on the highly osmotolerant *Zygosaccharomyces rouxii* and *Debaryomyces hansenii.* These yeast species can live in an environment with very high concentrations of salts and sugars and thus they become spoilage yeasts in many food processes or, on the other hand, they are involved in the production of some speciality salty fermented food. The high tolerance is thought to be due, besides other things, to specific transporters that are able to help to maintain potassium, water and pH homeostases under adverse conditions. First, we focused on alkali-metal cation transporters. *Z. rouxii* has one cation/proton antiporter, which in contrast to *S. cerevisiae*, does not recognize and transport potassium. Our study (site-directed mutagenesis, heterologous expression of mutated proteins in cells lacking their own cation transporters, phenotypic characterization, cation content and flux measurements and *in silico* protein structure modelling) revealed the existence of a hydrophobic filter which confers the cation selectivity of yeast plasma-membrane cation/proton antiporters (Kinclova-Zimmermannová et al., *J Mol Biol* **427**: 1681, 2015). Besides cation efflux systems, we also studied transporters that are involved in the accumulation of necessary potassium in growing and dividing cells. We characterized the Trk1 transporter in *Z. rouxii (Stribny et al., Curr Genet **58**: 255, 2012; Zimmermannová et al., FEMS Yeast Res revised version in preparation, 2015)* and contributed to the characterization of the Hak1 potassium transporter in *D. hansenii* (Martinez et al., *Fungal Genet Biol* **48**: 177, 2011). For both species, we described several physiological parameters in which they differ from the model yeast *S. cerevisiae* (Bubnova et al., *Yeast* **31**: 309, 2014; Michan et al., *FEMS Yeast Res* **13**: 180, 2013). Our knowledge on potassium and sodium transporters in non-conventional yeast species was summarized in an invited review article (Ramos et al., *FEMS Microbiol Lett* **317**: 1, 2011). In a long and fruitful collaboration with the group of Prof. M.C. Loureiro-Dias from Lisbon (post-doc J.M. Leandro from her group spent altogether two years in our team), we characterized specific fructose transporters in *Z. rouxii.* While *S. cerevisiae* is called a glucophilic yeast and it preferentially consumes glucose, *Z. rouxii* has a fructophilic character, prefers fructose over glucose and this is the reason why it sometimes spoils fruit products or the fermentation of musts with a high fructose content. We identified three genes encoding specific fructose transporters in this species, two facilitators and one fructose-proton symporter (Leandro et al., *Eukaryot Cell* **13**: 1371, 2014; Leandro et al., *Microbiology* **157**: 601, 2011; Leandro et al., *PLoS One* **8**: e68165, 2013). Our results may help to engineer *S. cerevisiae* industrial strains that consume fructose better. Our final results are geared towards the same aim. We have been cloning genes encoding glycerol transporters in these osmotolerant yeast species. Glycerol is usually accumulated as a compatible solute in yeast cells upon osmotic stress. To counterbalance the high external osmotic pressure it is produced in yeast cells in high quantities but is simultaneously lost from cells. Yeast species that have an efficient transporter mediating the reuptake of lost glycerol have an advantage over species with a less efficient glycerol uptake. Osmotolerant yeast species have been known for a long time for their high active uptake of glycerol. We described the role of glycerol transporters upon various stresses

(Duskova et al., *FEMS Microbiol Lett* **362**: doi: http://dx.doi.org/10.1093/femsle/fnu041, 2015) and cloned and characterized two glycerol transporters of osmotolerant *Z. rouxii* (Duskova et al., *Mol Microbiol* revised version in preparation, 2015). In this work, we performed most of the experiments, the collaborating team helped with desiccation-survival studies or estimation of glycerol-uptake kinetic parameters, respectively.

3) **INTERNAL COLLABORATION (within the Institute)**

Within the Institute of Physiology, the team (in total 7 members of the team) is involved in two large projects/centres: CBV and the Biocev start-up. We also partly participated in the Neuroscience Centre MSMT LC554 (2005-2011)

We collaborated with the team of V. Obšilová, Dept. Protein Structures on characterizing protein properties upon heterologous expression in yeasts (Veisova et al., *Biochem J* **443**: 663, 2012) and with the team of J. Pácha, Dept. Epithelial Physiology, who shared their Real-time PCR know-how with our team.

4) **DOMESTIC COLLABORATION (within the country)**

i) CAS institutes, universities and companies

The team has been involved in several projects with other institutes of the Academy and with teams from universities. These were the Centre of excellence on Yeast Research MSMT LC531(2006-2011) with the Faculty of Science and Faculty of Pharmacology, Charles University, and with the Institute of Microbiology CAS (MBU) and Institute of Organic Chemistry and Biochemistry CAS (UOCHB). This fruitful collaboration resulted in a subsequent project in applied research (TA CR, TA01011461, 2011-2014) with the Faculty of Science, UOCHB and MBU and the company LentiCats. The project dealt with the construction of recombinant yeast strains and their immobilization for the biotechnology industry. Our team was responsible for the cloning and heterologous expression of various laccases in yeast species, and for the optimization of yeast cultures for successful immobilization. Further, we collaborated in the development of a new soft-ware package, Ocellaris, for the analysis of images from a fluorescence microscope with MBU and the company DEL within the framework of another applied project (TA CR, TA01011467, 2011-2012). In that project we were involved in the development and validation of a new soft-ware package for estimating intracellular pH in single cells.

For several years, we collaborated with a group of biophysicists, experts in fluorescence techniques from the Faculty of Mathematics and Physics, Charles University; the research was focused on optimizing the measurement of intracellular pH. For the joint research effeort, we prepared the recombinant strains expressing pHluorin and the know-how of the relevant fluorescence measurements was provided by our partners (Maresova et al., *Yeast* **27**: 317, 2010)

We also collaborated with the Pichová team from UOCHB on the characterization of desaturases and secreted proteases in *Candida* species, where we used our know-how on cloning and the heterologous expression of enzymes from pathogenic yeasts in *S. cerevisiae (Bucek et al., PLoS One **9**: e93322, 2014; Vinterova et al., J Microbiol **51**: 336, 2013)*.

ii) Biomedicine oriented research

In 2013, we began a collaboration with the V. Čeřovský team in UOCHB on the development of new antifungal peptides. This collaboration resulted in a new project in applied research which started in the autumn of 2014 (TA ČR, TA01011467, 2014-2016). The team from UOCHB synthesizes new peptides with antifungal activities, and our role in the project is testing (via microbiological, biophysical and biochemical methods) their activity against various pathogenic yeast species.

5) INTERNATIONAL COLLABORATION

The team has participated in 5 large international projects and 5 smaller bilateral projects (cf. section 7). These collaborations resulted in 15 papers (referred to in section 2). Besides the collaboration within these projects we collaborated with the group of Prof. M. C. Loureiro-Dias from Inst. Agronomy, Lisbon, Portugal. The collaboration was aimed at characterizing specific fructose transporters in non-conventional yeast species and resulted in 3 joint papers (Leandro et al., *Eukaryot Cell* **13**: 1371, 2014; Leandro et al., *Microbiology* **157**: 601, 2011; Leandro et al., *PLoS One* **8**: e68165, 2013). A post-doc from the Loureiro-Dias group (M.J. Leandro) received a Portuguese national fellowship and in total spent 2 years in our team to clone and express the genes for *Z. rouxii* fructose transporters and prepare corresponding knock-outs. Another Portuguese team with whom we have been collaborating on the characterization of glycerol transporters, is the team of Prof. C. Lucas, Univ. Minho, Braga. A PhD student of our team (M. Dušková-Bubnová) spent 5 months in Braga to perform the necessary kinetic measurements of glycerol transport. Further, we collaborated with prof. Patrick Van Dijck, VIB and Univ. Leuven, Belgium on the characterization of general amino-acid permeases of the pathogenic yeast *Candida albicans*. A PhD student of our team (L. Kraidlova) spent about one year in the partner laboratory and her results were published in a joint paper (Kraidlova et al., *Eukaryot Cell* **10**: 1219, 2011). In 2012 we began a collaboration with Prof. Alexander Rapoport, Inst. Microbiology, Latvian Academy of Sciences, Riga. Latvia. A member of the Rapoport group spent three months in our team. As part of this collaboration we aimed to characterize the role of potassium and glycerol transporters in the survival of desiccation stress (Borovikova et al., *FEMS Microbiol Lett* **350**: 28, 2014; Duskova et al., *FEMS Microbiol Lett* **362**: doi: http://dx.doi.org/10.1093/femsle/fnu041, 2015). In 2013 we began a fruitful collaboration with Prof. Pierre Falson, CNRS, Lyon, France, who is a specialist in protein structure and who helped us with *in silico* modelling of the Nha1

protein (Kinclova-Zimmermannová et al., *J Mol Biol* **427**: 1681, 2015). Further, we began a collaboration with Prof. Linghuo Jiang, Jiangnan University, Wuxi, China with the aim of describing the linkage between calcium signalling and the regulation of alkali-metal-cation and pH homeostases in yeast cells (Papouskova et al., *FEMS Yeast Res* **in press** doi: 10.1093/femsyr/fov007 2015).

6) KEY METHODOLOGY AND CORE FACILITIES

Our team developed several new methods in 2010-2014. Besides using new strategies for the cloning and heterologous expression of enzymes and transporters in yeast, we have developed new tools for the genetic engineering of non-conventional yeast species. Thanks to a new post-doc (Marie Kodedová), who came from the Faculty of Mathematics and Physics, Charles university in 2012, we have significantly improved our use of modern fluorescence techniques. Using this new know-how, we are able to precisely monitor changes in intracellular pH, membrane potential and relative plasma-membrane damage both in batch cultures of yeast cells and via a high-throughput application (developed in our laboratory).

We are also responsible for an institutional core facility, the Casy Cell instrument for monitoring cell size and its changes.

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

In 2010-2014, the team has been involved in 16 national and 5 international projects.

At the national level, we have been partners in 4 large national projects financed from the MSMT (Ministry of Education), LC554, LC531, EE2.3.30.0025 and ED1.1.00/02.0109. These projects have been mainly aimed at training PhD students and post-docs. The activities involved consisted of teaching (special courses), tutoring and supervising (altogether 7 PhD students and post-docs from the team have been directly involved). Further, the team has participated in 3 projects of applied research (TACR TA01011467, TA01011461 and TA04010638) which are described briefly in section 4). For basic research, the team had 4 research projects from the national and CAS grant agencies (GA204/08/0354, IAA500110801, GAP503/10/0307 and GAP302/12/1151). All of these projects had no collaborating teams and their results are described in sections 2) and 9).

Five of the projects (CAS M200110901, MSMT ME10017, MSMT OC10012, MSMT LD13037 and MSMT LH14297) were smaller and served to promote bilateral collaborations with Spain, the USA, China or to participate in the European COST Action CM0902 Molecular machineries for ion translocation across Biomembranes (2009-2012).

As for international projects, the team has been involved as a partner in 2 EU projects (MC ITN), 2 ERA-Net (SysMo) projects and in one COST action. The 2 Marie Curie Initial Training Networks Cornucopia (PITN-GA-2010-

264717, Yeast Biodiversity as a Source of Innovations in Food and Health, 10 partners, 2011-2015) and ImResFun (PITN-GA-2013-606786, Molecular Mechanisms of Human Fungal Pathogen Host Interaction, 12 partners, 2013-2017) brought young foreign researchers (ESRS) to the team from Slovakia and Spain, respectively. Besides this, the team has been involved in the successful ERA-NET projects (call SysMo - Systems Biology of Microorganisms) Translucent I (7 partners, 2007-2010) and Translucent II (8 partners, 2010-2013). Unfortunately, the Czech Republic did not join this initiative and thus the team only had the status of an Associated partner in the project and had to cover the related research costs from national projects. Finally, the team has been one of the partners in the COST Action CM0902 Molecular machineries for ion translocation across biomembranes (23 partners, 2009-2012).

8) OTHER RELEVANT INFORMATION

In 2011, we organized Czech-Spanish workshop Yeast Physiology, 45 participants, 10 from Spain.
In 2010-2014, altogether 7 foreign PhD students or postdocs from 5 countries spent 6 weeks – 3 months in our laboratory to learn our unique techniques and perform specific experiments.

9) SUMMARY AND RESEARCH IMPACT

In summary, we have cloned the genes and characterized about 15 new yeast transporters, discovered the involvement of 4 new proteins in the regulation of intracellular cation homeostasis. We contributed to the characterization of metabolic and proteomic changes in yeast cells upon potassium starvation and upon osmotic stress. We have developed new tools for non-conventional and pathogenic yeast species and used these tools to characterize several transporters involved in the specific properties of these yeast species. We have a strong international collaboration and our laboratory has become famous for its unique collection of yeast mutant strains lacking cation transporters and for other developed tools for non-conventional and pathogenic yeast species (expression vectors, measurement of intracellular pH with pHluorin or relative membrane potential etc.).

List of papers mentioned above which are not in the list of published articles:

Duskova M, Borovikova D, Herynkova P, Rapoport A, and Sychrova H. The role of glycerol transporters in yeast cells in various physiological and stress conditions. *FEMS Microbiol Lett.* 2015;362(3): (dx.doi.org/10.1093/femsle/fnu041).

Duskova M, Ferriera C, Lucas C, and Sychrová H. Two glycerol uptake systems contribute to the high osmotolerance of *Zygosaccharomyces rouxii*. *Mol Microbiol.* 2015:revised version in preparation.

Kinclova-Zimmermannová O, Falson P, Cmunt D, and Sychrova H. A hydrophobic filter confers the cation selectivity of *Zygosaccharomyces rouxii* plasma-membrane Na^+/H^+ antiporters. *J Mol Biol.* 2015; **427**: 1681-1694 (dx.doi.org/10.1016/j.jmb.2015.02.012).

Papouskova K, Jiang L, and Sychrova H. Vcx1 and ESCRT components regulate intracellular pH homeostasis in the response of yeast cells to calcium stress. *FEMS Yeast Res.* 2015; in press (doi: 10.1093/femsyr/fov007)

Rosas-Santiago P, Lagunas-Gómez D, Barkla BJ, Vera-Estrella R, Lalonde S, Jones A, Frommer WB, Zimmermannova O, Sychrova H, and Pantoja O. Identification of rice Cornichon as a possible cargo receptor for the sodium transporter *Os*HKT1;3. *J Exp Bot.* 2015; in press (doi:10.1093/jxb/erv069.)

Zimmermannová O, Salzar A, Sychrova H, and Ramos J. The *Zygosaccharomyces rouxii* Trk1 is an efficient potassium transporter providing yeast cells with high lithium tolerance. *FEMS Yeast Res.* 2015:revised version in preparation.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the ASCR, v. v. i.

Scientific team	Neurochemistry

RESEARCH FOCUS

The topic of our interest is biochemical physiology and pharmacology of cholinergic neurons. Our research concentrates on (1) molecular pharmacology of muscarinic receptors (allosteric modulation of receptor activation, interaction of muscarinic receptors with G-proteins, modelling of muscarinic receptor signal transduction), (2) synthesis, storage, and release of acetylcholine and its presynaptic autoregulation, and (3) cholinergic mechanisms in the pathogenesis of Alzheimer´s disease, namely influence of beta-amyloid on acetylcholine metabolism and muscarinic transmission.

PERSONNEL

Senior scientists
Vladimír Doležal (team leader), neuroscientist, age 62, H-index 19
Jan Jakubík, PhD, pharmacologist, age 44, H-index 13

Junior scientists:
Pavel Michal, chemist, age 41, H-index 8
Vladimír Rudajev, biochemist, age 36, H-index 7

Postdoctoral fellows:
Glenda Alquicer, biologist (currently on maternal leave)
Eva Machová, neuroscientist (currently on maternal leave)

PhD students
Helena Janíčková (thesis defended in 2013, since 2014 postdoc at the Robarts Research Institute, University of Western Ontario)
Eva Dolejší
Alena Randáková
Pavel Zimčík

Laboratory assistants
Romana Ondřejová
Dana Ungerová

KEY RESULTS

General introduction to the topic

The main subject of our interest is the transmission of chemical signal mediated by muscarinic receptors. Natural agonist of muscarinic receptors is acetylcholine that is released from cholinergic

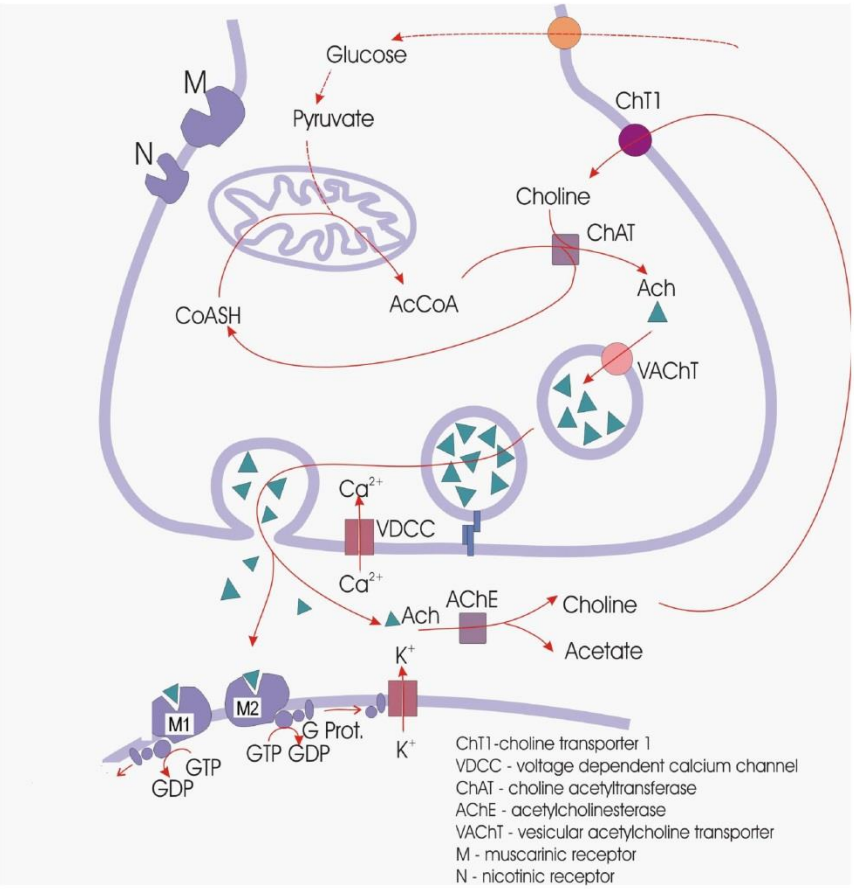


Figure 1: Cholinergic synapse

neurons (Figure 1). There are five subtypes of muscarinic receptors denoted as M1-M5 and encoded by five different genes. Individual muscarinic receptor subtypes share a high degree of homology in the transmembrane domains while extracellular and intracellular loops are less conserved (Figure 2). Intracellular C-terminus may form the fourth intracellular loop by means of glycosyl anchor. The N-terminal part of the third intracellular loop represents the contact domain for interaction with G-proteins. Higher variability of this domain enables selectivity of interaction with different G-proteins. The M1, M3, and M5 receptor subtypes preferentially activate Gq/11 G-protein intracellular signalling while the M2 and M4 subtypes prefer Gi/o G-proteins and activate their signalling pathways.

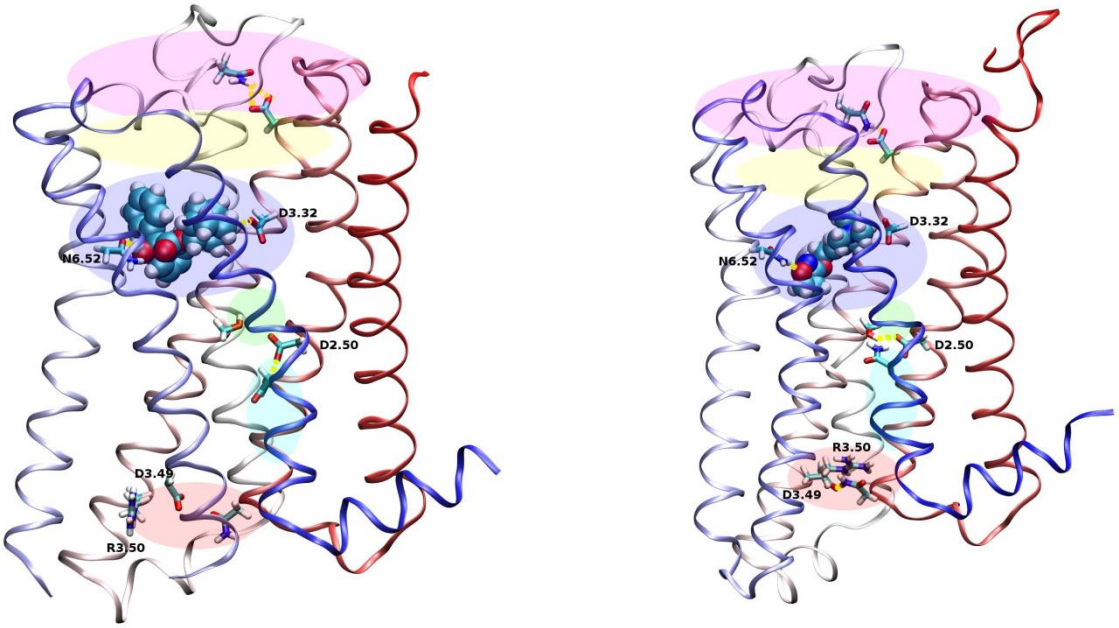


Figure 2: Models of the human M2 receptor in an inactive conformation with bound quinuclidinyl benzilate (left) and in an active conformation with bound iperoxo (right). Orientation: extracellular - top, TM VI and VII - front. Backbone of the receptor is colored by position in red to white to blue gradient. Cyan - carbon, blue - nitrogen, red - oxygen; yellow - hydrogen bonds. Domains: magenta - allosteric binding site, yellow - ectopic binding site, blue - orthosteric binding site, green - transmission switch, cyan - tyrosine toggle switch, red - ionic-lock switch = G-protein interface. Both ligands form hydrogen bonds with D3.31 and N6.52 in the orthosteric binding site. At inactive conformation D2.50 forms hydrogen bond with N7.49 in tyrosine toggle switch. Upon activation it forms hydrogen bond with S3.39. D3.49 and R3.50 in ionic-lock switch are free in inactive conformation and form hydrogen bonds with N2.39 upon receptor activation. Numbering according to Ballesteros and Weinstein.

Muscarinic receptors are widely expressed in both the central nervous system and in the periphery with distinct cellular as well as tissue localization of individual subtypes. They mediate various physiological roles ranging from complex higher nervous functions such as arousal, memory and alertness to vegetative processes such as regulation of heart rate and cardiac output, blood pressure, temperature regulation, perspiration, secretion of exocrine and endocrine glands, and motility of the gastrointestinal tract. In addition to these functions mediated by neuronal acetylcholine, muscarinic receptors also play a role in mediating local responses of non-neuronally derived acetylcholine, e.g. modulation of immune responses and regulation of local circulation.

In addition to physiological roles muscarinic receptors are also implicated in pathologic events like promotion of carcinoma cells growth, early pathogenesis of neurodegenerative diseases in the central nervous system like Alzheimer´s disease and Parkinson´s disease, schizophrenia, intoxications resulting in drug addiction, or overactive bladder in the periphery. All of these disturbances demonstrate involvement of specific muscarinic receptor subtypes and point to the importance of basic research in this field that is needful for development of selective pharmacotherapeutic interventions.

Because of the high homology of the orthosteric binding site of muscarinic receptor subtypes there is virtually no subtype selective agonist that binds to this site. Activation of specific receptor subtypes may be achieved by developing allosteric modulators of acetylcholine binding, since ectopic binding domains on the receptor are less conserved compared to the orthosteric site. Potentiation of the effects of acetylcholine by allosteric modulators would be beneficial in cases where acetylcholine release is reduced due to pathological conditions. When presynaptic function is severely compromised, the utilization of ectopic agonists can be a thinkable solution.

We followed two main lines of research of the muscarinic receptor-mediated signal transduction during evaluated period. One of them focused on molecular mechanisms of muscarinic receptor activation and its coupling with G-proteins, and the other on elucidation of adverse effects of amyloid-β fragments on muscarinic receptor-mediated signalling.

Molecular mechanisms of muscarinic receptor activation, coupling with G-proteins, and selective modulation

We studied one of a few known selective muscarinic agonists xanomeline that exhibit functional M1/M4 subtype selectivity. For experiments we availed CHO cells singly expressing human subtypes of the muscarinic receptor. Functional subtype preference of xanomeline among muscarinic receptors is rather puzzling. Its reversible binding and receptor activation occur with the same affinity and potency at all subtypes of muscarinic receptors. Also xanomeline wash-resistant binding occurs at all receptor subtypes with the same affinity. So far, the only observed qualitative exception from uniform behaviour of xanomeline at muscarinic receptors is functional antagonism by wash-resistant xanomeline at M5 receptors. There are also differences in kinetics of xanomeline binding and activation between the M1 and M2 receptors. Therefore we compared long-term and short-term effects of xanomeline at individual subtypes. Results were published in two articles, Grant et al., PLoS One 5:e15722, 2010 (First author is from collaborating laboratory at the University of Minnesota. Corresponding author is from the Department of Neurochemistry. Experimental work was partly carried out at the Department of Neurochemistry and partly at collaborating laboratory at the University of Minnesota) and Santruckova et al., PLoS One 9:e88910, 2014 (First and corresponding authors are from the Department of Neurochemistry. All experimental work was done at the Department of Neurochemistry. Co-worker from the University of Minnesota participated in experimental design, data analysis and ms preparation). In the first study we have shown that acute as well as chronic xanomeline exposure results in long-term changes in the M1 receptor binding and functional properties. Persistent binding of xanomeline elicits long-term changes in the receptor binding properties that are distinct from the profile obtained with carbachol. We have demonstrated that pretreatment with high and low concentrations of xanomeline result in differential modes of receptor regulation and that the effects observed at low concentrations of xanomeline are due, at least in part, to receptor down-regulation. In the second study, we show uniform xanomeline potency at individual receptor subtypes in releasing intracellular calcium. In contrast, data demonstrate higher efficacy of xanomeline in calcium signalling and longer lasting responses at the M1 and M4 receptors over the rest of the subtypes. Data suggest the existence of a distinct activation mechanism at the M1 and M4 receptor subtypes and show that functional selectivity of xanomeline is based on subtype differences in efficacy and long-term activation and is not due to differential receptor regulation at the cell level or in pharmacokinetics at the system level. However, further experiments are needed to delineate detailed molecular mechanism of xanomeline functional selectivity.

The biological activity of an agonist is a product of both affinity and efficacy. While affinity of an agonist for a receptor is strictly given by free binding energy, agonist efficacy in transducing signal across cell membrane depends on time-ordered complex conformational changes involving interactions among agonist, receptor, G-protein, and guanine nucleotides. These interactions and resulting conformational changes are less well characterized. We performed detailed analysis of allosteric interactions between guanine nucleotides and structurally distinct agonists exhibiting different potencies and efficacies, and analysed modes of coupling of muscarinic receptors to G-proteins. We published our results in two articles (Jakubik et al., Br J Pharmacol 162:1029, 2010 and Jakubik et al., PLoS One 6:e27732, 2011. Both articles have the first and corresponding author from the Department of Neurochemistry. All experimental work was done by the Department of Neurochemistry. Co-worker from the University of Minnesota participated in experimental design, data analysis and ms preparation). We demonstrated that the negative cooperativity between GDP and agonist binding plays a key role in signal transduction via muscarinic receptors. Agonist-induced low affinity conformation of the Gα G-protein subunit for GDP leads to accelerated dissociation of bound GDP that in turn accelerates binding of GTP and G-protein activation. Thus, stronger negative cooperativity between a given agonist and GDP binding leads to a bigger shift of the GDP/GTP affinity ratio resulting in a higher rate of GTP binding and agonist efficacy. Further, we showed that muscarinic receptors pre-couple with their preferential class of G-proteins in the absence of an agonist. In contrast to the M1 and M3 receptors that pre-couple both with preferential Gq/11 and non-preferential Gi/o G-proteins, the M2 and M4 receptors pre-couple only with their preferential Gi/o G-proteins. Lack of pre-coupling of the M2 and M4 receptors to Gq/11 G-proteins is not due to competition with preferential Gi/o G-proteins. None of muscarinic receptors pre-couples with Gs/olf G-proteins. Thus, the mode of coupling of given muscarinic receptor subtype is governed by a combination of the receptor subtype and the class of G-protein.

Unlike orthosteric ligands, a large number of allosteric modulators exert subtype selectivity. Several lines of evidence suggest that muscarinic allosteric ligands bind to the extracellular domain of muscarinic receptors. In contrast, virtually nothing is known on nature of the selectivity of orthosteric muscarinic ligands. Methoctramine is the M2 subtype-selective competitive antagonist of muscarinic acetylcholine receptors that exhibits allosteric properties at high concentrations. Results of our study on

mechanism of methoctramine binding were published in Molecular Pharmacology (Jakubík et al., Mol Pharmacol, 86:180, 2014. First and corresponding author is from the Department of Neurochemistry. All experimental and molecular modelling work was done by the Department of Neurochemistry. Co-worker from University of Minnesota participated in experimental design, data analysis and ms preparation). In this study we investigated whether extracellular domains of other muscarinic receptor subtypes are also involved in putative allosteric properties of methoctramine binding and if they take part in high affinity of the M2 receptor for methoctramine. To achieve this aim we availed differences of methoctramine binding between the M3 and M2 receptor subtypes. We modified gene of the M3 receptor in parts that encode the extracellular domains so that the resulting amino acid sequence resembles the one in the M2 receptor. Using this approach we demonstrated that methoctramine selectivity for the M2 receptor arises from its binding to glutamate residues in the second extracellular loop. We demonstrate that the high affinity methoctramine binding to the M2 receptors is due to the simultaneous interaction with both the orthosteric and allosteric binding sites. In orthosteric binding site methoctramine forms hydrogen bonds with D103 and N404, and interacts with Y403 via π-π stacking interaction. At the allosteric binding site methoctramine forms a hydrogen bond alternating between E172 and E175. Methoctramine can bind to the receptor occupied by antagonist N-methylscopolamine (NMS) with low affinity by interaction solely with the allosteric binding site. Although in such case the interaction between methoctramine and NMS is allosteric (is not mutually exclusive), NMS cannot leave the complex in the presence of methoctramine which physically prevents its dissociation. Lysine K523 in the o3 loop of the M3 receptor interacts with E219 in the o2 loop and hinders methoctramine binding to the allosteric site. It results in low affinity of methoctramine binding and lack of allosteric properties at the M3 receptors.

Intensive research in the field of muscarinic receptors has resulted in the discovery of new compounds that interact with muscarinic receptors in a novel manner. For example, the agonist xanomeline binds to muscarinic receptors in a wash-resistant manner and influences the receptor orthosteric binding site allosterically. The behaviour of such compounds is hard to elucidate without an appropriate molecular model. The crystallographic structure of muscarinic receptors was not available until year 2012. Several homology models of muscarinic receptors based on the crystal structure of rhodopsin expressed naturally in high levels have been published. With the newly available templates of class A of the G-protein coupled receptors it has become possible to design more reliable homology models. Therefore we evaluated the influence of homology modelling procedure and template on quality of homology model of the M2 receptor (Jakubik et al., J Comput Aided Mol Des 27:525, 2013. First and corresponding author is from the Department of Neurochemistry. All experimental and molecular modeling work was done by the Department of Neurochemistry. Co-worker from University of Minnesota participated in experimental design, data analysis and ms preparation). An inherent problem of homology models is the way in which their quality is evaluated. In this study we made 12 homology models of the M2 receptor and demonstrated a crucial role of the templates. We further showed insufficiency of the available tests to evaluate model accuracy. The data clearly show that: 1) Accuracy of homology models is determined by the template. 2) The influence of the template on the resulting model is the most apparent in parts that differ in the secondary structure, and these differences cannot be overcome by computing. 3) The model quality checks built into the programs are only approximate, and cannot be used for choosing the best model. 4) The best model cannot be determined on the basis of binding energy estimates of the docked ligands. This procedure can only distinguish between relatively good and bad models. 5) The only way to select an overall good model is visual inspection for known structural features, intramolecular interactions, hydrogen bond networks, etc.

Adverse effects of amyloid-β fragments on muscarinic receptor-mediated signalling

In studies on adverse effects of amyloid-β fragments on muscarinic neurotransmission we demonstrated the early deficit of cholinergic neurotransmission in cerebral cortex and hippocampus (but not in striatum) that is not present in young (2-month-old) mice and develops in parallel with the increase in soluble amyloid-β fragments in well established mouse model of Alzheimer´s disease (Machova et al., Neurobiol Dis 38: 27, 2010; Corresponding author is from the Department of Neurochemistry. Bulk of experimental work was done by the Department of Neurochemistry. Co-workers from the UEF in Kuopio took care of transgenic mice breeding, performed immunocytochemical experiments, and participated in ms preparation. Coworker from University of Minnesota participated in data analysis and ms preparation). We employed two experimental paradigms to probe ACh release in native tissue ex vivo. Using the first paradigm we addressed ACh release induced by electrical stimulation and its autoregulation in isolation from upstream events involving synthesis and storage of ACh. We found a significant increase in the evoked ACh release in cerebral cortex and a significant decrease in striatum

and hippocampus from 5-6-month-old transgenic animals. These changes are probably due to adaptive changes. The maximal extent of muscarinic autoregulation of ACh release (mediated by the M2 receptors in cortex and hippocampus, and by the M4 receptors in striatum) was preserved in all brain areas in transgenic animals. Using the second paradigm we examined the extent of maximal ACh release evoked by potassium depolarization under conditions that also involve ACh synthesis and storage as limiting steps. The significant decrease of evoked ACh release in cortex and hippocampus but not in striatum without change in tissue ACh content indicates a reduction of ACh loading to synaptic vesicles. Muscarinic receptor operation was further probed using carbachol-induced G-protein activation in cortical membranes that displayed decreased potency in adult animals but no change in young animals. These results indicate that functional pre- and postsynaptic cholinergic damage is not present in APPswe/PS1dE9 transgenic mice before two months of age, develop along with the increase in soluble β-amyloid concentration (mainly fragment Aβ1-42) in the brain, and demonstrate impaired operation of cholinergic synapses in cortex and hippocampus of transgenic APPswe/PS1dE9 mice at an early stage amyloid-β accumulation that does not yet demonstrate a major amyloid pathology.

To elucidate receptor subtype selectivity of the noxious effect of soluble amyloid-β fragment on muscarinic receptors we used CHO cells singly expressing individual receptor subtypes (Janickova et al., Neuropharmacology 67:272, 2013. First and corresponding author is from the Department of Neurochemistry. All experimental work was done by the Department of Neurochemistry. Co-worker from the UEF in Kuopio supplied transgenic mice and participated in ms preparation. Co-worker from the University of Minnesota participated in data analysis and ms preparation). We demonstrated that prolonged *in vitro* Aβ1-42 treatment of CHO cells expressing M1 muscarinic receptors changes characteristics of agonist binding and attenuates agonist-induced activation of phosphatidylinositol signalling. This effect, in line with the notion of the early involvement in pathogenesis of Alzheimer´s disease, is apparently mediated by low molecular mass Aβ1-42 (soluble) species. In concert with *in vivo* results, the treatment does not induce either changes in the expression of major G-proteins, and does not induce oxidative stress or cytotoxicity. These results implicate that the noxious effects of Aβ1-42 on the M1 receptor-mediated transmission involve a mechanism that develops within the plasma membrane and impacts receptor/G-protein/phospholipase C interaction. The attenuation of muscarinic receptor signalling previously reported in a rather early stage of amyloid overproduction in the transgenic mouse model of AD and demonstration of the M1 receptor-mediated signaling attenuation induced by generally non-toxic concentrations of Aβ1-42 *in vitro* evidence important role of the M1 muscarinic transmission in early pathogenesis of Alzheimer´s disease.

Membrane cholesterol level decreases during aging and in line with the established physiological role of Aβ1–40 fragment in inhibiting cholesterol synthesis the reduction is even more manifest in brains of a subset of Alzheimer's patients. In experiments on CHO cells singly expressing the M1, M2, or M3 receptor we investigated influence of membrane cholesterol concentration on activation of their signalling pathways (Michal et al., Neurochem Res DOI 10.1007/s11064-014-1325-z, 2014. First and corresponding author is from the Department of Neurochemistry. All experimental work was done by the Department of Neurochemistry. Co-worker from the University of Minnesota participated in planning of experiments, data analysis, and ms preparation). We demonstrated that increased membrane cholesterol reduces the maximal effect of M1 receptor stimulation but has no effect at M2 and M3 receptors. Reduced membrane cholesterol strongly inhibits maximal response of the M1 and M3 receptor stimulation but augments that of the M2 receptor stimulation. It shows that the most sensitive is signalling through the M1 subtype that is negatively influenced by both the increase and decrease in membrane cholesterol. Apparently, cholesterol membrane levels need to be carefully regulated to maintain fully functional muscarinic receptor signalling. In this context it is worth to note that the M1 muscarinic receptor subtype is not only involved in cognitive functions but also participates in the non-amyloidogenic cleavage of amyloid precursor protein.

In collaboration with the Danone Research, Centre for Specialised Nutrition (Wageningen) we studied influence of the dietary supplement FortasynTM Connect and its components on muscarinic transmission in PC12 and CHO cells (Savelkoul et al., J Neurochem 120:631, 2012. First and corresponding author is from the Danone Research, Centre for Specialised Nutrition. Experimental work on CHO cells was done by the Department of Neurochemistry and both co-authors from the Department of Neurochemistry participated in data analysis and ms preparation). Results of experiments on PC12 cells demonstrate that specific combination of nutrients acts synergistically in enhancing muscarinic responses, most likely through the M1 receptor based on sensitivity to selective antagonists. Using CHO cells singly expressing the M1 or M2 receptors we show that Fortasyn significantly increases maximal response at the M1 but not M2 receptor while it has no effect on either plasma membrane receptor density or EC50 of the carbachol-evoked response at both receptor subtypes. It prompts that the treatment may facilitate the M1 receptor-mediated Gq/11 G-protein activation.

INTERNAL COLLABORATION

Members of the Department have participated in projects Neuroscience Center LC544 (LC - Basic Research Centres, 2005-2011) supported by the Ministry of Education, Youth and Sports), Project of Excellence in the Field of Neuroscience GBP304/12/G069 (2012-2018) supported by the Czech Science Foundation, and Centre of Biomedical Research (2012-2015) supported by the Ministry of Education, Youth and Sports.

DOMESTIC COLLABORATION

Biology Centre, Academy of Sciences of the Czech Republic and Faculty of Science, University of South Bohemia (Kucerova et al., J Neurochem 121:383, 2012), Institute of Experimental Botany, Academy of Sciences of the Czech Republic (Jakubik et al., Mol Pharmacol 86:180, 2014), J. Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic and Department of Physiology, Faculty of Science, Charles University in Prague.

INTERNATIONAL COLLABORATION

The department has long-lasting collaboration with the University of Minnesota Medical School, collaboration with European countries namely Finland (University of East Finland in Kuopio), Germany (Saarland University, Saarbrucken), the Netherlands (Danone, Research Divisin, Wageningen) and with Izrael (University of Tel Aviv, Tel Aviv) within EU project LipiDiDiet (2008-2015), common project with Izrael (Univerzity of Tel Aviv) within the frame of bilateral cooperation. Informal scientific collaboration with Barry University, Florida, USA and Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Russia (Lyukmanova et al., J Biol Chem 286:106, 2011

KEY METHODOLOGY

Molecular biology approaches to prepare mutated receptors. Radioligand binding and functional studies to determine changes in ligand binding properties, efficacy and potency of signal transduction. Receptor homology modelling for determining molecular mechanisms of the relationship between structure and activity. Presently working on introduction of FRET, RICS and FLIM techniques for studies of ligand binding and receptor activation and receptor internalization, respectively. Cell culture and transfection techniques. Biochemical analyses of cholinergic neurotransmitter system using radioenzymatic, fluorometric and spectrophotometric measurements of metabolites and enzyme activities, western blotting and immunodetection of proteins, quantitative RT-PCR determination of gene expression, and ex vivo release of radiolabeled neurotransmitter from brain slices using superfusion technique.

INVOLVEMENT IN SIGNIFICANT PROJECTS

Large collaborative project GA FP7-211696 LipiDiDiet: Therapeutic and preventive impact of nutritional lipids on neuronal and cognitive performance in ageing, Alzheimer´s disease and vascular dementia. The project involves basic research, translational research, and clinical studies. Nineteen groups from seven countries participate in the project.

SUMMARY AND RESEARCH IMPACT

During evaluated time period we studied molecular mechanisms of muscarinic receptor activation and its coupling with G-proteins, and mechanisms of adverse effects of amyloid-β fragments on muscarinic receptor-mediated signalling. Main findings are listed in section “Key results”. They include findings that (1) functional selectivity of muscarinic agonist xanomeline depends on muscarinic receptor subtype differences in long-term activation and a distinct activation mechanism at the M1 and

M4 subtype, (2) negative cooperativity between a given agonist and GDP binding leads to a bigger shift of the GDP/GTP affinity ratio resulting in a higher rate of GTP binding and agonist efficacy, (3) the mode of coupling of given muscarinic receptor subtype is governed by a combination of the receptor subtype and the class of G-protein, (4) the high affinity methoctramine binding to the M2 receptors is due to the simultaneous interaction with both the orthosteric and allosteric binding sites that is located in the o3 loop, (5) the accuracy of homology models of the M2 muscarinic receptor subtype depends on used template, model quality checks built into the programs are only approximate, the best model cannot be determined on the basis of binding energy estimates of the docked ligands, and the only way to select an overall good model is visual inspection for known structural features, intramolecular interactions, hydrogen bond networks, etc., (6) functional pre- and postsynaptic cholinergic damage is not present in APPswe/PS1dE9 transgenic mice model of Alzheimer´s disease before two months of age and develops along with the increase in soluble β-amyloid concentration (mainly fragment Aβ1-42) earlier than noticeable amyloid pathology, (7) soluble Aβ1-42 fragment impairs receptor/G-protein/phospholipase C interaction specifically at the M1 receptor, (8) signalling via the M1 receptor is attenuated by both increase and decrease in membrane cholesterol concentrations, and (9) specific supplements (Fortasyn) amplifies signalling via the M1 but not M2 muscarinic receptor subtype.

Results of our experimental work were published in thirteen peer-reviewed articles. Nine articles have the corresponding author and eight articles the first author from the Department of Neurochemistry. Articles appeared in following Journals:

Jakubik et al., Mol Pharmacol 86:180, 2014 (2013 IF 4.12, Q1 in Pharmacology and Pharmacy)
Michal et al., Neurochem Res. DOI 10.1007/s11064-014-1325-z, 2014 (2013 IF 2.55, Q3 in Neurosciences)
Jakubik et al., Physiol Res 63: S177, 2014 (IF 1.49, Q4 in Physiology)
Santruckova et al., PLoS One 9:e88910, 2014 (2013 IF 3.53, Q1 in Multidisciplinary Sciences)
Jakubik et al., J Comput Aided Mol Des 27:525, 2013 (2013 IF 2.78, Q1 in Computer Science, Interdisciplinary application)
Janickova et al., Neuropharmacology 67:272, 2013 (2013 IF 4.82, Q1 in Neurosciences)
Kucerova et al., J Neurochem 121:383, 2012 (2013 IF 4.24, Q2 in Neurosciences)
Savelkoul et al., J Neurochem 120:631, 2012 (2013 IF 4.24, Q2 in Neurosciences)
Jakubik et al., PLoS One 6:e27732, 2011 (2013 IF 3.53, Q1 in Multidisciplinary Sciences)
Lyukmanova et al., J Biol Chem 286:106, 2011 (2013 IF 4.60, Q1 in Biochemistry and Molecular Biology)
Grant et al., PLoS One 5:e15722, 2010 (2013 IF 3.53, Q1 in Multidisciplinary Sciences)
Jakubik et al., Br J Pharmacol 162:1029, 2010 (2013 IF 4.99, Q1 in Pharmacology and Pharmacy)
Machova et al., Neurobiol Dis 38:27, 2010 (2013 IF 5.20, Q1 in Neurosciences)

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the ASCR, v. v. i.

Scientific team	Neurophysiology of Memory

Characteristics of main results achieved by the team in the evaluated period

In the description of the results achieved in collaboration with other teams, the share of the team in its creation must be clearly specified (i.e. what specific activity the team contributed to the result). Maximum length of 10 pages.

The Department of Neurophysiology of Memory of the Institute of Physiology, CAS, has long-term experience in testing behavioral, mnemonic and cognitive functions in laboratory animals. More recently, the lab has focused on neural mechanisms of spatial cognition, role of brain structures in navigation in a dynamic world and neurochemistry of spatial navigation. We also study cognitive deficits and declines in brain disorders with behavioral pharmacology. We are engaged in drug research and development, as well as development of early diagnosis methods for neuropsychiatric disorders.

Using neuroanatomical, neuropharmacological, cellular, molecular, and behavioral approaches, we investigate brain function in relation to behavior, both in health and disease. In intensive collaboration, we also work on the development of novel therapeutics focused on brain diseases. Scientists take many approaches in search for neural substrates of cognitive functions, learning and memory, ranging from inactivations of brain areas to e.g. immediate-early-gene imaging or advanced electrophysiology. Each of these techniques has different explanatory values and resolutions; each displays to a different level of understanding.

Our focus is to integrate research at molecular, cellular and systems levels in understanding cognitive functions in mammals, mainly learning and memory, behavioral flexibility, working memory, recognition of position and other cognitive domains. Our aim is to elucidate the mechanisms by which memories and other representations are formed and to search for treatments for resistant cognitive deficits in Alzheimer´s disease, schizophrenia, obsessive-compulsive disorder and others.

The Department of Neurophysiology of Memory has a special focus on spatial navigation as a model of declarative memory in both healthy and diseased brains. The basic branch of research targets the neuronal and neurochemical mechanisms of navigation in dynamic environments, and, using the data from the animal models of neuropsychiatric disorders, this research is translated into development of novel real and virtual diagnostic tests for patients with neuropsychiatric disorders. Further, we are engaged in neuropharmacological *in vivo* screening, as well as research of new therapeutic approaches. Taken together, in course of evaluated period, we have published more than 30 papers and participated in 4 patents in close collaboration with domestic and international partners.

Our fields of interest can be divided into four principal areas:

A) Spatial navigation in dynamic environments and behavioral flexibility

This is a research topic of the laboratory focused on relatively unknown aspects of spatial cognition in rodents, their neuroanatomical substrate, developing appropriate behavioral tests and their cross-species comparability.

The first line of investigations pursued the rodents' ability to navigate efficiently in environments in which the relevant navigational cues are moving (dynamic environment). Following the previous work in the laboratory, a novel behavioral paradigm, an enemy avoidance task, was introduced. It requires a rat to organize its behavior with respect to a moving spatial cue represented by a small mobile car-like robot. We are the firs to document the dorsal hippocampus is essential for avoidance of the moving robot, but not when the robot is stationary (Telensky et al., *PNAS* **108**: 5414, 2011). Further experiments showed that female rats are equal to males in acquisition of the robot avoidance task, but females exhibited different, more anxiety-like pattern of behavior (Svoboda et al., *Physiol Res* **61**: 659, 2012).

Other experiments have focused on the ability of laboratory rats to discriminate position of a distant object presented on a computer screen. Previous studies have shown that the rats without a functional dorsal hippocampus did not discriminate the reward position from the non-reward positions. Then the same rats were trained to discriminate light and dark conditions.

The hippocampal inactivation did not disrupt the ability to discriminate these two conditions. It indicated that the inactivation itself had no major effect on the operant behavior and its control by visual stimuli. We conclude that rats use the dorsal hippocampus for recognizing positions of objects located in an inaccessible part of the environment (Klement et al., *Behav Brain Res* **207**: 480, 2010).

Importantly, these studies showed the feasibility of using a computer screen for presenting spatial stimuli to rats. This allowed us to focus further research on developing and implementing tests in virtual reality environments that would be virtual analogies to real tasks.

Such virtual-reality-like tests would enable us to study spatial cognition independent of locomotion and to facilitate cross-species comparisons. Virtual tests have significant advantages: the stimuli, timing, and other factors of the experimental design can be easily adjusted; their results can be straightforwardly compared between various animal species and people; also, cognitive component can be studied without potentially confounding locomotor activity. In addition, the virtual test can be used in electrophysiological and in some pharmacological studies where motor functions could be affected and potentially could confound the results.

In one such test, based on previous rat experiments and developed for rhesus monkeys, we showed also that the mental strategies that monkeys used for orientation in one spatial frame according to the map presented in the other spatial frame depended on the type of stimulus manipulation. We demonstrated that for monkeys there was a difference between solving "mental rotation" and "mental translocation" in this experimental design. We showed that humans were able both to mentally rotate and translocate the displayed stimuli. However, the mental rotation was more difficult for them than mental translocation. These experiments help us to understand how the monkeys perceive the abstract spatial information, create the representation of space and how they transform the information about the position obtained from one spatial frame into another. The comparison between humans and monkeys allows us to study this cognitive ability in phylogeny (Nekovarova et al., *Behav Brain Res* **240**: 182, 2013).

The most relevant papers:

Blahna et al., *Behav Brain Res* **216**, 2011;

Fajnerova et al. *Behav Brain Res.***267**:126, 2014

Klement et al., *Behav Brain Res* **207**: 480, 2010

Levcik et al., *Hippocampus* **23**: 153, 2013

Levcik et al., *Physiol Res* **62**: 561, 2013

Nekovarova et al., *Behav Brain Res* **240**: 182, 2013

Nekovarova et al., *Behav Brain Res* **240**: 182, 2013

Svoboda et al., *Physiol Res* **61**: 659, 2012

Telensky et al., ***Proc Natl Acad Sci USA*** **108**: 5414, 2011

Reviews:

Stuchlík A, et al., ***Physiol Res.*** **62**: S1, 2013

Stuchlik A. *Front Behav Neurosci* **8**:106 2014

Stuchlik et al., *Physiol Res*. **63**: S237, 2014

Team contribution: The entire research was conducted in the laboratory of Neurophysiology of Memory.

Supported by:

Czech Science Foundation: GA309/09/0286, GA14-03627S

Ministry of Education, Youth and Sports: 1M0517, LC554

Grant Agency of Academy of Sciences grant KJB500110904, M200111202, M200111271,

Ministry of Education, Youth and Sports: 1M0517, LC554, LH14053

Marie Curie Reintegration Grant PIRG-GA-2009-256581

B) Animal models of neuropsychiatric diseases

The animal models of brain disorders are indispensable tools in the study of neuropathological mechanisms of the diseases as well as in testing potential novel therapies. Our group has experience with different schizophrenia models, especially rats after acute application of the psychotomimetic drug dizocilpine (Lobelova et al., *Behav Brain Res* **246**: 55-62, 2013; Zemanova et al., *Pharmacol Biochem Behav* **106**: 117-123, 2013; Kubik et al., *Front Behav Neurosci.* **75**, 2014) and Nogo-A knockdown transgenic rats (Krištofiková et al., *Front Behav Neurosci*, 2013; Petrasek et al., 2014), model of obsessive-compulsive disorder (OCD) or study of the neurobiology of depression.

We have studied the predictive validity of the acute dizocilpine (MK-801) animal model in the behavioral test with high cognitive demands. This model is based on a glutamatergic hypothesis of schizophrenia. This hypothesis posits that dysregulation of the glutamatergic neurotransmission lies beneath the pathogenesis of this disease. Our results, following previous works, help us to understand the neurobiology of the cognitive processes in schizophrenia and also to evaluate predictive validity of the model. For example, one study investigated the effects modulating glutamatergic neurotransmission by metabotropic glutamate receptor (mGluR) agonists, in search of new therapeutic options for schizophrenia (Vales et al., *Eur J Pharmacol* **639**: 91-98, 2010).

In a recent study, we have demonstrated that a key cognitive deficit in schizophrenia, disrupted discrimination of relevant and irrelevant information, is straightforwardly accessible in the animal model. Place avoidance on a rotating arena (Carousel) was impaired after systemic dizocilpine, an established model of psychosis, in the presence, but not in the absence, of task-

irrelevant information. The drug also increased similarity between hippocampal ensemble representations of experience in distinct, but not same, environments (**Kubik et al., *Front Behav Neurosci.* 75, 2014**).

We took part in an international effort to characterize a novel model of schizophrenia, a transgenic rat with decreased expression of the Nogo-A protein. Nogo-A is a molecule that regulates growth and regeneration of axons, as well, synaptic plasticity, and dysregulation of its function or expression is suspected to play a role in the pathogenesis of schizophrenia. In two papers (Petrasek et al., Front Behav Neurosci, 2014; Petrasek et al., Neurobiology of Learning and Memory, 2014) we explored the impact of decreased Nogo-A expression on behavior. A cognitive deficit specific for the Carousel Maze suggested that the transgenic animals had difficulties in managing conflicting sources of spatial information, which closely resembles the cognitive symptoms of schizophrenic patients. Validity of the model was corroborated by changes in emotionality, and also by a biochemical analysis of the brains, reported in Krištofiková et al. (Front Behav Neurosci, 2013).

In order to assess deficits in spatial cognition in schizophrenia patients and compare their performance with the data obtained in the animal models of schizophrenia, we designed a virtual analog of two animal tasks: a) the Morris water maze task (MWM) analogue called the virtual Four Goals Navigation (vFGN) task; and an analog to the Carousel maze task modified to its preference version called the Active Allocentric Place Preference task. We demonstrated the deficit of spatial cognition in the group of schizophrenia patients compared to the matched group of healthy volunteers. Despite some limitations of the study, our results correspond well with the previous studies in animal models of schizophrenia and support the decline of spatial cognition in schizophrenia, indicating the usefulness of the two virtual tasks in future comparative research (Fajnerova et al., *Front Behav Neurosci.* **8**, 2014). In the next study we introduced an entirely novel concept of the dysfunctional switching between default mode and central executive networks related to the disrupted activity of the salient network. This model can represent a unitary mechanism of a wide range of symptoms in schizophrenia including the deficit of self and theory of mind. This concept can contribute to our understanding of mechanisms underlying schizophrenia and can serve as a basis for novel therapeutic approaches (Nekovarova et al., *Front Behav Neurosci.*, 2014a). Likewise in the next theoretical work we discussed the possible common mechanism of chronic pain and depression with respect to the effect of antidepressants. We suggested that the association between depression and chronic pain is not just at the level of experience, but has a common neural substrate. Therapeutic intervention at this level could improve overall patient quality of life. Such a theoretical framework is ready to be incorporated into clinical practice (Nekovarova et al., *Front Behav Neurosci.*, 2014b).

In the field of animal models of obsessive-compulsive disorder (OCD) we have showed an impairment in cognitive flexibility in an animal model induced by the sensitization of dopamine D-2 like receptors with quinpirole. Sensitization alone was not sufficient to induce a cognitive flexibility deficit. A drugged state with quinpirole was needed.; However, subtle alterations of reversal learning were seen even in an undrugged but sensitized state. Results parallel human studies where a cognitive flexibility deficit was observed in OCD patients with checking symptoms. This work adds to the face validity of this animal model of OCD (Hatalova et al., *Front Behav Neurosci.*, 2014).

Papers:

Entlerova et al., ***Physiol Behav.*** **120**: 11, 2013

Fajnerova et al. *Behav Brain Res.* **267**: 126, 2014

Fajnerova et al., *Front Behav Neurosci.* **8**: 157, 2014

Hatalova et al., ***Front Behav Neurosci* . 8**: 122, 2014

Krištofiková et al., ***Front Behav Neurosci.* 7**: 90, 2013

Kubík et al., ***Front Behav Neurosci.* 8**: 75, 2014

Lobellova et al., ***Behav Brain Res.* 246**: 55, 2013

Mikulecka et al., *Front Behav Neurosci.* **8**: 101, 2014

Nekovarova et al., *Front Behav Neurosci*. **8**: 99, 2014

Nekovarova, et al., *Front Behav Neurosci.* **8**: 171, 2014

Petrasek et al., ***Front Behav Neurosci.* 8**: 90, 2014

Petrasek et al., ***Neurobiol Learn Mem.*** 42-9, 2014

Prokopova et al., *Pharmacol Biochem Behav.* **102**: 151, 2012

Rambousek et al., *Front Behav Neurosci*. **8**: 180, 2014

Stuchlik and Sumyoshi, *Front Behav Neurosci.* **8**: 444, 2014

Vales et al., ***Behav Brain Res.* 235**: 82, 2012

Vales et al., ***Eur J Pharmacol*. 639**: 91, 2010

Zemanova et al., ***Pharmacol Biochem Behav.* 106**: 117, 2013

Reviews:

Stuchlik *Front Behav Neurosci* **8**: 106 2014

Stuchlik et al., *Physiol Res*. **63**: 237, 2014

Book Chapters

- Nekovarova et al., Cognitive deficits in pharmacologic animal models of schizophrenia. In Schizophrenia Research: Recent Advances. Tomiki Sumiyoshi (Ed), ISBN: 978-1-61942-459-3, Nova Publishing, 2011
- Stuchlik, et al., Behavioral Tests for Evaluation of Information Processing and Cognitive Deficits in Rodent Animal Models of Neuropsychiatric Disorders, Schizophrenia in the 21st Century, Dr. T.H.J. Burne (Ed.), ISBN: 978-953-51-0315-8, InTech, 2012

Team contribution: Publications have focused on OCD models and dizocilpine models of schizophrenia. The experiments were conducted in collaboration with the Prague Psychiatric Centre. Both teams focus on schizophrenia and OCD and their mechanisms. The Department of Neurophysiology of Memory is mostly concerned with the animal models of schizophrenia and their use in novel treatment of schizophrenia.

Nogo-A-knockdown studies were part of international collaboration with the following institutions: Department of Molecular Biology, Central Institute of Mental Health, Brain Research Institute, University of Zürich, and Department of Biology, ETH Zürich, Division of Molecular Mechanisms of Tumor Invasion, German Cancer Research Center, Prague Psychiatric Center and also in intramural collaboration with Department of Neurohumoral Regulations, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic. Our laboratory designed the experiments focused on cognitive and motoric function described in the papers, the international partners prepared and provided the transgenic animal model.

The study assessed deficit in spatial cognition in patients suffering from schizophrenia. It was performed in collaboration with two other institutions: 1) the Prague Psychiatric Center, clinical

base of the Third faculty of medicine Charles University was responsible for recruitment and testing of schizophrenia patients, and 2) the Faculty of Mathematics and Physics, Charles University collaborated on the development of virtual applications. The team's contribution was mainly in the design and development of spatial tests and recruitment of healthy volunteers.

Supported by:

Internal Grant Agency of Ministry of Health: NT13386, NT13403

Czech Science Foundation: GA309/09/1053, GPP303/10/P191, GCP303/10/J032, GA14-03627S

Ministry of Education, Youth and Sports: 1M0517, LC554

Marie Curie Reintegration Grant PIRG-GA-2009-256581

B) Spatial navigation deficits in Alzheimer's disease and mild cognitive impairment

The next line of research in our laboratory focuses on alterations in spatial navigation in cognitively impaired patients, mainly due to Alzheimer's disease (AD). A human analogue of the Morris water maze, called Blue Velvet Arena (BVA), was developed in our laboratory to distinguish allocentric and egocentric spatial deficits in patients. We found significant relationship between the volume of hippocampal cortex and spatial navigation impairment in Alzheimer's disease patients and even mild cognitive impairment patients. The patients with lower right hippocampal volume reached lower scores in allocentric navigation, both in real space and on a computer monitor. The hippocampal deficits are among the primary symptoms of Alzheimer's disease. Strong correlation between the right hippocampal volume and allocentric navigation efficiency in BVA was found in patients suffering from mild cognitive deficit and AD using total brain and hippocampal volumetric MRI. This relationship marks the role of the right hippocampus in spatial navigation and likely results from hippocampal atrophy associated with development of AD. Importantly, the link between right hippocampal volume and navigation was observed also in a computerized overhead 2D version of the Morris water maze suitable as a clinical diagnostic test for AD.

We also demonstrated, in human patients documenting spatial performance, deficits associated with apolipoprotein E allele ε4. The carriers of this allele with mild cognitive impairment (MCI) are known to be at higher risk of developing Alzheimer's disease (AD). The ε4 positive group resembled the mild AD group in their spatial navigation impairment but was similar to other MCI patients in global cognitive functioning.

Papers:

Gazova et al., *Frontiers Aging Neuroscience* **4**: 1, 2012

Laczo et al., *Neurodegener Dis* **8**: 169, 2011

Laczo et al., *Neurodegener. Dis*. **10**: 153, 2012

Laczo et al., *Neurodegener. Dis*. **7**: 148, 2010

Laczo et al., *Neuropsychology.* **28**: 676, 2014

Nedelska et al., *Proc Natl Acad Sci USA* **109**: 2590, 2012

Sheardova et al., *PloS one* **9**: e105623, 2014

Reviews:

Stuchlik et al., *Physiol Res*. **63**: 237, 2014

Vlcek and Laczo , *Frontiers in Behavioral Neuroscience* **8**: 1, 2014.

Team contribution:

The research was conducted in close collaboration between the Dept. of Neurophysiology of Memory and 2nd Faculty of Medicine, Charles University. The spatial navigation task for patients was developed in the Dept. of Neurophysiology of Memory. Particularly, Kamil Vlček participated in the study design, statistical analysis and writing of manuscripts.

Supported by:

Czech Science Foundation: GA309/09/0286, GA309/09/1053, GA14-03627S, GA309/07/0341

Ministry of Education, Youth and Sports: 1M0517, LC554

D) Drug development

Significant advances have been achieved in the field of development and screening of new neuropsychiatric therapeutics based on steroidal compounds. We have started development and testing of new specific NMDA receptor inhibitors based on 3αC substituted derivatives of pregnanolone. 3α5βpregnanolone glutamate (PG), a synthetic analogue of pregnanolone sulfate, selected as a lead structure.

PG crosses the blood-brain barrier, does not induce psychotomimetic symptoms (such as a hyperlocomotion and sensorimotor gating deficit), and reduces excitotoxic damage to brain tissue and consequent behavioral impairment in rats. Follwing experiments demonstrate a lack of the neurotoxicity of the PG in the intact early postnatal period too. Neuroprotective properties were also confirmed in the model of the focal ischemia in the early postnatal period.

Despite being a use-dependent NMDA receptor inhibitor, PG also exerts a paradoxical "antipsychotic-like" effect in an animal model of schizophrenia by acute systemic MK-801. The procognitive properties were evaluated using place avoidance on the Carousel. In addition to the place avoidance behavior, we evaluated effects of PG on locomotor activity and anxiety. PG alone altered neither spatial learning nor locomotor activity in control animals. In the model animals, PG reversed the MK-801-induced cognitive deficit without reducing hyperlocomotion. The highest dose of PG also showed mild but significant anxiolytic properties.

Finally the last part of research deals with how PG treatment affects behavioral response to chronic and acute stress in an animal model of depression. Repeated social defeat and forced swimming tests were used as animal models of depression. The effect of the drugs on the locomotor/exploratory activity in the open-field test was also tested together with an effect on anxiety in the elevated plus maze. Results showed that pregnanolone glutamate (PG) did not induce hyperlocomotion, whereas both dizocilpine and ketamine significantly increased spontaneous locomotor activity in the open field. In the elevated plus maze, PG displayed anxiolytic-like properties. In forced swimming, PG prolonged time to the first floating. Acute treatment of PG disinhibited suppressed locomotor activity in the repeatedly defeated group-housed mice. Aggressive behavior of isolated mice was reduced after the chronic 30-day administration of PG. PG showed antidepressant-like and anxiolytic-like properties in the used tests, with minimal side-effects.

Taken together, this branch of research gives rise to the possibility of obtaining the drugs with clinical potential and minimal side effects, i.e., with a more favorable risk/benefit ratio.

Papers:

Holubova et al, ***Front Behav Neurosci*** **8**: 130, 2014

Kapras et al., *Steroids* **77:**282, 2012

Kleteckova et al., *Neuroscience letters* 11, 2014;

Rambousek et al., *Neuropharmacology* **61**: 61, 2011;

Vales et al., *Behav Brain Res* **235**: 82, 2012.

Reviews:

Korinek et al., *Steroids* **76:** 1409, 2011

Stuchlik et al., ***Physiol Res***. **63**: 237, 2014

Patents:

- Czech Patent (UPV) No.: 303037:- Pregnanolone derivatives substituted in position 3alpha, process for their preparation and use.

- Czech Patent (UPV) No.: 303443: Pregnanolone derivatives substituted in position 3alpha with cationic group, process of their preparation, their use and composition containing them.

-US patent No.: US8575376: Steroid anionic compounds, method of their production, usage and pharmaceutical preparation involving them

-PCT Patent Application WO/2010/136000: Steroid anionic compounds, method of their production, usage and pharmaceutical preparation involving them

Team contribution: The project is a result of the close collaboration between Dept. of Neurophysiology of Memory, Department of Cellular Neurophysiology and Institute of Organic Chemistry and Biochemistry. These partners established frontiers group in the field of transfer of technology in Czech Republic. Our team contributes mainly at the conceptual and neurobehavioral level.

Supported by:

Internal Grant Agency of Ministry of Health: NS10365

Czech Science Foundation: GAP303/12/1464, GA14-20613S

Technology Agency: TE01020028

The Dept. of Neurophysiology of Memory uses a modern multidisciplinary approach with a focus on neural mechanisms of spatial cognition, role of brain structures in navigation in dynamic world and neurochemistry, study cognitive deficits and declines in brain disorders and their animal models, behavioral pharmacology and drug research and development, as well as early diagnosis methods development. The team has a good age- and gender-balanced and suited for intensive cooperative output. The laboratory has extensive experience with behavioral testing, selective lesions and inactivations, behavioral pharmacology, molecular imaging and neurogenesis experiments

Our findings will also stimulate directed applied studies, potentially leading to applied results which eventually should lead to novel and more sensitive diagnostic screening tests and treatment for neurodegenerative and memory disorders, where early detection is a significant predictor of better therapeutic outcome.

The results are represented by lectures and posters presentations at scientific conferences and by scientific papers. The results are published mainly in impacted journals with a significant trend of the increased caliber of the papers. Also an important impact of this research is the scientific training of graduate and PhD students, who will actively participate in designing and executing the research, analysis and presentation of the results. We report the research not only to scientific community but also distribute the results to the public via popularization events.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Neurohumoral Regulations

TEAM DESCRIPTION

RESEARCH FOCUS

The Department of Neurohumoral regulations has been involved in the field of chronobiology, i.e., research on the temporal regulation of physiological processes, for more than 40 years. During the early period, the Department gained recognition as one of the leading groups in the field of circadian control of the rhythmic melatonin production in the pineal gland by light and photoperiod (Illnerová, *Suprachiasmatic Nucleus: The Mind's Clock* 197, 1991). In the 90', our results provided crucial evidence that photoperiod (season of the year) modulates the circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Sumová et al., *Proc Natl Acad Sci U S A* **92**: 7754, 1995). This finding established a novel concept that the SCN serves in our body not only as the clock that keeps track of the daytime but also as a calendar. Thereafter, other major contributions in the field have been made in elucidation of the mechanisms of how the circadian clock in the SCN develops during ontogenesis (Sládek et al., *Proc Natl Acad Sci U S A* **101**: 6231, 2004).

During the past decade, advances in the understanding of the complex structure of the circadian system and its integrative role in physiology have identified the circadian control as one of the general regulatory mechanisms governing homeostasis in mammals. The research focus of our team has naturally been mirroring the enormous expansion of the field. Currently, we aim to unravel the mechanisms of how the circadian system controls physiological processes and also to deepen our understanding how malfunctioning of this regulation affects our health. Namely, we study 4 topics: **1) Central and peripheral circadian clocks and their entrainment with external environment**, including the mechanism of photic, photoperiodic and non-photic entrainment; **2) Ontogenesis of the circadian system** and the mechanism of how maternal and environmental factors affect development of the system in mammals; **3) Circadian regulation of physiological functions**, with the main focus on the circadian regulation of the gastrointestinal tract; **4) Circadian system in humans in health and diseases**, mostly studying the relationship between the circadian system and neuropsychiatric diseases.

PERSONNEL

Currently (2014-5), the research team is composed of 2 scientists (and 1 emeritus), 3 PhD/2 MSc/2 BSc students. Also, 1 research and 2 laboratory assistants are appointed.

The group leader, **Dr. Alena Sumová, DSc. (AS),** aged 54, is the a senior scientist (PI) who has been the head of the Department since 1999. She is an expert in neurobiology and chronobiology (H-index 22). **Prof. Helena Illnerová (HI),** aged 77**,** is a senior scientist and emeritus of the Czech Academy of Sciences. She is a biochemist with deep knowledge in physiology (H-index 32). She served both as Vice-President (1993-2001) and President (2001-2005) of the Academy of Sciences of the

Czech Republic. Due to her life-long dedication to the field, she has been very active in science popularization. **Martin Sládek, PhD (MS)**, aged 36, is a junior scientist (formerly a post-doctoral fellow in the Department). He is an expert in molecular biology (H-index 12). **(Zdenka Bendová, PhD (ZB),** aged 48, had been a senior scientist in the team until 2012 specializing in neurobiology).

The PhD students currently (2014-5) affiliated to the Department include: Lenka Polidarová (LP; supervisor AS), Lucie Olejníková (LO; supervisor AS) and Zuzana Novosadová (ZN; supervisor AS, consultant MS). Group's 2010-14 alumni include Kristýna Matějů (KM), Marta Nováková (MN) and Serhiy Sosniyenko (SS); all of them were PhD students supervised by AS.

Currently, 2 MSc. students (Karel Tejkal and Karolína Šuchmanová) and 2 BSc students are working on their theses. Additionally, Lucia Paušlyová and Kamila Weissová were part of the team as MSc. students during 2010 - 2014. All of these students are/were supervised by AS.

Pavel Houdek (formerly a MSc student in the Department) is currently a research assistant (appointed since 2014). Ivan Hájek (IH) and Daniela Parkanová and were holding this position till 2013 and 2014, respectively. There are two technicians in the team (Eva Suchanová and Lucie Heppnerová).

KEY RESULTS ACHIEVED 2010 - 2014

1. Central and peripheral circadian clocks and their entrainment with external environment

1.1. Photoperiodic modulation of the circadian system

We have previously shown that changes in the photoperiod modulate function of the central clock located in the SCN. At that time, there was only faint knowledge on photoperiodic modulation of the peripheral clocks. Therefore, we studied the mechanisms of how photoperiod modulates the entire circadian system. These studies were aimed to answer the question whether the peripheral clocks change their functional state depending on photoperiod due to signals derived from the central clock in the SCN, or via SCN-independent pathways. By measuring the dynamics of adjustment to the changes in photoperiod of clock gene expression daily profiles in the mouse SCN and liver and of the SCN-driven daily locomotor activity in mice, we demonstrated that the mechanisms of photoperiodic modulation differ between the SCN and liver. In the SCN, adjustment from long to short days proceeded gradually in the separate SCN regions (rostral, medial, caudal) and the short photoperiod-like state was attained mostly via advancing the gene expression decline. The adjustment of the SCN gene expression profiles paralleled the adjustment of the locomotor activity which proceeded by gradually advancing the activity onset. In the liver, the expression profiles adjusted differently, by advancing the *Rev-erbα* expression rise and *Per2* decline. The result suggested for the first time that photoperiodic modulation of the hepatic clock might be mediated via a complex mechanism, involving the SCN-derived signals (affecting *Per2* expression) and behavioural signals, likely related to feeding (affecting *Rev-erbα* expression) **(Sosniyenko et al., *Am J Physiol Regul Integr Comp Physiol* 298: R959, 2010**). Therefore, in our subsequent study, we investigated whether the photoperiodic modulation of the hepatic clock, as detected by the *Per2* and *Rev-erbα* expression profiles, is mediated by SCN-mediated modulation of the feeding rhythm or by other SCN-driven pathways. The results suggested that the photoperiod-modulated SCN clock affects waveforms of gene expression profiles in the liver by food-independent signals **(Parkanová et al., *Eur J Neurosci* 35: 1446, 2012)**. *These studies were supported by EU, 6FP EUCLOCK No. 18741 and by Czech Science Foundation (305090321 and 303110668) (AS was the PI in all projects).*

1.2. Photic entrainment of the circadian clock in the SCN

The circadian clock in the SCN is dominantly entrained by light. The SCN cells respond to light stimulation by activation of NMDA receptors (NMDAR), which leads to elevation of intracellular Ca(2+) levels, phosphorylation of extracellular-signal-regulated kinases 1/2 (ERK1/2) and cyclic AMP-responsive element binding protein (CREB) and finally it results in transcriptional activation of the clock genes *Per1* and *Per2*. We found that in the SCN, NMDAR 2B subunit (NR2B) exhibits a circadian rhythm at both the mRNA and protein level with a peak at night. Selective NR2B inhibitor, ifenprodil, inhibited the glutamate-induced phosphorylation of CREB (pCREB) and ERK1/2 (pERK1/2). This proved the involvement of NR2B in activating the signalling cascade involved in photic resetting of the circadian clock **(Bendová et al., *Neurochem Int* 61: 43, 2012)**; *the study was done with major contribution of our team (ZB was the PI), one collaborator from the Department of Cellular and Molecular Neuroendocrinology helped technically with the tissue cultures).*

The study was supported by Czech Science Foundation (303101227).

2. Ontogenesis of the circadian system

Our significant contribution to the field of ontogenesis of the circadian system have been acknowledged by an invitation of AS to contribute a peer review on this topic to "The Neurobiology of Circadian Timing", a part of a book series of "Progress in Brain Research" **(Sumová et al., *Prog Brain Res* 199: 83, 2012)**. Our recent research focused on the development of the individual compartments of the circadian system with a special emphasis on their development from the early state up to the matured functional one.

2.1. Development of retinal photic sensitivity

We addressed the question when during the ontogenesis do the specific cell subpopulations in the retina of early postnatal Wistar rats become photosensitive and are thus able to relay the photic information to the developing SCN. We found that retina is photosensitive since birth, i.e., well before the eyes are opened. During postnatal development, the spatial distribution of light-induced gene expression in the individual cellular layers gradually changes **(Matějů et al., *J Comp Neurol* 518: 3630, 2010)**.*The study was supported by the Czech Science Foundation 309080503, Grant LC554 and EU, 6FP EUCLOCK No. 18741 (AS was the PI in all projects)*

2.2. Development of the SCN clock

Using the most sensitive method to detect low abundant transcripts in the fetal SCN, we challenged our own earlier findings that the circadian rhythms in clock gene expression are undetectable at the very early stage in vivo. We demonstrated that even in the absence of the canonic clock gene expression rhythms, other genes were rhythmically expressed at the early fetal stage. These results provided the first evidence that gene expression rhythms might be present in the SCN even before the clock fully develops. These rhythms are likely driven by maternal cues. We found that *Rev-erbα* (*Nr1d1*) is the very first clock gene expressed rhythmically and might thus provide the link communicating the information about daytime from the maternal to the fetal clock **(Houdek and Sumová, *PLoS One* 9: e107360, 2014)**. *The study was supported by the Czech Science Foundation 303121108 (AS was the PI).*

2.3. Development of the peripheral clock in the colon

By detecting daily profiles of clock gene expression in the colon of rat pups at various developmental stages, we found that the daily rhythms became apparent since embryonic day 20. However, the clock was unlikely to be matured yet because the mutual phasing among the profiles, necessary for the clock to be functional, was established gradually later and the adult-like state was achieved around postnatal day (P)20. A study with foster mothers revealed that during the prenatal period, the maternal circadian phase may partially modulate the development of the colonic clock. The presence or absence of rhythm in maternal care affected the phasing of the clock gene expression profiles in pups at P10 and P20. The rhythmic signals from the pup SCN were not necessary for reversal in the colonic clock phase between P10 and P20. The data demonstrated for the first time the ontogenetic maturation of the colonic clock and stressed the importance of prenatal and postnatal maternal rhythmic signals for its development. These data may contribute to the understanding of colonic function-related diseases in newborn children **(Polidarová et al., *Am J Physiol Gastrointest Liver Physiol* 306: G346, 2014**; *the concept and majority of the work was performed by members of our team, participation of collaborators from the Dept. of Epithelial Physiology (assistance with the sampling of colonic tissue, expertise in colonic physiology)*. *The study was supported by the Czech Science Foundation 303121108 (AS was the PI).*

2.4. Maternal entrainment of the fetal SCN

We demonstrated that the fetal SCN may be synchronized a by regular feeding schedule imposed to pregnant rats that were arrhythmic due to a prolonged exposure to constant light. The feeding regime did not affect the fetal SCN clock in case the mothers were rhythmic and synchronized with the external light/dark cycle. The finding provided an important evidence for the presence of an entraining pathway, different to melatonin, ensuring a communication between the fetal and maternal circadian systems. The results highlight the importance of a regular feeding regime for proper entrainment of the circadian clock during the prenatal period, especially in conditions when the maternal circadian system is disrupted **(Nováková et al., *J Biol Rhythms* 25: 350, 2010)**. *The study was supported by the Czech Science Foundation 309080503 and Ministry of Education Grant LC554 (AS was the PI in both cases).*

3. <u>Circadian regulation of physiological functions</u>

3.1. Feeding and circadian clocks

Feeding regime is a powerful entraining cue for the peripheral clocks whereas the central clock in the SCN of adult rats is insensitive to it. We studied the effect of a feeding regime on the circadian system and found that under specific conditions, the feeding regime may entrain the SCN. It happened when the rats were exposed to constant light and their SCN was thus unable to drive circadian rhythm in behaviour. Imposing a regular feeding regime via restriction of food availability for 6 h during the day not only established a behavioural rhythm in these rats, but also synchronized the rhythms in clock gene expression in the SCN. The results provided evidence that the SCN may be sensitive to changes in behavioural and metabolic states related to regular feeding **(Nováková et al., *Neuroscience* 197: 65, 2011)**. *The study was supported by the Czech Science Foundation 305090321, 303110668 and Ministry of Education Grant LC554 (AS was the PI in all cases)*

In collaboration with Department of Epithelial Physiology, we focused on the circadian clocks in the gastrointestinal tract (GIT) (reviewed in **Pácha and Sumová, *Acta Physiol (Oxf)* 208: 11, 2013)*;*** *both authors contributed equally*). The peripheral clocks are entrained via complex mechanisms, involving the SCN-dependent and independent pathways. We found that the circadian clocks in different parts of the GIT differ in their sensitivity to signalling from the SCN. The circadian clock in the duodenum was less dependent on the presence of the SCN signals than was the clock in the colon. Regular feeding was more efficient as an entraining cue in the duodenum and liver than in the colon. The stronger dependence of the colonic clock on the SCN signals might be linked to the observation of higher incidence of colonic diseases, including colorectal cancer, in patients with disrupted circadian clocks **(Polidarová et al., *Chronobiol Int* 28: 204, 2011**; *the concept and majority of the work was performed by our team, with a participation of collaborators from Dept. of Epithelial Physiology (assistance with the sampling of the colonic tissue, expertise on colonic physiology*). In another collaborative study, we demonstrated that along the colonic crypt axis, the daily profiles of clock gene expression did not differ whereas expression of genes coding for ion transporters and channels was specific to either the crypt mouth or the crypt base. The results also indicated that the transporters and channels involved in NaCl absorption, but likely not in NaCl secretion, undergo diurnal regulation, thus suggesting a role of the intestinal clock in the coordination of colonic NaCl absorption **(Soták et al., *Am J Physiol Gastrointest Liver Physiol* 301: G1066, 2011**; *the work was performed mostly by the collaborators. We provided the animal room facility suitable for circadian studies, AS provided the expertise on designing the experiment and LP performed the sampling of the colonic tissue). These studies were supported by the Czech Science Foundation grants 303110668, 305090321 and LC554 and 6FP EUCLOCK 018741 grants awarded to AS and the Czech Science Foundation grant 303100969 to JP.*

3.2. Circadian clock and colorectal cancer

In collaboration with the Department of Epithelial Physiology, we studied the link between the circadian clock and the cell cycle in cancer (reviewed in **Soták et al., *Ann Med* 46: 221, 2014***; the review was prepared by the collaborators, from our team AS, contributed to the concept, wrote the part concerning the circadian clocks, and provided critical comments on the early version of the manuscript*). Results of the collaborative study provided evidence that in mice bearing the chemically induced primary colorectal tumors, the circadian clocks were disrupted selectively in the tumor, only marginally in the near-tumor surrounding tissue and were intact in the healthy colonic tissue. Interestingly, the clock gene expression rhythms were shifted in livers of tumor-bearing mice, although the tissue was devoid of tumors. The results point at a possible link between the colonic clock disruption and carcinogenesis and tumor progression **(Soták et al., *Int J Cancer* 132: 1032, 2013**; *both of the teams contributed equally to this study*). *The project was supported by the Czech Science Foundation grant 13-08304S awarded to JP (PI); AS, MS and LP are the co-researchers, and by grant awarded by the Ministry of Health NS9982-4/2008 to JP.*

3.3. Circadian system in spontaneously hypertensive rats (SHR)

Malfunction of the circadian timing system may result in cardiovascular and metabolic diseases, and conversely, these diseases can impair the circadian system. We thus focused on the circadian system of spontaneously hypertensive rats (SHR), i.e., an animal model spontaneously developing the pathology. Our study was the first to

analyze the function of the SHR circadian system in its complexity, i.e., both the central clock in the SCN and the peripheral clocks. We found that SHR exhibited an early chronotype, because the central SCN clock was phase advanced relative to light/dark cycle and the SCN driven behavioural rhythm ran faster compared to Wistar rats. Moreover, the output rhythm was dampened. The SHR peripheral clock responded to the dampened SCN output in a tissue-specific manner. In the colon of SHR, the clock function was severely altered whereas the differences were only minor in the liver. The changes resulted in a mutual desynchrony of circadian oscillators within the circadian system of SHR, thereby potentially contributing to a metabolic pathology of the strain **(Sládek et al., *PLoS One* 7: e46951, 2012)**. Next, we tested the hypothesis that the aberrant peripheral clock entrainment of SHR resulted from a compromised sensitivity of the peripheral clock to the daily temperature cycles. Using the cultured Wistar rat and SHR fibroblasts transfected with the circadian luminescence reporter *Bmal1*-dLuc, we demonstrated a slight resistance of SHR fibroblasts to the temperature entrainment **(Sládek et al., *PLoS One* 8: e77010, 2013)**. Our results also revealed that compared with control rats, SHR were more sensitive to restricted feeding (RF) and developed earlier and more pronounced food anticipatory activity. In SHR, the higher behavioural sensitivity to RF was correlated with greater phase advances of the hepatic clock in response to RF. In contrast to the controls, RF did not suppress the amplitude of the hepatic clock oscillation in SHR. Moreover, the results suggested the possible involvement of the *Bmal2* gene in the higher sensitivity of the hepatic clock to RF in SHR because, in contrast to the Wistar rats, the rhythm of *Bmal2* expression was advanced similarly to that of *Bmal1* under RF. Altogether, the data demonstrated a higher behavioural and circadian responsiveness to RF in the rat strain with a cardiovascular and metabolic pathology and suggested a likely function for the *Bmal2* gene within the circadian clock **(Polidarová et al., *PLoS One* 8: e75690, 2013)**. *These projects were supported by the Czech Science Foundation grants 303110668 (awarded to AS) and 30510P244 (awarded to MS).*

3.4. Circadian clock and brain functions

As a result of our recently established collaboration with Department of Neurohysiology of Memory, we described a deviation in the circadian regulation of behaviour in Nogo-A-deficient transgenic rats. The animal model has been designated as a valuable model of schizophrenia [**(Petrásek et al., *Front Behav Neurosci* 8: 90, 2014)**; *our team designed (AS) and performed (KW and MS) the chronobiology aspect of the study and performed the molecular assessment of the rat genotype by qRT-PCR and Western blot (MS and KW)*]. *The circadian study was supported by the Centre of Excellence in Neuroscience and the Czech Science Foundation grant 30412G069 (AS was the PI).*

4. Circadian system in humans

4.1. Circadian system in healthy subjects

Individuals differ in their chronotype, i.e., the preferred timing of sleep and activity. This varies widely; extremely early chronotypes may wake up when the extremely late chronotypes fall asleep. Therefore, the extreme chronotypes may not be synchronized with the rest of the society, i.e., with social time. Our study represented the first demonstration that in humans, the individual chronotype modulates the peripheral circadian clock. Moreover, the effect was detected under field study conditions. The classification of the subjects according to their chronotype was confirmed by a significant correlation between the phase of their melatonin profiles in saliva and timing

of mid-sleep. The circadian phases of the clock gene (*Per1*, *Per2* and *Rev-erbα*) expression profiles in the oral mucosa of the early chronotypes were advanced compared to the late chronotypes and significantly correlated with the mid-sleep phase of the tested subjects. Our results thus demonstrated that the individual chronotype in humans living in real-life conditions affected not only the phasing of the daily melatonin rhythm but also the phasing of the peripheral clocks which are responsible for control of tissue specific programs. The data deepen our understanding of the mechanisms underlying human chronotypes in real life **(Nováková et al., *Chronobiol Int* 30: 607, 2013)**. The finding attracted attention of The New York Times in their article "Everyday Jet Lag" published on 20October, 2013 (http://well.blogs.nytimes.com/2013/10/17/everyday-jet-lag/?_r=0).
The study was supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic, grant No. NT11474-4/2010 (awarded to AS).

4.2. Circadian system and neuropsychiatric diseases

Disruption of the circadian system has been attributed to various diseases. In our study, we assessed the functional state of the circadian system in children with attention-deficit/hyperactivity disorder (ADHD), employing the saliva melatonin levels as the relevant marker. Comparison between children of different ages (6-7, 8-9 and 10-12 years) revealed significant differences in the melatonin rhythm waveforms between the ADHD and the control groups. In the ADHD group, the melatonin signal duration was shorter than in the control group but the difference was present only in the oldest, but not in the younger children. The results also demonstrated that whereas in healthy children, the entire melatonin profile phase-delayed with age, in the ADHD group, duration of nocturnal melatonin levels shortened with age. The data may indicate a shortening of the subjective night in the ADHD group, which might contribute to poor sleep quality and development of some behavioural symptoms of the disease [**(Nováková et al., *Chronobiol Int* 28: 630, 2011)**, *the study was performed in collaboration with the Department of Psychiatry, 1st Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic, where the subjects were diagnosed and sampled. Contribution of our team was major - we designed the study (AS), helped with the sampling (IH and MN), performed all melatonin assays (IH and MN), analyzed and interpreted the data (AS), wrote and communicated the manuscript (AS)*]. *The study was supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic, grant No. NR /9543 (awarded to IP).*

In children with a rare genetic disease, the Smith-Magenis syndrome (SMS), we assessed the functional state of the circadian system using melatonin levels in saliva and clock gene expression the buccal mucosa, using a newly introduced method for their detection in human samples. This was the first study to use this marker for assessing the human circadian system in a disease. Our original approach made it possible to demonstrate that the aberrancies in the temporal regulation of melatonin levels, typical for this disease, are likely caused by the aberrant circadian system. The results established a hypothesis that in SMS, the central circadian clock in the brain is unable to provide rhythmic signals to the periphery, which leads to aberrations in control of melatonin levels, peripheral clock gene and sleep rhythms [**(Nováková et al., *J Clin Endocrinol Metab* 97: E312, 2012)**; *the study was performed in collaboration with the Department of Neurology, Charles University, 1st Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic, where the subjects were diagnosed and sampled. Contribution of our team was major - we designed the*

study (AS), organized sampling of controls (MN), performed all melatonin (IH and MN) and clock gene expression (MN) assays, analyzed the data (AS) and wrote and communicated the manuscript (AS)]. *This study was mainly supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic, grant No. NT11474-4/2010 (awarded to AS).*

INTERNAL COLLABORATION (within the Institute)

Chronobiology is a highly integrative discipline studying high-order of regulations in physiology. Therefore, our position at the Institute of Physiology CAS provides ideal opportunity to establish collaboration with experts in the specific fields of physiology. Apart from the above described collaborations, which already yielded the published results (Departments of Epithelial Physiology, Neurophysiology of Memory and Cellular and Molecular Neurophysiology), there are ongoing informal collaborations with other groups, namely the Departments of Adipose Tissue Biology (joint grant applications), Genetics of Model Diseases (animal model SHR), Experimental Hypertension (collaboration on SHR model), etc.

Participation in centers of excellence coordinated by our Institute:

Centre of neurosciences (LC554)
Centre of Physiology of Animal Cell (GD305/08/H037)
Centre of excellence in the field of neuroscience (GBP304/12/G069)

DOMESTIC COLLABORATION (within the country)

One of our major focus is related to human circadian rhythms. Therefore, collaboration within the biomedical research institutions is essential for our work. The results of the collaboration with the 1st Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic (Prof. I. Paclt) and Department of Neurology, Charles University, 1st Faculty of Medicine and General Teaching Hospital, Prague, Czech Republic (Prof. S. Nevšímalová) have already been mentioned above, because these studies were published. Apart from them, we also collaborate with the Department of Psychiatry, University Hospital Olomouc, Czech Republic (Prof. J. Praško) within a joint grant awarded to AS by the Ministry of Health (NT11474) to study circadian system in bipolar patients; result of the study will be published in 2015 in Bipolar Disorders (in press). We also collaborate with the Charles University in Prague, Third Faculty of Medicine, Department of Neurology, Prague, Czech Republic and National Institute of Mental Health, Klecany, Czech Republic (A. Bartoš) to study circadian system in Alzheimer patients (grant support P304/12/G069).

INTERNATIONAL COLLABORATION

Within the Europe, we have a long-term collaboration with the **MRC, Laboratory of Molecular Biology, Cambridge, UK** (Prof. Hastings) which is realized by repeated visits of team members and joint publications (e.g., Zhang et al., *Curr Biol* **23**: 1863, 2013). We also collaborate with the **University of Manchester, UK** (Dr. Quin-Jun Meng) within a grant project awarded to AS by the Czech Academy of Sciences in a Program of support of international collaboration (M200111202). We also have a collaboration with the **University of Zurich, Switzerland** (Prof. S. Brown) which was supported by the Sciex grant 2012.

Outside the Europe, we have an informal collaboration with the **University of Toronto, Canada** (Prof. M. Ralph) which has been realized by repeated visits and initiation of experimental collaboration.

KEY METHODOLOGY AND CORE FACILITIES

Our methodological approach is to study the circadian system at all levels of its complexity, i.e., ranging from the system level via the tissue and cellular levels up to the gene expression level studied in cell and tissue cultures in vitro. We use various models, namely rats (Wistar, SHR), mice (WT and transgenic PER2::LUC) and human subjects.

Starting from the top level of complexity, in humans, we examine the **circadian behaviour** via questionnaires (MCTQ) and automated monitoring of daily activity (Actiwatch). In animal models, we perform detailed behavioural analysis via the cage-mounted infrared detectors (CAMS, Clock lab sw) for automated recording of activity and infra red cameras for inspection of specific behavioural patterns (feeding, maternal care). At the organ and cellular levels, we prepare *in vitro* **tissue explants** (hypothalamus, peripheral tissues like pancreas, lung, colon, etc.) and **primary fibroblasts** from animal tissues or from human skin biopsies and maintain them in culture. To interfere with the circadian system at the system level we namely use **environmental** (e.g. constant light, timed restricted feeding, diet), **surgical** (e.g. pinealectomy, xenografts) and **pharmacological interventions**. At the tissue and cellular levels, the interventions are the temperature cycles or application of receptor agonists and inhibitors.

We analyze bioactive molecules and metabolites (glucocorticoids, melatonin, glucose, etc.) in blood of laboratory rodents and saliva of human subjects by **radioimmunoassay**. To analyze protein levels in animal tissues and cells, we use **Western blotting**, **immunohistochemistry** and **immunofluorescence**. To detect the gene expression, we use **in situ hybridization** in animal brain sections and **reverse transcription quantitative real-time polymerase chain reaction** (RT qPCR, ViiA7 or LightCycler480) in peripheral tissue extracts and brain samples precisely separated by **laser capture microdissection** (Leica LMD6000). **We introduced and have been successfully using a modified method to analyze the clock gene expression in buccal smears of human subjects by RT qPCR.**

While the above mentioned methods require regular sampling intervals to get the temporal information, we also use methods which enable direct examination of rhythms at molecular levels by **real time analysis of luminescent reporters** either in tissue explants from transgenic animals (Per2::Luc mice), or from cell lines and primary cell cultures transfected by plasmid constructs and transduced by lentiviral reporters.

Lately, we started using **methods of genetic engineering** (overexpression and knockdown of target genes via both siRNA and shRNA, all coupled with lentiviral delivery). There is also a number of accompanying methods such as cloning, nucleic acid isolation and quantification (e.g. on Agilent Bioanalyzer), electrophoretic methods, basic histology and fluorescent microscopy (Juli Cell analyzer in lab, Institutional microscope core facility), selection of monoclonal cell lines, lentiviral preparation (ultracentrifugation) and animal breeding.

INVOLVEMENT IN SIGNIFICANT PROJECTS

Our Department had been involved in the **6FP EU Project (EUCLOCK) 018741** (coordinated by Prof. T. Roenneberg, Ludwig-Maximilians-University, Institute of Medical Psychology, Munich, Germany) which integrated 27 European laboratories to study entrainment of the circadian system. The project finished in 2011 and provided us with great opportunity for networking within the European chronobiological laboratories.

OTHER RELEVANT INFORMATION

Awards of the scientific achievements of team members during 2010-2014:
MS - Otto Wichterle Prize, awarded by the Czech Academy of Sciences (2010)
AS - Laufberger Medal awarded by the Czech Physiological Society (2011)
HI - Honorary G. J. Mendel medal, awarded by the Czech Academy of Sciences (2013)
HI - J.E. Purkyně Medal, awarded by the Czech Physiological Society (2013)
HI - Laurel Medal, the Crystal category, awarded by the Chamber of commerce (2013)
HI - Silver Medal of the City of Prague (2014)

SUMMARY AND RESEARCH IMPACT

In modern society, majority of people experience extensive socio-economic pressure to change their lifestyle which may disrupt their circadian system. Such situation may happen for example due to shift work which has often been associated with significant increase in incidence of various diseases, including gastrointestinal and cardiovascular diseases and colorectal cancer. No exceptions are being made for pregnant women who also are often exposed to changes in their working schedules. Keeping the endogenous circadian system strong and in a proper phase with the outside world and the entire society is an important pre-requisite for good health. Understanding the mechanisms of how our organism maintains its organ's functions temporally synchronized, especially those relevant to metabolism and cell cycle, is currently of utmost importance and, therefore, recognition of the pathways entraining the circadian clocks with external environment represents currently a hot topic in chronobiology. Although the aberrant circadian system has been attributed to various diseases, the causal relationship for many of them has not yet been proved. We believe that our results have the potential for clinical translation. They are important not only for understanding the etiology of the diseases but potentially also for precaution and new strategies of their therapy (chronotherapy).

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Computational Neuroscience

1) TEAM DESCRIPTION

RESEARCH FOCUS:

a) Analysis of stochastic neuronal models.

Main methodology is provided by mathematical modelling based on the theory of stochastic processes and differential equations, including extensive numerical simulations. Advanced statistical analysis of simulated as well as real experimental data is performed in order to estimate biophysically relevant parameters of the studied models. The results permit detailed comparison of models and data, and consequently they contribute to the knowledge of the mechanism how signal is processed by neurons.

b) Information processing in sensory neurons and neuronal models.

There is an increasing support for the opinion that different forms of noise play an important and positive role in signal transmission within the nervous system. Methods of information theory and statistical estimation theory are applied to analyse the neuronal coding efficiency, based on both simulated and experimental data. Of particular interest is the notion of efficient coding hypothesis for insect olfactory sensory neurons, and biophysical modelling of ligand-receptor interaction in pheromone reception.

c) Neural control of rhythmic motion

Nonlinear dynamics and sensory neuronal models are applied to investigate rhythmic motion in insects (mechanosensory control of wingbeat) and in humans (physiological and essential tremor). We focus in particular on the dynamical interplay between the neural activity and the biomechanics of the periphery. The computational models are constrained by extensive kinematic data from custom-designed experiments.

d) Biophysical modeling of neural development

The connectivity of the nervous system crucially depends on precisely guided growth of axons during development. We use biophysical models to analyze the dynamics of axon growth and the resulting connectivity patterns, based on videomicroscopy observations and micromanipulation experiments from collaborating laboratories. We investigate the consequences of axon bundling for neuronal activity and information processing.

PERSONNEL

– **Scientists**

–Doc. RNDr. Petr Lansky, DrSc. (team leader 2010-2013), expert in stochastic processes and neuronal modeling, age 66, h-index 26

– Lubomir Kostal, PhD (team leader 2014-), expert in information and estimation theory, age 36, h-index 7

– RNDr. Martin Zapotocky, PhD, expert in biophysics and statistical physics, age 48, h-index 9

– **Postdoctoral fellows**:

-Ondrej Pokora, PhD (team member 2011-2012)
-Stevan Pilarski, PhD (team member 2012-2014)
-Ryota Kobayashi, PhD (2011)
-Shinsuke Koyama, PhD (2012)
-Jan Bartussek, PhD (2012-2013)

The last three postdocs were completely supported by their own grants. Their stays resulted in 2 publications so far and in promising continuing collaboration. See also:
http://www.fgu.cas.cz/en/departments/computational-neuroscience
http://comput.biomed.cas.cz/pmwiki.php/Main/Lide

- PhD students

- Pavel Sanda (defended in 2012, since 2013: postdoctoral stay with Maxim Bazhenov, University of California, Riverside)
- Marie Levakova
- Jakub Cupera (defended in 2013)
- Kamil Rajdl
- Soma Chakraborty
- Daniel Smit
- Stepan Kortus

In addition two PhD students (Achileas Koutsou, University of Cyprus in 2012; and Massimiliano Tamborrino, University of Compenhagen in 2011) were supervised by Petr Lansky during their 6-month stay in our department.

2) **KEY RESULTS**

a) Kostal, L., Lansky, P. and Pokora, O. (2013) Measures of statistical dispersion based on Shannon and Fisher information concepts. *Inform. Sciences*, **235**, 214–223.

Kostal, L., Lansky, P. and Pokora, O. (2011) Variability measures of positive random variables. *PLoS ONE*, **6**, e21998

Frequently, the dispersion (i.e., the “spread” or “variability”) of measured data needs to be described in analyses of experimental data. In the case of neurons, these data are often given in the form of interspike intervals or times-to-first spike after the stimulus

onset. Although standard deviation is used ubiquitously for quantification of variability, such approach has limitations. For example highly variable data might not be random at all if it only consists of “extremely small” and “extremely large” measurements. Although the probability density function (histogram) of the data provides a complete view, one needs quantitative methods in order to make a comparison between different experimental scenarios. We propose two alternative measures of dispersion, with the same units as the standard deviation, which are sensitive to distinct features of the probability density function, such as the overall spread or modes. The proposed measures are of broad interest, whenever the “variability” or “randomness” of the probability description is analyzed.
This work was supported by the Centre for Neuroscience P304/12/G069 to P.L., the Grant Agency of the Czech Republic Projects P103/11/0282 to P.L. and O.P. and P103/12/P558 to L.K..
Both papers were entirely done in the Institute of Physiology and all the authors were members of the team.

b) Kostal, L. and Lansky, P. (2010) Information transfer with small-amplitude signals. *Phys. Rev. E*, **81**, 050901 – *Rapid Communication*.

We consider the problem of information transmission under low signal-to-noise ratio conditions. The main novel result is represented by the formula for mutual information in the limit of vanishing input amplitude, never previously discussed in the literature. Our result holds for a broad class of information channels (both biologically-inspired and artificial) with memory, and hence it is of interest to biophysicists, (optical) communication researchers, computational neuroscientist, and information theorists. The inclusion of memory effect is an important point since many realistic systems exhibit this property on various time scales, as demonstrated in the followup paper (Kostal, L., *Phys. Rev. E*, 2010).
This work was supported by the Centre for Neuroscience LC554.
This paper was entirely done in the Institute of Physiology, all the authors were members of the team and it was published in the prestigeous „Rapid Communications“ section of the *Phys. Rev. E*.

c) Kostal, L., Lansky, P. & McDonnell, M. D. (2013) Metabolic cost of neuronal information in an empirical stimulus-response model. *Biol. Cybern.*, **107**, 355–365.

The limits on maximum information that can be transferred by single neurons help us to understand how sensory and other information is being processed in the brain. Neurons use significant amount of energy for the spiking activity, and thus energy usage should be coupled to considerations about the efficiency of neuronal information transfer. In this study, we consider the classical empirical neuronal model subject to metabolic cost constraints, and we find that the optimal information per metabolic cost ratios may occur for on a surprisingly small stimulus range.
L. Kostal and P. Lansky were supported by the Centre for Neuroscience P304/12/G069 and the Grant Agency of the Czech Republic projects P103/11/0282 and P103/12/P558. M. D. McDonnell’s contribution was supported by the Australian Research Council under ARC grant DP1093425 (including an Australian Research Fellowship).

The majority of the research was performed by the team members (Kostal and Lansky). The research was started during the visit of dr. McDonnell in our department. This paper was entirely done in the Institute of Physiology.

d) Levakova, M., Ditlevsen, S., and Lansky, P. (2014) Estimating latency from inhibitory input, *Biol. Cybern*., **108**, 475-493.

Estimation of the stimulus response latency in neuronal recordings represents a key problem in temporal coding analysis. The response latency has been explored and estimation methods proposed mostly for excitatory stimuli, which means that the neuron reacts to the stimulus by an increase in the firing rate. This paper focuses on the important case of inhibitory activity instead, although the methodology can mostly by applied to the excitatory scenario as well. By employing the advanced methods of statistical estimation theory, new state-of-the-art estimators are proposed and tested against simulated data.
M.L. and P.L. were supported by the Grant Agency of the Czech Republic, project P304/12/G069. S.D. was supported by the Danish Council for Independent Research |Natural Sciences. Thework is part of theDynamical Systems Interdisciplinary Network, University of Copenhagen.
The majority of the research was performed in our team, during the visit of prof. Ditlevsen. This paper was entirely done in the Institute of Physiology.

e) Bartussek, J., Mutlu, A.K., Zapotocky, M., and Fry, S.N. (2013) Limit-cycle-based control of the myogenic wingbeat rhythm in the fruit fly Drosophila. *J. Roy. Soc. Interface* **10**, 20121013.

This work is the first nonlinear dynamics based investigation of the control of wing motion in flies. Using a combination of novel experiments and computational analysis, it is shown that a mechanosensor-activated miniscule steering muscle entrains the limit cycle oscillation of the flight power muscle, permitting to achieve wingbeat frequency control at the time scale of 10 msec. This paper also forms the basis for our subsequent work (Bartussek, Haberkern and Zapotocky, 2015), in which we systematically investigate the neurally mediated interaction between the wing system and the external mechanical environment, and we develop a novel computational model that captures the resulting dynamics.
This work was supported by the Volkswagen foundation (I/80984 – 986), Swiss National Science Foundation (CR23I2-130111/1) and the Czech Republic P304/12/G069.
The data analysis and theoretical work was done mainly in Prague, while the experiments were performed in Fry's laboratory in Zurich. During the project, Bartussek moved from ETH Zurich (where he completed his Ph.D.) to Prague for a postdoctoral stay in our team.

Besides appearing in the publications, our results were presented at a number of international symposia, workshops and seminars during 2010-2014. Many of these talks were invited (Kostal gave 8 invited talks, Lansky 5, Zapotocky 6), with the organizers covering most of the expenses.

3) INTERNAL COLLABORATION (within the Institute)

In 2014, Zapotocky collaborated with the Department of Cellular Neurophysiology, which resulted in a joint paper (currently in press). In addition starting in 2014, we collaborate with prof. David Sedmera (Department of Cardiovascular Morphogenesis) and with Oleksandr Chernyavskiy (Department of Biomathematics), as part of the Czech Science Foundation grant „Biophysical modeling of axon fasciculation and targeting due to adhesive interactions“ (PI: Martin Zapotocky).

4) DOMESTIC COLLABORATION (within the country)

We have a fruitful collaboration with the Masaryk University in Brno, Faculty of Science (9 publications) and Charles University of Prague, Faculty of Mathematics and Physics, department of Probability and Mathematical Statistics (2 publications) within the period 2010-2014. In the Krc campus, we have a close collaboration with the Department of Molecular Neurophysiology at the Institute of Experimental Medicine (Czech Academy of Sciences), which allows one of our Ph.D. students to work on a project that is half theoretical and half experimental. We also collaborate with the Neurology Department of the First Medical Faculty, Charles University in Prague (joint manuscript currently in preparation).

5) INTERNATIONAL COLLABORATION

Thanks to the scientific activity and global visibility of the key members of the research team (see the Participation of the team members in the activities of the scientific community) there are several successfully running collaborations, which contribute to the publication activity:
(see http://comput.biomed.cas.cz/pmwiki.php/Main/Publikace)

- Dr. Jean-Pierre Rospars (Institut National de la Recherche Agronomique, Versailles, France): modelling and experimental data on the insect olfactory sensory system, numerous publications also before 2010 (Rospars et al., Brain Res., 2013; Kostal et al., *PLoS Comput. Biol.*, 2008),

- Prof. Susanne Ditlevsen and Dr. Massimiliano Tamborrino (Department of Mathematical Sciences, University of Copenhagen): advanced statistical methods and stimulus identification (e.g., Ditlevsen & Lansky, *Neural Comput.*, 2011; Tamborrino et al., *Phys. Rev. E*, 2012)

- Dr. Mark McDonnell (Institute For Telecommunications Research, University of South Australia): the balance of energy vs. information encoding efficiency (Kostal et al., *Biol. Cybern.*, 2013),

- Dr. Shinsuke Koyama (Institute of Statistical Mathematics, Tokyo, Japan): the problem of coding efficiency under different encoding schemes (Koyama & Kostal, *Math. Biosci. Eng.*, 2014)

- Prof. Laura Sacerdote, Dr. Cristina Zucca, Dr. Federico Polito (Department of Mathematics, University of Torino, Italy): aspects of network connectivity and communication, stimulus identification and response properties (Polito et al., *J. Phys. A*, 2014).

- Dr. Ryota Kobayashi (National Institute of Informatics, Tokyo, Japan): detailed modeling of induced energetic costs, neurons and their networks, e.g., Kobayashi et al., Neural Comput, 2011.

- Dr. Jan Bartussek (University of Rostock, Germany), mechanosensory control of wing motion in flies (Bartussek et al., *J. Roy. Soc. Interface* 2013).

Besides the above mentioned international collaborations, which resulted in publications during 2010-2014, there is a research in progress with:

- Dr. Tomoki Fukai (RIKEN, Tokyo, Japan): key experimental data on in-vivo information coding in cortical neurons

- prof. Toby Berger (University of Virginia, USA), the renowned information theorist and the recipient of Shannon Award 2002 and Hamming Medal 2011. The topic of common interest is the joint source-channel information coding in neural systems

- dr. Sydney Lehky (The Salk Institute, USA), information processing in the visual system

- prof. Priscilla Greenwood (The University of British Columbia, Vancouver, Canada), methods of mathematical statistics in neuroscience.

- dr. Henry Tuckwell (Max Planck Institute for Mathematics in the Sciences, Leipzig, Germany). Dr. Petr Lansky has a long-lasting collaboration with both prof. Greenwood and dr. Tuckwell, see the publication list at: http://www2.biomed.cas.cz/~lansky/

- Prof. Alain Trembleau (Universite Pierre et Marie Curie, Paris, France) – an extensive collaboration in the past 3 years, leading to joint conference presentations in 2014 and first joint papers in 2015, topic: Axon growth and bundling in neuronal cell culture

- Prof. Frederic Pincet (Ecole normale superieure, Paris, France) – biophysics of axon bundling

6) KEY METHODOLOGY AND CORE FACILITIES

The global methodology is provided by the information and estimation theory, (stochastic) differential equations and biophysical modeling. More detailed technical description of the methodology is beyond the scope of this report.

The department already employs its own computer cluster (a Supermicro SuperServer rack system) for numerically intensive calculations. We own multiple licenses for MATLAB, PGI compilers, and the mathematical analysis software Mathematica (Wolfram Research). Much of our work is done using open source computational tools (Linux, R, ImageJ / Fiji, Python).

The department does not have its own experimental facilities. Our biologist collaborators rely on the lab equipment in their own departments, and on the central facilities of the Krc campus.

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

The team was receiving funding from the following significant grant projects during 2010-2014 (only foreign grants and block grants are listed):

- Volkswagen Foundation, Germany, I/83 892 (2010), PI: Martin Zapotocky

- Office of Naval Research Global, N62909-11-1-1111 (2011-2012), PI: Lubomir Kostal

- European Office of Aerospace Research and Development , FA8655-12-1-2006 (2012-2013), PI: Lubomir Kostal

- The Neuroscience Centre LC554 (2006-2010) from the Czech Ministry of Education, Youth and Sports

- Project of Excellence in the Field of Neuroscience GBP304/12/G069 (2012-2018) from the Czech Science Foundation

9) SUMMARY AND RESEARCH IMPACT

The field of computational neuroscience has experienced a dramatic increase during the last three decades, attracting a number of scientists from different disciplines, especially with background in mathematics and physics. New topics have emerged alongside the traditional neuronal modeling approaches. The longstanding problem of neuronal coding and information processing, together with the description of memory, learning and development now receive the most attention. Consistently with the increased interest, separate programs, departments and labs of computational neuroscience have been started in many prestigious universities and institutes (Salk Institute, The Swartz Foundation in Harvard and Yale, Arizona State University or Humboldt University to name a few). Furthermore, the computational approach to neuroscience has been selected as a flagship project of European Union for years 2013–2023 with a budget of 1 billion EUR (*Human Brain Project*). The computational neuroscience approach has become mainstream and modern approach to investigate various neuroscientific topics.

The department of computational neuroscience in Prague, although formally established in 2010, has in fact a much longer history, represented by the activity of Dr. Petr Lansky, an internationally renowned scientist in the field. The global visibility of the department is documented not only by the quality publication output but also by the constant flux of visitors from foreign departments (Japan, USA, Denmark, etc.), the resulting collaborations, successful grant applications, and organization of international workshops.

The focus of our research during 2010-2014 can be divided into several categories.

Our investigation of the information efficiency of individual neural coding schemes led to the definitions of novel measures of statistical dispersion coefficients. These measures allow higher resolution in the quantification of the experimentally predicted neural coding schemes (Kostal et al. *PLoS ONE*, 2011; Kostal and Pokora, *Entropy*, 2012; Kostal et al., *Inform*. *Sci*. 2013).

The key result lies in the prediction of the influence of the metabolic workload on the information processing in neural systems (Kostal et al, *Biol Cybern*., 2013; Kostal and Lansky, *BioSystems*, 2013). We calculated the relevant optimality conditions that allow for future comparison with experimental data (Kostal, *Phys. Rev. E*, 2010; Kostal and Lansky, *Phys. Rev. E*, 2010). Our new results hence open the door for the innovative and highly focused research to follow. The imminence and timeliness of our topic is also documented by the EU Human Brain Project and USA BRAIN Initiatives, which demonstrate the interest and global priority given to the topics of computational (theoretical) neuroscience.

Different neuronal coding schemes were analyzed by employing the methods of information theory and estimation theory (Pawlas et al., *Neural Comput*., 2010; Lansky and Ditlevsen, *Neural Comput*., 2011). For this purpose both approximate and numerical approaches were developed as intended (Levakova et al., *Biol. Cybern*, 2014; Rajdl and Lansky, *Math. Biosci. Eng*, 2014; Kobayashi et al, *Neural Comput*, 2011; Lansky and Pawlas, *Phys. Rev. E*, 2011).

The details of our approach, including methodology and results, can be found in the respective papers. The number of invited talks in the last year of the project further guarantees the recognition and international visibility of our results and the newly established contacts at the top institutes promise fruitful collaborations in the future.

Overall, **33 papers** were published during 2010-2014 in impacted journals with **19** having both the first and corresponding author as the team member. These include publications in the prestigious and field-specific journals such as *Information Sciences, Physical Review E, Neural Computation and Biological Cybernetics*. There was in total collaborations with 17 institutions and with 24 researchers who were not members of the team.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the ASCR, v. v. i.

Scientific team	Protein Structures

1) TEAM DESCRIPTION

i. RESEARCH FOCUS

Research in our group is focused on providing a mechanistic understanding of how the biological activity of protein complexes is regulated using various biochemical and biophysical approaches. In particular, we are interested in the regulation of selected signalling proteins whose functions are controlled through protein-protein interactions and, especially, through phosphorylation-dependent interactions. Several proteins were described to specifically bind phosphorylated motifs and among them the 14-3-3 proteins were the first to be shown to recognize motifs containing phosphoserine or phosphothreonine. The 14-3-3 proteins regulate the function of its binding partners through number of mechanisms which interest us, such as direct conformational change and masking of structural features on the surface of the target molecule.

During the period under the review, we have been focusing on several projects including:

- Structural studies of the 14-3-3 protein complexes with neutral trehalase Nth1, regulator of G protein signalling 3 and phosducin
- Structural characterization of DNA-binding domain of transcription factor FOXO4 and its interaction with the target DNA
- Structural study of the thioredoxin-binding domain of ASK1 kinase and its interaction with thioredoxin
- Characterization of interaction between cytoplasmatic domains of TRP channels and their binding partners

ii. PERSONNEL

Senior scientists:

RNDr. Veronika Obšilová, Ph.D. (**head of the department/team leader**; Ph.D. in medicinal biophysics; expert in biochemistry, biophysics; age 42; H-index 15)

Ing. Jan Teisinger, Ph.D. (full time till 12/2014; Ph.D. in biochemistry; biochemist; age 70; H-index 23)

Prof. RNDr. Tomáš Obšil, Ph.D. (part time; Ph.D. in physical chemistry; professor of physical chemistry; expert in biophysics, crystallography, biochemistry; age 42; H-index 22)

Junior scientist:

Mgr. Lenka Gryčová, Ph.D. (full time 1/2010-9/2012; since 10/2012 on maternity leave; Ph.D. in medicinal biophysics; biophysicist, biochemist; age 34, H-index 7)

Postdoctoral fellow: none at the moment

Research assistant: Mgr. Dana Kalábová (part time)

PhD/MSc/BSc students (7/4/1), laboratory assistants (1)

Current Ph.D. students:

Internal: M.Sc. Miroslava Kopecká
M.Sc. Kristýna Boušová
M.Sc. Michaela Jirků
M.Sc. Salome Kylarová

External: M.Sc. Dalibor Košek
M.Sc. Miroslava Kacířová
M.Sc. Olívia Petrvalská

Current Undergraduate students: Bc. Vojtěch Dolejš; Bc. Aneta Šmídová; Bc. Katerína Pšenáková; Bc. Jiří Šimůnek; Kateřina Jarosilová

Laboratory assistant: Simona Krausová

Defended past Ph.D. students:

RNDr. Lenka Řežábková, Ph.D. (defended in 2012; now postdoctoral researcher at Paul Sherrer Institute, Villigen, Switzerland)
Mgr. Hana Janoušková, Ph.D. (defended in 2013; now postdoctoral researcher at Institute for Research in Oncology, Bellinzona, Switzerland)
Mgr. Dana Veisová, Ph.D. (defended in 2013; now on maternity leave)
Mgr. Eva Macáková-Slivenecká, Ph.D. (defended in 2013; now at Institute of Microbiology CAS)

Past Ph.D. students whose defences are planned in 2015:

M.Sc. Jan Bílý (now at Czech University of Life Sciences, Prague)
M.Sc. Blanka Holendová-Holakovská (currently on maternity leave)
M.Sc. Petr Vácha (now at SEQme s.r.o., Dobříš)

2) KEY RESULTS

a) Molecular mechanism of the 14-3-3-protein-dependent regulation of yeast neutral trehalase Nth1

The overall goal of this project was to understand the molecular mechanism of the 14-3-3 protein–dependent regulation of yeast neutral trehalase (Nth1). This enzyme catalyzes the hydrolysis of trehalose (non-reducing sugar found in a wide variety of organisms) and its enzymatic activity is regulated in the phosphorylation and the 14-3-3 protein-dependent manner. In the pilot study, we performed biophysical characterization of yeast 14-3-3 isoforms Bmh1 and Bmh2 and showed that their C-terminal segments behave differently compared to isoforms from other eukaryotes, in particular they do not possess autoinhibitory function (Veisova et al., *Biochemistry* **49**: 3853, 2010). Next, we identified key phosphorylation sites in neutral trehalase Nth1 from S. cerevisiae that are responsible for its binding to 14-3-3 and thus its activation using site-directed mutagenesis, enzyme kinetics measurements and mass

spectrometry (Veisova et al., *Biochem J* **443**: 663, 2012). In two following studies we performed structural characterization of the Nth1:Bmh1 complex. Hydrogen/deuterium exchange coupled to mass spectrometry and chemical cross-linking experiments revealed that the 14-3-3 protein binding induces significant structural changes within several regions of Nth1 including the catalytic trehalase domain (Macakova et al., *Biochim Biophys Acta* **1830**: 4491, 2013). SAXS-based low-resolution solution structures of Nth1 alone and the Nth1:14-3-3 complex (Fig. 1) clearly showed structural rearrangement of Nth1 upon the complex formation with Bmh1 (Kopecka et al., *J Biol Chem* **289**: 13948, 2014). Our results provided a first structural view on the 14-3-3 protein-dependent activation of yeast neutral trehalase Nth1, which is relevant to understand not only the process of Nth1 activity regulation but also the role of the 14-3-3 proteins in the regulation of other enzymes.

Supported by Czech Science Foundation (Project P207/11/0455, PI: Veronika Obšilová) with the major involvement of group of V. Obšilová/T. Obšil and their PhD students: DV, EM, MK, LR and DK. In collaboration with Institute of Microbiology that contributed by MS measurements and Faculty of Science, Charles University that contributed by AUC measurements and Institute of Physics, Charles University that contributed by time-resolved fluorescence measurements.

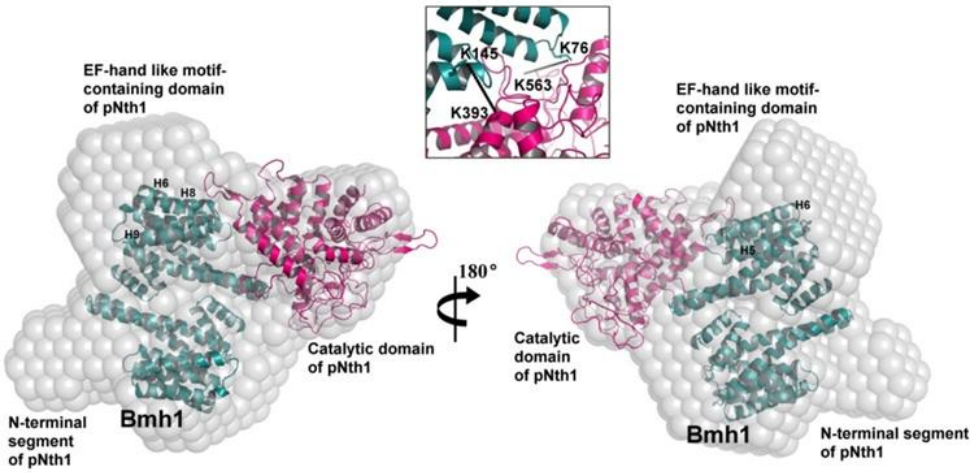


Fig. 1. Overlay of the rigid body model of the Nth1:Bmh1 dimer complex with SAXS based molecular envelope. The envelope is shown in gray, the catalytic domain of Nth1 (sequence 291-721) is shown in magenta, and Bmh1 dimer (sequence 4-236) is shown in cyan (Kopecka et al., J Biol Chem **289**: 13948, 2014).

b) Role of the 14-3-3 proteins in the regulation of proteins involved in the modulation of G-protein signalling

In this project we investigated how the 14-3-3 protein binding affects the structure and thus the function of two proteins involved in the regulation of G protein signalling: regulator of G protein signalling (RGS) RGS3 and phosducin. In the first study we solved the crystal structure of the RGS domain of RGS3 at 2.3 Å resolution and characterized interaction between 14-3-3 and RGS3 using fluorescence spectroscopy. The data obtained from the resolution of the structure of the RGS domain suggest that the 14-3-3 protein-induced conformational change affects the region within the Gα-interacting portion of the RGS domain (Rezabkova et al., *J Struct Biol* **170**: 451, 2010). This can explain the inhibitory effect of the 14-3-3 protein on GAP activity of RGS3. In the next study, we provided a structural mechanism for 14-3-3-dependent inhibition of RGS3-Gα interaction and determines the low resolution solution structure of the 14-3-

3ζ:RGS3 complex using SAXS and HDX-MS (Rezabkova et al., *J Biol Chem* **286**: 43527, 2011). The SAXS-based low resolution structure of the 14-3-3:RGS3 complex (Fig. 2) suggests that the 14-3-3 protein binding affects the structure of the G interaction portion of RGS3 as well as sterically blocks the interaction between the RGS domain and the G subunit of heterotrimeric G proteins. Another protein that interests us is phosducin (Pdc). Pdc plays an important role in the regulation of G protein signalling, transcriptional control, and modulation of blood pressure and its function is negatively regulated by phosphorylation followed by binding to 14-3-3. To gain insight into the role of 14-3-3 in the regulation of Pdc function, we first studied interaction between Pdc and 14-3-3 using analytical ultracentrifugation, dynamic light scattering and time-resolved fluorescence spectroscopy. Our data revealed that both phosphorylation sites Ser-54 and Ser-73 are required for Pdc binding to 14-3-3 and that phosphorylated Pdc undergoes a conformational change upon the binding to 14-3-3. These changes involve the Gtβγ binding surfaces and thus could explain the inhibitory effect of 14-3-3 on Pdc function (Rezabkova et al., *Biophysical Journal* **103**: 1960, 2012).

In addition, we were invited to discuss our results concerning the 14-3-3-dependent regulation of protein function in a review article (Obsil et al., *Semin Cell Dev Biol* **22**: 663, 2011).

All these studies were supported by Grant Agency of the Academy of Sciences of the Czech Republic (grant # IAA501110801, PI: Tomáš Obšil, co-PI: Veronika Obšilová) and Ministry of Education, Youth and Sports of the CR (Centre of Neurosciences LC554) with the major involvement of group of T. Obšil and V. Obšilová and their PhD students: LR and MK. In collaboration with Faculty of Science, Charles University that contributed by AUC and DLS measurements and Institute of Physics, Charles University that contributed by time-resolved fluorescence measurements.

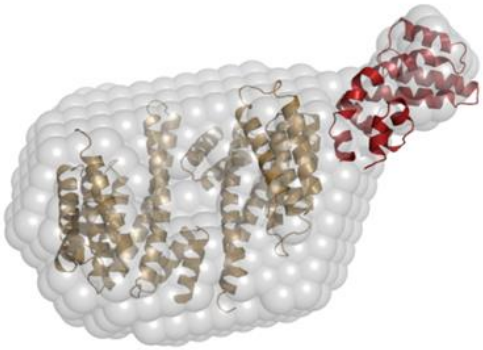


Fig. 2. Structure of the complex of the 14-3-3 protein (light brown) with the regulator of G-protein signalling 3 (red) proposed on the basis of SAXS (small angle X-ray scattering) measurement. The molecular envelope calculated from SAXS data is shown in gray (Rezabkova et al., *J Biol Chem* **286**: 43527, 2011).

c) Structural studies of DNA-binding domain of transcription factor FOXO4 and its interaction with the target DNA

In this project we investigated in detail the interaction between DNA-binding domain of transcription factor FOXO4 (FOXO4-DBD) and its target DNA. The forkhead box transcription factors are a family of structurally related transcriptional activators involved in embryogenesis, tumorigenesis, and the metabolism control. FOXO factors play an important role in cellular proliferation, survival, and in mediating effects of insulin and growth factors on metabolism. We solved the crystal structure of FOXO4-DBD bound to a 13 bp DNA duplex containing a FOXO consensus binding sequence with 1.9 Å resolution (Fig. 3). In contrast to other FOXO-DBD–DNA structures, the structure of the FOXO4-DBD–DNA complex suggested that the loop between helices H2 and H3 has a different conformation and participates in DNA binding as well as that both direct water–DNA base contacts and the unique water-network interactions

contribute to FOXO-DBD binding to the DNA in a sequence specific manner (Boura et al., *Acta Crystallogr D Biol Crystallogr* **66**: 1351, 2010). Next, we studied the real-time kinetics of the interaction between FOXO4-DBD and the DNA by using surface plasmon resonance (SPR) and time-resolved tryptophan fluorescence anisotropy decay measurements. We found out that the interaction between FOXO4-DBD and DNA can be described using a conformational change model, which suggests a structural change of FOXO4-DBD upon binding to the DNA. This was further confirmed by significant reduction of segmental dynamics of FOXO4-DBD upon binding to the target DNA. Moreover we showed that non-specific contacts are important for binding affinity and specificity of FOXO4 (Vacha et al., *Biophys Chem* **184**: 68, 2013).
In addition, the structural characterization of FOXO proteins, the mechanisms of DNA recognition and the role of posttranslational modifications in the regulation of FOXO DNA-binding properties were summarized in a review article as a part of a Special Issue entitled: PI3K-AKT-FOXO axis in cancer and aging (Obsil et al., *Biochim Biophys Acta* **1813**: 1946, 2011).
Supported by Grant Agency of the Academy of Sciences of the Czech Republic (grant # IAA501110801, PI: Tomáš Obšil, co-PI: Veronika Obšilová) and Ministry of Education, Youth and Sports of the CR (Centre of Neurosciences LC554) with the major involvement of group of T. Obšil and V. Obšilová and PhD student PV and a master student IZ. In collaboration with Institute of Microbiology that contributed by SPR measurements and Institute of Physics, Charles University that contributed by time-resolved fluorescence measurements.

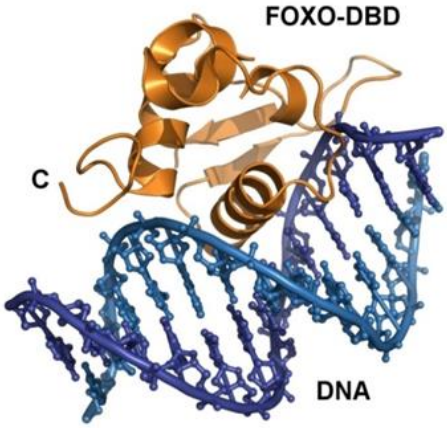


Fig. 3. The crystal structure of DNA-binding domain of forkhead transcription factor FOXO4 bound to the DNA (PDB code 3L2C) (Boura et al., Acta Crystallogr D Biol Crystallogr **66**: 1351, 2010).

d) Structural characterization of thioredoxin-binding domain of protein kinase ASK1 and its interaction with thioredoxin

We recently started a new project focused on understanding of mechanism of apoptosis signal-regulating kinase 1 (ASK1) regulation. ASK1, a mitogen-activated protein kinase kinase kinase, plays a key role in the pathogenesis of multiple diseases. Its activity is regulated through very complex mechanism involving binding to several other proteins including 14-3-3 and thioredoxin (TRX). To better understand the role of TRX binding in the regulation of ASK1, we performed a biophysical and structural characterization of the TRX1-binding domain of ASK1 (ASK1-TBD) and its complex with reduced TRX1. We showed that ASK1-TBD is a monomeric and rigid domain that forms a stable complex with reduced TRX1 with 1:1 molar stoichiometry. SAXS data revealed a compact and slightly asymmetric shape of ASK1-TBD and suggested reduced TRX1 interacts with this domain through the large binding interface without inducing any dramatic conformational change as seen in Fig.4 (Kosek et al., *J Biol Chem* **289**: 24463, 2014).

Supported by the Czech Science Foundation (grant # 14-10061S, PI: Tomáš Obšil, co-PI: Veronika Obšilová) with the major involvement of group of T. Obšil and V. Obšilová and their PhD students: DK, SK and a master student KP. In collaboration with Department of Physical and Macromolecular Chemistry that contributed by AUC measurements and Institute of Physics, Faculty of Mathematics and Physics, Charles University, Prague that contributed by the time-resolved fluorescence measurements.

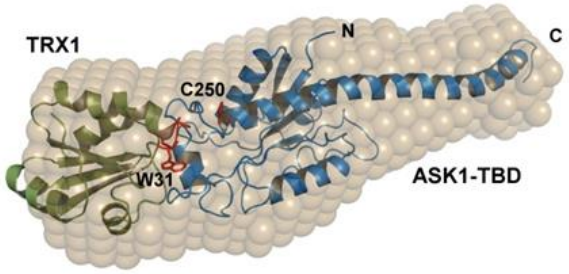


Fig. 4. Superposition of the SAXS envelope with the model of the Ask1-TBD:Trx1 complex (Kosek et al., J Biol Chem 289: 24463, 2014)

e) Mapping of the intracellular domains of TRP channels as a calmodulin/S100A1/phosphatidyl inositol-4,5-bisphosphate interaction sites.

Transient Receptor Potential (TRP) channels form a diverse family of cation channels that respond to a variety of signals. For example, some are involved in sensory perception and are directly activated by chemical ligands and/or physical sensory stimuli such as temperature, mechanical and osmotic stresses. The 28 mammalian TRP channels identified so far can be divided into several subfamilies according to their primary structure: TRPV, TRPC, TRPA, TRPM, TRPP, TRPML and TRPN. All are predicted to have six transmembrane domains with a pore region between the fifth and the sixth segment. Cytosolic N-/C-tails are responsible for regulation of TRPs, which carry binding sites for signal molecules like Ca^{2+}-binding proteins calmodulin (CaM) and S100A1 or phospholipids e.g. phosphatidyl inositol-4,5-bisphosphate (PIP2). In this project, we focused on identification of binding sites for these signal molecules in intracellular N- and C- termini of selected TRP channels using steady-state fluorescence anisotropy binding assay, surface plasmon resonance measurements and homology modeling. We determined the binding sites for CaM and S100A1 in intracellular N- and C- termini of channels TRPM3, TRPV1, TRPV2, TRPV5 and TRPC6. We described the putative binding sites for PIP2 in N-terminus of TRPM3 and C-terminus of TRPV1 and identified key amino acid residues involved in the CaM and S100A1 binding in intracellular N-terminus of channel TRPM3 (Holakovska et al., *J Biol Chem* **287**: 16645, 2012) and C-terminus of channels TRPC6 (Bily et al., *PLoS One* **8**: e62677, 2013; Friedlova et al., *Neurochem Int* **56**: 363, 2010), TRPV2, and TRPV5 (Holakovska et al., *Amino Acids* **40**: 741, 2011). We also elucidated PIP2 binding sites in N-terminus of channel TRPM3 (Holendova et al., *Channels (Austin)* **6**: 479, 2012) and in both N- and C-termini of channel TRPV1 (Grycova et al., *PLoS One* **7**: e48437, 2012). Moreover we have shown that these regions overlap with previously localized CaM/ S100A1 binding sites.

Supported by the Czech Science Foundation (grant 301/10/1159, PI: Jan Teisinger and grant P205/10/P308, PI: Lenka Grycova) with the major involvement of group of J. Teisinger, his PhD students: JB, BH, MJ, KB and L. Gryčová. In collaboration with Institute of Microbiology that contributed by MS measurements and surface plasmon resonance measurements.

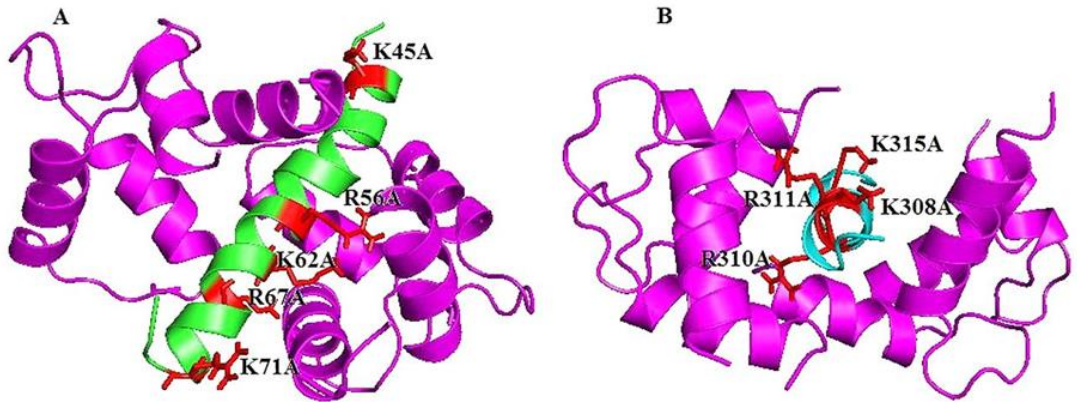


Fig. 5. Computer homology models of TRPM3 41–70 (A) in green and TRPM3 302–324 (B) in blue with Ca^{2+}-CaM in magenta; mutated basic amino acids in red. According to the homology modeling the binding domains occupy α-helical structure that runs through the central cavity of Ca^{2+}-CaM (Holakovska et al., J Biol Chem **287**: 16645, 2012).

3) INTERNAL COLLABORATION (within the Institute)

Membrane Transport: We collaborated with *Hana Sychrová* on the preparation of yeast constructs for in vivo expression experiments supported by 1 common publication (Veisova et al., *Biochem J* **443**: 663, 2012).
Cellular and Molecular Neuroendocrinology: Collaboration with *Hana Zemková* on homology modelling of P2X receptors supported by 2 common publications (Rokic et al., *Physiol Res* **59**: 927, 2010; Stojilkovic et al., *Cell Mol Neurobiol* **30**: 1251, 2010).
Cellular Neurophysiology: Collaboration with *Viktorie Vlachová* on cloning and mutagenesis of TRPV1 and TRPA1 channels supported by 5 common publications (Boukalova et al., *J Biol Chem* **285**: 41455, 2010; Boukalova et al., *Biochim Biophys Acta* **1833**: 520, 2013; Samad et al., *Biochem J* **433**: 197, 2011; Touska et al., *Curr Pharm Biotechnol* **12**: 122, 2011).

4) DOMESTIC COLLABORATION (within the country)

Faculty of Science, Charles University, Prague: cooperation with the group of prof. Tomáš Obšil (*Dept of Physical and Macromolecular Chemistry*) – close collaboration on all projects in terms of joint laboratories, access to AUC, DLS and ITC.
Faculty of Mathematics and Physics, Charles University, Prague: established collaboration with assoc.prof. Petr Heřman and assoc. prof. Jaroslav Večeř (*Institute of Physics*) – access to time-resolved fluorescence spectroscopy supported by 6 joint publications (Kosek et al., *J Biol Chem* **289**: 24463, 2014; Rezabkova et al., *J Struct Biol* **170**: 451, 2010; Rezabkova et al., *Biophysical Journal* **103**: 1960, 2012; Rezabkova et al., *J Biol Chem* **286**: 43527, 2011; Vacha et al., *Biophys Chem* **184**: 68, 2013; Veisova et al., *Biochemistry* **49**: 3853, 2010).
Institute of Microbiology CAS: established cooperation with *Lab of Molecular Structure Characterization* (Petr Novák, Petr Man and assoc. prof. Miroslav Šulc) and *Lab of Molecular Biology of Bacterial Pathogens* (Ladislav Bumba) – access to MS facilities and SPR supported by 12 joint publications (Friedlova et al., *Neurochem Int* **56**: 363, 2010; Grycova et al., *PLoS One* **7**: e48437, 2012; Haladova et al., *J Struct Biol* **179**: 10, 2012; Holakovska et al., *J Biol Chem* **287**: 16645, 2012; Kopecka et al., *J Biol Chem* **289**: 13948, 2014; Krasny et al., *J Mass Spectrom* **47**: 1294, 2012; Macakova et al., *Biochim Biophys Acta* **1830**: 4491, 2013; Rezabkova et al., *J Struct Biol* **170**: 451, 2010; Rezabkova et al., *Biophysical Journal* **103**: 1960, 2012;

Rezabkova et al., *J Biol Chem* **286**: 43527, 2011; Vacha et al., *Biophys Chem* **184**: 68, 2013; Veisova et al., *Biochem J* **443**: 663, 2012).

5) INTERNATIONAL COLLABORATION

Dr. Stanko S. Stojilkovič, NICHD, Bethesda, USA: Collaboration on modelling of P2X receptors supported by 2 common publications (Coddou et al., *Pharmacol Rev* **63**: 641, 2011; Stojilkovic et al., *Cell Mol Neurobiol* **30**: 1251, 2010)

6) KEY METHODOLOGY AND CORE FACILITIES

Following methods are used as principle tools:
Molecular biology: cloning; site-directed mutagenesis
Biochemistry: recombinant protein expression (*E. coli,* yeast); protein purification; enzyme kinetics measurements
Biophysics: steady-state and time-resolved fluorescence spectroscopy; circular dichroism (CD); analytical ultracentrifugation (AUC); dynamic light scattering (DLS); small angle X-ray scattering (SAXS); protein crystallography; hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS); surface plasmon resonance (SPR); differential scanning fluorimetry (DSF)
Structural bioinformatics: protein structure homology modelling; protein-protein and protein-small molecule docking; molecular dynamics simulations

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

List of domestic grants in which several institutes from The Czech Academy of Sciences and/or Charles University participate(d):
Neuroscience Centre - LC554; Ministry of Education, Youth and Sport (2005 -2011) - Institute of Experimental medicine CAS and Institute of Physiology CAS (two institutes, 8 laboratories in the Institute of Physiology CAS)
Centre of Physiology of Animal Cell GD305/08/H037; Grant Agency of the Czech Republic, that supported collaboration between 25 students of different subject fields in biomedicine at the level of their doctoral study programs at the Czech Academy of Sciences and Charles University in Prague.
Project of excellence in the field of neuroscience - GBP304/12/G069; Czech Science Foundation (2012-2018) - main coordinator from Institute of Physiology - (four institutions, 8 laboratories in the Institute of Physiology CAS)

8) OTHER RELEVANT INFORMATION

Postdoctoral stays and prizes of students that defended their Ph.D. at our department, Institute of Physiology CAS.
Lenka Řežábková, Ph.D. (at our department till 2012) - **prize of dean and prize of rector** for the best master thesis (2010); Ph.D. defended in 2012; now postdoctoral researcher at Paul Sherrer Institute, Villigen, Switzerland)
Hana Janoušková, Ph.D. (at our department till 2013) - Ph.D. defended in 2013; now postdoctoral researcher at Institute for Research in Oncology, Bellinzona, Switzerland; **Medal of Josef Hlavka** for the best Ph.D. thesis in 2nd Medical faculty (2014)

9) SUMMARY AND RESEARCH IMPACT

Almost all cellular functions are dependent on signal transduction pathways which relay signals from outside of the cell to the inside. Signalling pathways are highly

complex and involve many proteins that interact with each other and through these tightly regulated binding interactions modulate their functions. Defects or dysregulation of signalling pathways are responsible for the pathogenesis of many diseases including cancer or diabetes. Main goal of our research is to provide better understanding of structure-function relationship of selected proteins and protein complexes involved in biomedically relevant signalling pathways. For example, FOXO forkhead transcription factors are important tumor suppressors, protein kinase ASK1 is involved in cancer, diabetes, cardiovascular and neurodegenerative diseases, TRP channels are involved in neurodegenerative disorders. We use various biochemical and biophysical approaches to study structure, function, and interactions of selected proteins with the goal to understand molecular mechanisms of their regulation. This report also shows that we solve relevant experimental questions attractive to young students and that the education and skills they learn in our laboratory allow them to gain competitive positions in respected foreign laboratories.

During the period under the review 2010-2014, we have been working on several projects and achieved many interesting and important results:

- *Structural studies of the 14-3-3 protein complexes*–These studies provided structural insight into mechanisms of the 14-3-3 protein-mediated regulation of yeast neutral trehalase Nth1, regulator of G protein signalling 3 (RGS3) and phosducin.
- *Structural characterization of the interaction between DNA-binding domain of transcription factor FOXO4 and DNA*–These studies enabled detailed understanding of interaction between FOXO4-DBD and its target DNA.
- *Structural study of the thioredoxin-binding domain of ASK1 kinase and its interaction with thioredoxin*–This study provided structural basis of the interaction between ASK1 and reduced thioredoxin.
- *Characterization of interaction between cytoplasmic domains of TRP channels and their binding partners*–These studies provided important structural insight into the regulation of selected TRP channels through interaction with signalling molecules CaM, S100A1 and PIP2.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Developmental Epileptology

Report on the scientific activity of the team in 2010–2014

1) TEAM DESCRIPTION

RESEARCH FOCUS

Our long-term research goals are (1) to better understand the pathophysiology of epilepsy, (2) to develop new approaches to epilepsy treatment and (3) to improve diagnostic tools for epilepsy.

Our research plan is complex, so we have adopted an interdisciplinary approach involving close collaboration with experts in various research fields including mathematics, physics and drug development.

Composed primarily of basic scientists, our department focuses on developmental aspects of epilepsy and age-specific epilepsy treatments. In all age groups, we study mechanisms of ictogenesis, epileptogenesis and epilepsy related comorbidities. Also, in close collaboration with clinical epilepsy centers, we work to develop new diagnostic techniques for epilepsy. Beyond basic research, we work to a limited extent with the pharmaceutical industry to search for age-specific anti-seizure drugs and to ameliorate the potential adverse side effects of these drugs.

Our primary research topics are:

- Mechanisms of ictogenesis, epileptogenesis and epilepsy-related comorbidities in mature and immature brain
- Development of new diagnostic techniques for epilepsy
- The long term impact on brain development of early pharmacological intervention in neurotransmitter systems**.**
- The role of oxidative stress in the pathogenesis of epilepsy and seizures during the development
- Developmental pharmacology of classical and potential anti-seizure drugs

PERSONNEL

Our research team is comprised of four senior scientists: Dr. Kubova, head of the department, Dr. Otahal, Dr. Mikulecka and Dr. Jiruska, who joined the department in 2012; one junior scientist, Mgr. Tsenov; and two emeritus scientists, Dr. Folbergrova, Dr. Druga (part time) and Dr. Mares. The team is multidisciplinary and its members have different expertise. Both pre- and post-graduate students are actively involved in research projects and our scientists regularly mentor Bachelor, Master and Doctoral theses. Within the last five years, the department hosted the following students and

fellows: Erasmus student Jeni Virta (University Turku, Finland; 2 mo), Fulbright student Thuy Hua (Occidental College, Los Angeles, USA; 1y), post-doc Cecilia Zavala Tecuapetla (Ciudad de Mexico, Mexico; 2y) and Kerry Thompson, PhD (Occidental College, Los Angeles, USA) who spent his 6 mo sabbatical in our department.

The quality of our research team and its achievements are reflected by the following awards to its members: The Jan Evangelista Purkyně Honorary Medal for Merit in the Biomedical Sciences to Dr. Folbergrova (2012) and Prof. Mares (2013); and the Jessenius prize for contribution to medical research, Czech Patient Union, (2014) to Dr. Jiruska. In addition, Prof. Mares and Prof. Druga are members of the Czech Medical Academy (FCMA)

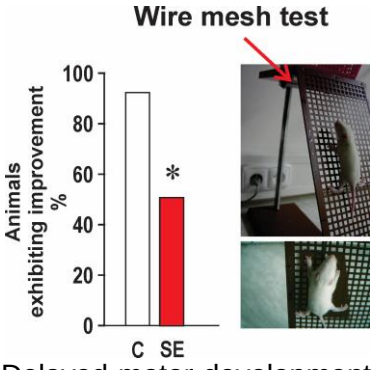
Members of the team:

- Hana Kubova, PharmD, DSc; associate professor, Charles Uviversity, Czech Republic and University of Eastern Finland, Kuopio, Finland (senior scientist, head of department, 56y, pharmacology, developmental epileptology, morphology, h-index 23),
- Jakub Otahal, MD, PhD; associate professor, Charles University, Czech Republic (senior scientist,40y, h-index 10),
- Premysl Jiruska, MD, PhD; associate professor, Charles University, Czech Republic (senior scientist, 39y, h-index 9) (team member since 1/2012)
- Anna Mikulecka, PhD (senior scientist, 66y, behavior, ethology, h-index 11),
- Grygoriy Tsenov, PhD (junior scientist, 35y, electrophysiology, developmental epileptology, h-index 5),
- Pavel Mares, MD, DSc, professor, Charles University (emeritus, 78y, developmental epileptology, developmental pharmacology, h-index 30),
- Jaroslava Folbergrova, DSc (emeritus, 82y, biochemistry, h-index 35)
- Rastislav Druga, MD, DSc Charles University (part time, emeritus, neuroanatomy, 75y, h-index 18)
- PhD/MSc/BSc students (16/39/4), 7 PhD students defended their thesis
- laboratory assistants (3; tissue processing + histology/electrophysiology + animal surgery + tissue preparation)

2) KEY RESULTS

Mechanisms of ictogenesis, epileptogenesis and epilepsy-related comorbidities in mature and immature brain.

Age-related effects of status epilepticus: We have performed studies on age-related effects of status epilepticus (SE). SE is known to have different effects at different stages of development and we have demonstrated that these effects begin in infancy where SE produces hippocampal damage in 12-day-old rats (Druga et al, *Brain Res.* **1355:** 174-179, 2010). Furthermore, as the age at which animals are subjected to SE increases, so does the resulting damage. Regardless of the age at which animals experience SE, they later in life exhibit chronic epilepsy, cognitive deficits and atrophy of temporal lobe structures. Notably, however, we have shown that regardless of age, the severity of hippocampal damage correlates with the extent of cognitive impairment but not with the severity of epilepsy. (Kubova and Mares, Neuroscience **235:** 232-249, 2013; paper awarded by the Czech League Against Epilepsy).



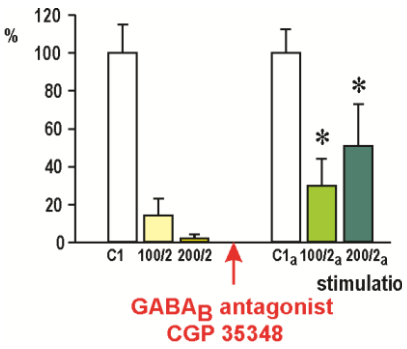
Delayed motor development in animals with early onset epilepsy

Book chapters: Kubová, H, In: *Developmental Neurotoxicology Research: Principles, Models, Techniques, Strategies, and Mechanisms*, Experimental models of epileptogenesis. Eds. Wang C, Slikker, W, Jr. (2011), pp 581-601; Kubova H et al, In *Pediatric Epilepsy Surgery. Advances and Technical Standards in Neurosurgery.* New insight on the mechanisms of epileptogenesis in the developing brain. Eds. Akalan, N and Di (2012) pp 3-44.

Support: Czech Science Foundation (GACR) P302/10/0971 and P304/12/G069 and Ministry of Education (KONTAKT II - collaborative US-Czech grant) ME08045 (HK)

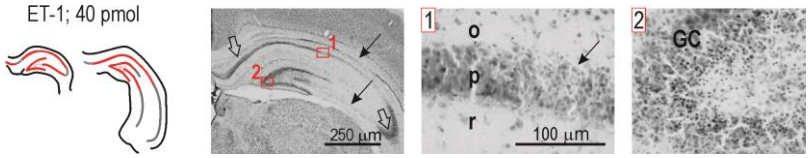
Age dependent properties of post-seizure refractory period: In adult animals, there is a refractory period immediately following a seizure during which it is more difficult to elicit a new seizure. We have shown that this refractoriness is due, at least in part, to activation of $GABA_B$ receptors (Mares and Kubova, *Neuropharmacology* **88:** 99-102, 2015, Epub 2014 Sep 16). The refractory period could be suppressed by a $GABA_B$ antagonist but not by a $GABA_A$ antagonist. This result is consistent with the ineffectiveness of a $GABA_A$ receptor antagonist in a model of cortical epileptic afterdischarges (Tabashidze and Mares, *Brain Res.* **1412:** 102-107, 2011). Furthermore, we have studied the maturational development of refractoriness and demonstrated that it is absent in 12-day-old rats and only appears later during the course of the third postnatal week (Mares and Kubova, *Epilepsia* **56:** e10-e14, 2015, Epub 2014 Dec 3). These findings at least partly explain the high incidence of SE in infants.

Support: Czech Science Foundation (GACR), No. P302/10/0971 and P304/12/G069 and Ministry of Education of the Czech Republic No.LH11015 (HK, PM)



$GABA_B$ receptor antagonists partially suppresses postictal refractoriness in juvenile rats.

Role of endothelin receptors in focal ischemia and acute seizures: Using the model of focal cerebral ischemia induced by the intrahippocampal infusion of endothelin-1 (ET-1) in 12-day-old rats, we examined the role of the endothelin receptors in the development of focal ischemia, symptomatic acute seizures and neurodegeneration. Our results indicate that the activation of ETA receptors is crucial for the development of ischemia, but that either ETA or ETB receptor can mediate the development of seizures following the application of ET-1 in immature rats. The dissociation between the ischemia-producing and seizure-producing processes suggests that damage is not necessary to induce seizures, although it may exacerbate them. (Tsenov et al, Exp.Neurol. **265:** 40-47, 2015, Epub 2014 Dec 24).



Hippocampal damage induced by the intrahippocampal infusion of ET-1 in immature animals (schematic drawings and microphotographs of Nissl-stained sections).

Support: Czech Science Foundation (GACR) P304/11/P386 and P304/12/G069 (GT)

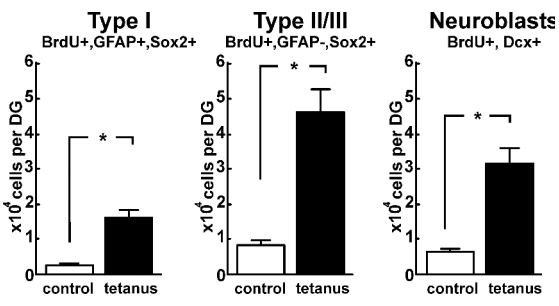
Team's contribution: Experiments were designed and performed in Dpt. Developmental Epileptology. J. Burchfiel, Ph.D (Medical Center, University of Rochester) participated in EEG analysis and data interpretation

Role of nNOS in status epilepticus and early epileptogenic event. We performed studies demonstrating that the administration of L-NPA, a highly selective nNOS inhibitor, delayed the onset and reduced the severity of KA-induced SE. There were reductions in the following perameters: duration of convulsions, the power of the gamma-band EEG associated with seizure severity, the frequency of epileptiform spikes, and the expression of c-Fos. These findings suggest that nNOS is involved in seizure susceptibility and in the manifestations of convulsive seizures in the KA model. In the early epileptogenic period, L-NPA treatment reduced the frequency of epileptiform spiking, suppressed synaptophysin expression in the dentate gyrus and suppressed gliosis in the CA3 region of the hippocampus. These observations suggest a potential disease-modifying action of L-NPA on the epileptogenic process by modulating the initiating event. (Beamer et al, *Eur. J. Neurosci.* **36:** 3194-3203, 2012).

***Support*:** Czech Science Foundation (GACR) GAP303/10/0999 (JO)

***Team's contribution*:** Electrophysiological part of this study was performed in our department by Ed Beamer under supervision of Dr. Otahal.

Neurogenesis in non-lesional temporal lobe epilepsy Temporal lobe epilepsy (TLE) alters adult neurogenesis and it has been suggested that it plays an important role in both the pathophysiology of TLE and the associated cognitive decline. We demonstrated that repeated spontaneous brief temporal lobe seizures are sufficient to promote increased hippocampal neurogenesis in the absence of status epilepticus and severe cell loss. These results provide a potential rationale for



Increased precursor cell proliferation-survival (Type I-III) and neurogenesis in tetanus toxin model of temporal lobe epilepsy.

developing pharmacological strategies directed at modulating neurogenesis as a treatment for reversing learning and memory deficits in TLE. (Jiruska et al, *Neurobiol. Dis.* **54:** 492-498, 2013).

Support: Czech Science Foundation (GACR P303/10/0999) (PJ)

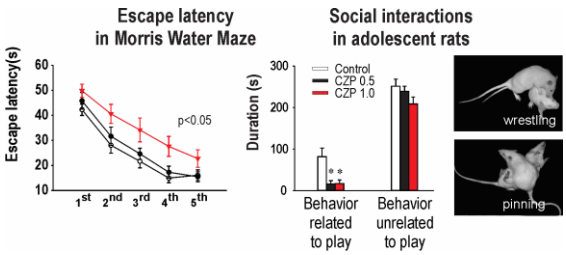
Team's contribution: Dr. Jiruska designed and performed experiments and data analysis.

Cellular and network mechanisms of cognitive decline*:* Intellectual disability affects 2–3% of the population and mutations of the X-chromosome are a major cause of moderate to severe cases. We brought an innovative observation that links molecular consequences of the mutation to impaired cognitive function. Our results demonstrate that abnormal neurotransmission is responsible for failure to generate brain oscillations required for normal cognitive functions. Furthermore, these findings raise the possibility of drug treatments for affected individuals and patients with cognitive decline associated with other neurological disorders including epilepsy. (Powell et al, *PLoS ONE* **9:** e95871, 2014).

Team's contribution: Dr. Jiruska participated in experiments and data analysis.

Long term impact of early pharmacological intervention in neurotransmitter systems on brain development.

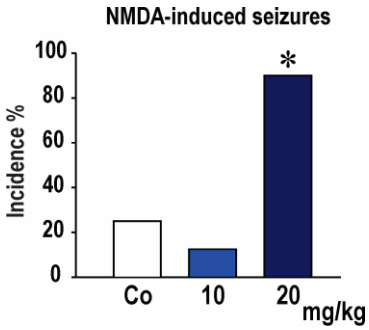
Enduring effects of early benzodiazepine administration: In our program on developmental pharmacology we studied both acute and long term effects of enhancement of GABAergic inhibition in neonatal animals. Although our data did not reveal any qualitative differences in the immediate effectiveness of benzodiazepines (BZD) between immature and mature brain (Mikulecka et al, Epilepsy Behav. **20:** 12-19, 2011; Kubova and Mares, *Physiol.Res.* **61:** 319-323, 2012), we demonstrated that exposure to BZD during infancy produced persistent alterations in multiple brain functions later in life including impaired cognitive abilities, disturbances in emotional/motivation responsiveness and deficits in social behavior. (Mikulecka et al, Front. Behav. Neurosci. **8:**101, 2014; Front. Behav. Neurosci. **8:**169, 2014). These findings suggest that early exposure to prescription medication may increase the risk of later-appearing behavioral deficits or even psychiatric symptoms.



Early benzodiazepine exposure leads to the permanent impairment of cognitive and social functions

***Support*:** Czech Science Foundation (GACR) 305/09/0846 and P304/12/G069 (HK)

Enduring effects of early caffeine exposure: We have demonstrated that neonatal exposure to caffeine, an adenosine receptor antagonist, can have persistent effects on neuronal excitability and sensitivity to seizure-inducing processes. Animals receiving caffeine in early development showed the following alterations later in life: (1) Seizure threshold was reduced to glutamate receptor agonists, kainic acid and N-methyl-D-aspartate (Tchekalarova et al, *Epilepsy Res.* **88:** 231-238, 2010a). (2) Following the administration of pentylenetetrazol, minimal clonic seizures were potentiated (Tchekalarova et al, *Pharmacol.Rep.* **65:** 847-853, 2013), whereas the incidence of non-convulsive seizures (a model of human absence seizures) was decreased (Tchekalarova et al, *Epilepsy Res.* **88:** 231-238, 2010a). (3) The threshold was increased for elicitation of epileptic afterdischarges by electrical stimulation of cerebral cortex (Tchekalarova et al, *Brain Res.* **1356:** 121-129, 2010b). Some of these changes were present even in adult animals (Tchekalarova et al, *Pharmacol.Rep.* **65:** 847-853, 2013). These results indicate that agents administered early in life can have significant and complex effects on later life seizure susceptibility.



Enduring alteration of seizure susceptibility in animals exposed to caffeine early in life

***Support*:** Ministry of Health (IGA MZd) NR/9184-3, Ministry of Education (KONTAKT II - collaborative US-Czech grant) LH11015 (PM)
***Team's contribution*:** Experiments were designed by Dr. Mares (Dpt. Developmental Epileptology). Dr. Tchekalarova (visiting scientist, Bulgarian Academy of Sciences) performed experiments during her visits of Dpt. Developmental Epileptology.

The role of oxidative stress in pathogenesis of epilepsy and seizures during the development:

1) We have studied the role of oxidative stress in immature brain during the acute phase of seizures and also during the long periods of survival (up to 5 weeks) following seizures. The widely-held opinion has been that oxidative stress during seizures is age-dependent and it does not occur in immature brain. We have demonstrated for the first time that oxidative stress does occur in immature brain during seizures and it is apparently due to both increased free radical production (Folbergrová et al., *Exp. Neurol* **233:** 421-429, 2012) and limited antioxidant defenses (Folbergrová et al., *Int.J.Devl. Neuroscience* **31:** 123-130, 2013; Folbergrová, *Physiol.Res* **62 (Suppl.1):** S39-S48, 2013).
2) We have demonstrated mitochondrial dysfunction in immature brain during and following seizures.There was pronounced inhibition of mitochondrial complex I activity, which persisted during long periods of survival, corresponding to the period of epileptogenesis (Folbergrová et al., *Neurochem. Int.* **56:** 394-403, 2010). This inhibition was associated with significant increases of three mitochondrial markers of oxidative damage: 3-nitrotyrosine, 4-hydroxynonenal and protein carbonyl groups. The findings suggest that oxidative modification of complex I is very likely responsible for the sustained deficiency of complex I activity. The persisting inhibition of complex I may lead to the enhanced production of ROS and/or RNS, contributing not only to neuronal injury, but also to epileptogenesis. The increased production of superoxide anions and the

inhibition of complex I activity could be completely suppressed, or at least substantially attenuated, by scavengers of free radicals, particularly by SOD mimetics (Folbergrová et al., *Neurochem. Int.* **56:** 394-403, 2010; *Exp. Neurol* **233:** 421-429, 2012). Our findings suggest that substances with antioxidant properties, combined with conventional therapies, might be a beneficial treatment for epilepsy.

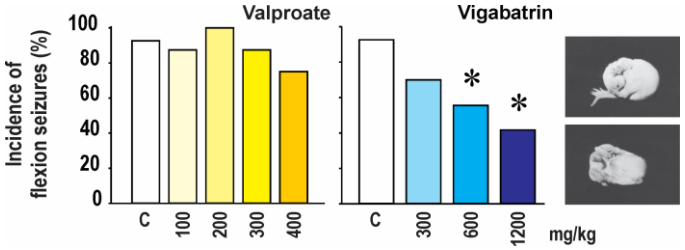
Review*:** Folbergrová and Kunz, *Mitochondrion* **12**: 35-40, 2012; ***Otahal et al, Int. Rev. Neurobiol **114**: 2014

***Support*:** Czech Science Foundation (GACR) GA309/05/2015, 309/08/0292 and GAP303/10/0999, Ministry of Education IM683 780 5002 and ERC CZ LL 1204 from MEYES CR (JF, JO)

***Team's contribution*:** Experiments were designed and performed in Dpt. Developmental Epileptology. Members of Dpt. Bioenergetics (Dr. Jesina, Dr. Houstek) performed measurement of the content of mitochondrial enzymes and participated in data interpretation.

Developmental pharmacology and pharmacology of anti-seizure drugs:

We have shown that the effects of drugs affecting GABA receptors can be both model-specific and age-specific. In immature rats, vigabatrin (an inhibitor of GABA catabolism) suppressed NMDA-induced flexion seizures, which is a model of age dependent infantile spasms. On the other hand, valproate was ineffective against these seizures. Both drugs, however, at least partially suppressed generalized tonic-clonic seizures in this model. These results suggest that NMDA-induced flexion seizures can be used to screen for age-specific anti-seizure drugs (Kubova and Mares, *Epilepsia* **51:** 469-472, 2010). Other studies demonstrated that the following drugs were more effective in seizure models in immature animals than in adult animals: (1) The neuroactive steroid, ganaxolon, a positive allosteric modulator of GABAA receptors, is more efficient in blocking cortical epileptic afterdischarges – a model of myoclonic seizures (Mares and Stehlikova, *Neurosci. Lett.* **469:** 396-399, 2010). (2) Valproate and its derivatives were more efficient in suppressing pentetrazol-induced seizures (Mares et al, *Epilepsy Res.* **106:** 64-73, 2013). (3) The positive allosteric modulator of $GABA_B$ receptors, CGP7930, was more efficient in both models (Mares *Epilepsy Res.* **100:** 49-54, 2012, Mares et al, *Epilepsy Behav.* **28:** 113-120, 2013).



Vigabatrin suppresses NMDA-induced flexion seizures in a model of infantile spasms

***Support*:** Czech Science Foundation (GACR) 305/06/0713, P304/10/1274, and Ministry of Education LC554 (PM, HK)

Anticonvulsant effects of 5% CO_2: CO_2 has long been recognized for its anticonvulsant action. In our multicenter study, we demonstrated that it suppressed electrically induced cortical afterdischarges in rats and macaques, and bicuculline-induced epileptiform activity in macaques. In a pilot study carried out in patients, inhalation of 5% CO_2 rapidly terminated electrographic seizures, suggesting that medical carbogen with 5% CO_2 could be used as an acute treatment in epileptic patients.(Tolner et al, Epilepsia **52:** 104-114, 2011)

Support: Ministry of Education LC554 (HK)

Team's contribution: Dr. Tolner performed and evaluated experiments with cortical afterdischarges in rats in Dpt. Developmental Epilepsy under supervision of Dr. Kubova.

Development of new diagnostic techniques for epilepsy: This study examined the significance of high-frequency oscillations in brain electircal activity (a proposed marker of epileptogenic tissue) in the presurgical diagnosis of neocortical epilepsy. The results demonstrated that favourable outcome was associated with resection of neocortex in which there was a high rate of these high-frequency oscillations. The major advantage of this approach is that it is highly unbiased, patient-oriented and compensates for high inter-patient variability. Therefore, it can be implemented prospectively into presurgical examination (Cho et al. *Epilepsia* **55:** 1872-1883, 2014).

Editorial: P. Jiruska was one of editors of *International Review of Neurobiology*, Vol. 114, Modern Concepts of Focal Epileptic Networks (2014)

Support: Grants from Neuron Fund for Support of Science (Czech Republic, 2012/10) and the Ministry of Health of the Czech Republic (IGA NT/14489-3) (PJ).

Team's contribution: Dr. Jiruska was involved in patients' data analysis

3) **INTERNAL COLLABORATION (within the Institute)**

- Collaboration with Dpt. Adipose tissue biology (1 *joint paper*)
- Collaboration with Dpt. Neurophysiology of Memory (1 *joint grant, 2 joint papers, common training of pre- and postgradual students, support in electrophysiological methods,*)
- Collaboration with Dpt. Epithelial physiology (*1 joint paper*, 1 *common grant*)
- Collaboration with Dpt. Bioenergetics (2 *joint papers and common grant*)

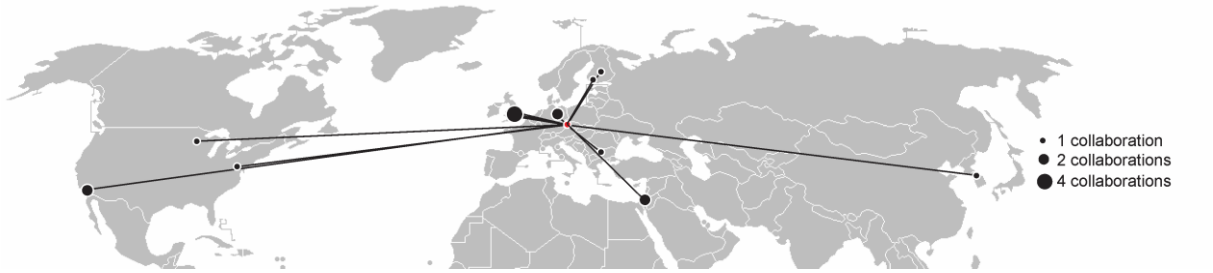
Participation in centers of excellence coordinated by our Institute:
Centre of Neurosciences (LC554)
Centre of Physiology of Animal Cell (GD305/08/H037)
Centre of Project of excellence in the field of neuroscience (GBP304/12/G069)

4) **DOMESTIC COLLABORATION (within the country)**

- ***Members of department chair or co-chair three OPPK projects NeuroImage, Biomodels and Brain view***
- Faculty of Electrical Engineering, Czech Technical University in Prague
- 1st Faculty of Medicine, Charles University in Prague, Institute of Anatomy (*1 joint paper*)
- 2nd Faculty of Medicine, Charles University in Prague, Motol University Hospital (*1 joint paper and 3 proceedings papers, joint grant and informal collaboration*),
- 3rd Faculty of Medicine, Charles University in Prague, Department of normal, pathological and clinical physiology (*pharmacology of cortical epileptic phenomena in developing brain* - 4 *joint papers*)
- Institute of Computer Science, Academy of Sciences of Czech Republic (joint grant),
- Member of the team is one of the founding members of Intracranial Signal Analysis Research Group Prague http://isarg.feld.cvut.cz/index.html

- Institute of Organic Chemistry and Biochemistry (*1 joint paper and patent CZ302050; 2010*)
- University of chemistry and technology, Institute of Chemical Technology Assoc. Laboratory of Medicinal Diagnosis (*Prof. Kačer – development of analytic methods - informal joint project*)
- The Military University Hospital Prague, Department of Neurosurgery *(Dr. Ostry,– 1 joint paper; Dr.Vanek cerebrospinal fluid dynamics after brain injuries - informal joint project)*
- Czech Technical University, Faculty of electrical engeneering department of graphics and computer vision *(Ing Sporka; electromyographic approach for text input into the computer and control of myoelectric prosthesis - common project TextAble)*

5) INTERNATIONAL COLLABORATION



- Prof. M. Bialer, Hebrew Univ Jerusalem, Inst Drug Res, Sch Pharm, Fac Med, Israel (*developmental pharmacology of new antiepileptic drugs -1 joint paper*)
- Prof. K. Kaila, University of Helsinki, Finland (*common study „anticonvulsant effect of CO_2" - 1 joint paper*)
- Prof. Asla Pitkanen, University of Eastern Finland, Kuopio, Finland (*long lasting effects of SE and epileptogenesis in immature brain 1 joint paper*)
- Prof. C. Wasterlain, Brain Research Institute, UCLA, LA, California, USA (*collaborative project - White matter injury as a consequence of status epilepticus in immature rats, Ministry of Education*)
- Prof. R. Sankar, David Geffen School of Medicine at UCLA, LA, California, USA (*collaborative project - Study of immediate changes after epileptic seizures elicited at different stages of postnatal development, Ministry of Education*)
- Dr. J. Tchekalarova, Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria (*visiting scientist*, *6 joint papers; enduring effects of caffeine exposure during early postnatal life*)
- Prof. John. G.R. Jefferys Department of Pharmacology, University of Oxford, United Kingdom (studies on the mechanisms of seizure genesis, cognitive decline; *6 joint papers*)
- Prof. Eckehrad Scholl, Institut für Theoretische Physik, Technische Universität Berlin, Germany *(Collaborative Research Center SFB 910, joint project on the role of synchronization during seizures)*
- Prof. Brian Litt, Dept. of Bioengineering, University of Pennsylvania, *USA (work on International EEG Database, www.ieeg.org)*

- Prof. Annette Dolphin, Neuro, Physiology & Pharmacology, University College London, United Kingdom *(1 joint paper, the role of calcium channel subunits in pathophysiology of epilepsy)*
- Prof. Attila Sik, School of Clinical and Experimental medicine, University of Birmingham, United Kingdom *(1 joint paper, functional and structural effect on tetanus toxin on Vesicle Associated Membrane Proteins)*
- Prof. Gregory Worrell, Mayo Clinic, USA *(1 joint paper, development of spike detector)*
- Prof. Heung Song Bong, Samsung Medical Center, Sungkyunkwan University School of Medicine, Korea *(1 joint paper, study on neocortical high-frequency oscillations and their role in presurgical evaluation)*
- Prof. William P. Gray, National Institute of Neuroscience and Mental Health Research, United Kingdom *(1 joint paper, study focused on the abnormal neurogenesis in temporal lobe epilepsy)*
- Dr. Richard Kovacs, Charite Berlin, Institute for Physiology, Germany (*common project "Vulnerability of inhibitory basket cells to oxidative stress during maturation and its impact on schizophrenia and epilepsy", 1 joint papers*)
- Dr.Nicola Maggio, Talpiot Medical Leadership Program, The Chaim Sheba Medical Center, Tel HaShomer, Israel (*1 joint paper*)

6) KEY METHODOLOGY AND CORE FACILITIES

Our department possesses state-of-the-art multichannel video/EEG monitoring system, systems for in vitro and in vivo electrophysiology, a behavioral laboratory with top-ranking software for behavioral analysis (Noldus, Netherlans), Laser Doppler flowmeter (PeriFlux 5000, Perimed), Oxyprobe (OxyLab, OxfordOptronic) and equipment for tissue processing (cryocut Leica 180, rotary microtome Leica RM2245, glow box -80C. We also maintain microscopic systems (microscope Olympus AX70 with fluorescence, cameras and Cell*Imaging Software for image analysis), a system for microdyalysis (m-dialysis and CMA microdialysis, Sweden). There is a ***NeuroImage*** laboratory (OPPK project, coordinator Dr. Kubova) equipped with a microscopic system for stereology, density measurement and neuronal structure analysis (MBF Bioscience) and *Laboratory of behavioral analysis* (part of project ***Biomodels,*** OPPK project, coordinator Dr. Kubova) equipped with Phenotyper (Noldus, Netherlands) and software for behavioral analysis (Biobserve, Germany).

7) INVOLVEMENT IN SIGNIFICANT PROJECTS

Centre of Neurosciences (LC554)
Centre of Physiology of Animal Cell (GD305/08/H037)
Centre of Project of excellence in the field of neuroscience (GBP304/12/G069)

8) OTHER RELEVANT INFORMATION

Collaboration with business sector: Research grant from UCB BiopharmaSPRL; *Anticonvulsant effects of potential antiepileptic drug in immature animals.*

9) SUMMARY AND RESEARCH IMPACT

During 2010-2014, the Department of Developmental Epileptology contributed substantially to the understanding of mechanisms of seizure generation and termination in immature animals. Our long term studies showed that

pharmacological interference with inhibitory systems during critical periods of early postnatal development (enhancement of GABAergic and antagonism of adenosinergic inhibition) leads to persistent behavioral deterioration and changes of brain excitability. We demonstrated age-dependent changes in the time course and pattern of chronic epilepsy developing after status epilepticus. In addition, we showed that status epilepticus produced significant behavioral impairments later in life and that these impairments correlated with the severity of structural damage. Our studies have substantially contributed to elucidating of the role of oxidative stress and mitochondrial dysfunction in immature brain during experimentally-induced seizures. This work suggests that substances with antioxidant properties, in combination with conventional therapies, might be a beneficial treatment for epilepsy

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Developmental Cardiology

RESEARCH FOCUS

Ischaemic heart disease is the main cause of mortality worldwide. We study cardiac tolerance to injury caused by acute oxygen deprivation from the molecular level to the whole organism using animal models. Our research is focused mainly on the study of mechanisms that underlie:

a) increased cardiac tolerance to injury conferred by adaptation to chronic hypoxia and regular exercise training,

b) high cardiac tolerance during early ontogeny and permanent consequences of early developmental interventions for cardiac tolerance in adulthood,

c) and altered cardiac tolerance associated with various pathological states.

Ad a) Cardioprotective mechanism of chronic hypoxia and exercise

Prolonged exposure to hypoxic environment or regular exercise training leads to adaptation which is associated with improved cardiac tolerance to acute oxygen deprivation. We study molecular mechanisms underlying the long-lasting cardioprotective effects of chronic hypoxia and exercise on various manifestations of ischaemic injury.

Chronic hypoxia is the main pathophysiological feature of several disease states, but it also occurs naturally in high-altitude residents or during development *in utero*. Organisms react to hypoxia by activating various adaptive responses aiming to compensate the lack of oxygen and maintain homeostasis.

Already in the late 50th of the last century, epidemiological observation indicated that the incidence of ischaemic heart disease is much lower in high-altitude dwellers than in lowlanders. At the same time, experimental studies proved that the hearts of rats adapted to chronic hypoxia has improved tolerance to injury caused by acute ischaemia or anoxia. Precise molecular mechanism underlying the cardioprotective effects of chronic hypoxia is still unclear; only recently we began to reveal step by step some factors that play a role. In view of the fact that the protected cardiac phenotype persists long after the cessation of the stimulus, understanding its mechanism may potentially contribute to a development of new strategies in prevention and therapy of ischaemic states. Our recent and ongoing projects focus mainly to the role of following factors: mitochondrial potassium channels (K_{ATP} and BK_{Ca}), reactive oxygen and nitrogen species, pro-inflammatory and anti-inflammatory cytokines (TNF-α, IL10), protein kinase C (isoforms δ a ε) and β-adrenergic and a opioid receptors.

Similarly as chronic hypoxia, regular physical activity also affords long-lasting cardiac tolerance to acute ischaemic injury. The aim of our new project is to find out, whether these two cardioprotective phenomena utilize the same or different molecular mechanisms and whether the salutary effects of chronic hypoxia can be further potentiated by regular exercise (running on a treadmill).

Ad b) Developmental aspects of cardiac ischaemic tolerance

The immature heart is highly tolerant to ischaemic injury, but chronic oxygen deficit during early ontogeny may have negative consequences that persist till adulthood. We study mechanisms responsible for developmental changes of cardiac ischaemic tolerance and for late consequences of hypoxia acting during prenatal and early postnatal life.

Cardiac tolerance of the immature heart to acute oxygen deficiency is significantly higher as compared with the adult myocardium. The developmental changes are sex-dependent: cardiac tolerance is similar in males and females up to the end of the weaning period; the adult female heart is, however, more resistant to oxygen deprivation than the male heart. The mechanisms of the higher resistance of the neonatal heart to oxygen deprivation have not yet been satisfactorily clarified. Human epidemiological studies have shown a clear association of adverse perinatal hypoxic environment and increased risk of ischaemic heart disease in later adult life. Experimental studies on late effects of hypoxia have confirmed the clinical data: the cardiac muscle of males exposed perinatally to chronic hypoxia is less tolerant to ischaemic injury as compared with control animals. We focus mainly on the analysis of mechanisms determining sex-dependent late myocardial effects of hypoxia experienced in early life.

The importance of developmental approach for experimental and clinical cardiology is indisputable. It offers new possibilities in studies of pathogenesis, prevention and therapy of serious cardiovascular diseases. Retrieval of developmental mechanisms participating on changes in cardiac tolerance to hypoxia/ischaemia is the best example for this view. The experimental results may be utilized in the clinical practice, particularly in paediatric cardiology and cardiac surgery.

Ad c) Cardiac ischaemic tolerance in diseased states

Systemic hypertension, dyslipidemia and diabetes are major risk factors of ischaemic heart disease and its acute form, myocardial infarction. These pathological states diversely interfere with mechanisms that protect the heart against acute oxygen deprivation. We study the effects of various forms of hypertension on the heart function and ischaemic tolerance.

The rapid progress in molecular biology and genetics enabled to create many experimental animal models, which are suitable for analysis of individual factors participating in the development of systemic hypertension. However, their effects on myocardial functions and cardiac ischaemic tolerance are not yet clearly defined. Recently, the role of these factors was revealed partially, suggesting that not only impaired cardiac ischaemic tolerance but also activation of cardioprotective mechanisms may occur in hypertensive hearts.

Our ongoing projects involve genetic models of systemic hypertension and hypertension models of renal origin. The aims of these projects are to clarify the role of following factors in cardiac ischaemic tolerance: mitochondrial genome, epoxyeicosatrienoic acids and other arachidonic acid metabolites, reactive oxygen and nitrogen species, C reactive protein and pro-inflammatory cytokines. We also investigate effects of chronic hypoxia on cardiac ischaemic tolerance in rats with systemic hypertension in order to find out whether different or the same molecular mechanisms identified earlier in normotensive individuals are involved.

KEY RESULTS

Cardioprotective mechanism of chronic hypoxia

We showed that long-term adaptation of rats to chronic continuous normobaric hypoxia (without any periodic exposure to room air) protected against lethal myocardial injury caused by the acute ischaemia/reperfusion (I/R) insult. This protective effect was demonstrated both in the open-chest model of myocardial infarction and in freshly isolated ventricular myocytes. However, the tolerance to different end points of heart injury developed in a reciprocal manner.

While the infarct size-limiting effect of chronic hypoxia needed several weeks to develop, the susceptibility to ventricular arrhythmias was most pronounced in the early phase of adaptation, vanishing with its prolongation. The considerably delayed appearance of the protective effect of chronic hypoxia against infarction suggests that this phenomenon is not just a form of hypoxic pre-conditioning. Another novel observation was that daily interruption of the hypoxic exposure with 60-min normoxic episodes blunted the cardioprotection likely by a mechanism, which attenuated antioxidant defence and resulted in oxidative stress (Neckář et al., *Curr Pharm Des* **19**: 6880, 2013).

Further attention was paid to a dual role of reactive oxygen species (ROS), which on one hand contribute to the development of myocardial I/R injury and on the other hand activate adaptive responses resulting in the improved ischaemic tolerance of chronically hypoxic hearts as we showed earlier. ROS can modulate ischaemic tolerance by affecting a number of redox-sensitive signalling components including mitochondrial potassium channels. We focused on voltage-dependent calcium-activated potassium channels with high unitary conductance (BK_{Ca}) that are located in the inner mitochondrial membrane and mediate the influx of potassium into the matrix (reviewed in Kolář, in: Pierce, Mizin, Omelchenko, eds. *Advanced Bioactive Compounds Countering the Effects of Radiological, Chemical and Biological Agents*, Dordrecht: Springer, pp. 163-175, 2013). Using isolated ventricular myocytes exposed to a simulated I/R in the presence of selective pharmacological activators and inhibitors, we demonstrated that the opening of BK_{Ca} channels limited cell injury associated mainly with the reperfusion (re-oxygenation) phase. The salutary effects (i.e. better cell survival and lower lactate dehydrogenase release) were mediated by superoxide generated by mitochondrial respiratory chain, but not by the PI3-kinase/Akt pathway, which is a common mediator of short-lasting cardioprotective mechanisms (Borchert et al., *Exp Biol Med* **238**: 233, 2013). Moreover, we found that the activity of these channels plays a major role in the protected phenotype of myocytes isolated from chronically hypoxic hearts (Borchert et al., *Am J Physiol Heart Circ Physiol* **300**: H507, 2011). Brief daily interruption of hypoxic exposure prevented the activation of BK_{Ca} channels and completely abolished the cardioprotection most likely due to oxidative stress as a consequence of the insufficient antioxidant capacity. Chronic hypoxia-induced marked de-glycosylation of the native regulatory β1-subunit of the channel, which is known to increase its open probability and mean open time, does not seem to play a role in this form of cardioprotection (Neckář et al., *Curr Pharm Des* **19**: 6880, 2013). At present, we attempt to reveal factors that control the activity of these channels under various modes of chronic hypoxia. These studies were supported by the Grant Agency of the Academy of Sciences of the Czech Republic (grant # IAA500110804, PI: Gudrun H. Borchert; the Czech Science Foundation (grant # 303/12/1162, PI: František Kolář). The contribution of team members to these results was strongly predominant.

We also found that chronic hypoxia led to the up-regulation and activation of mitochondrial superoxide dismutase which showed a close negative correlation with the reduction of myocardial infarct size, suggesting that this enzyme contributed to the improved ischaemic tolerance. Animals treated with the antioxidant *N*-acetylcysteine during the adaptation period did not show these effects and were not protected against I/R injury, further supporting the view that ROS-dependent signalling plays an important role in this form of cardioprotection (Balková et al., *Physiol Res* **60**: 467, 2011).

Other results of studies concerning the cardioprotective mechanism of chronic hypoxia addressed the role of protein kinase C (Hlaváčková et al., *Mol Cell Biochem* **345**: 271, 2010), protein kinase B (Akt) and hexokinase (Wasková-Arnoštová et al., *Cell Physiol Biochem* **31**: 66, 2013 and **33**: 310, 2014) and signalling *via* opioid receptors (Maslov et al., *Life Sci* **93**: 373, 2013). Contributions of collaborating teams from the Faculty of Science, Charles University in Prague and Institute of Cardiology in Tomsk to these results were essential.

Developmental aspects of cardiac ischaemic tolerance

We have shown that cardiac tolerance of the immature heart to acute I/R injury is higher as compared with the adult myocardium (Charvátová et al., *Physiol Res* **61**(S1): S19, 2012;

Ošťádal et al., *Can J Physiol Pharmacol* **92**: 566, 2014). The developmental changes are sex-dependent: cardiac tolerance is similar in males and females up to the end of the weaning period but the adult female heart is more tolerant to oxygen deprivation than the male heart (reviewed in Ošťádal and Ošťádal, *Br J Pharmacol* **171**: 541, 2014). The mechanisms of the higher resistance of the neonatal heart to oxygen deprivation have not yet been satisfactorily clarified. Still unclear is the possible role of mitochondria in spite of the fact that mitochondria are responsible for cellular oxygen handling.

We have observed significant ontogenetic differences in the role of mitochondrial permeability transition pore (MPTP) in myocardial I/R injury. Whereas the blockade of MPTP by sanglifehrin in perfused rat heart had a protective effect on I/R-induced damage in the adult myocardium, as has already been demonstrated earlier, it had no effect in the neonatal heart (Milerova et al., *Mol Cell Biochem* **335**: 147, 2010). For the explanation of this difference the possible lower sensitivity of MPTP in the neonatal heart to pore-opening factors has to be taken into consideration. Indeed, we have found that in cardiac mitochondria isolated from neonatal rats, calcium-dependent and cyclosporine-sensitive MPTP is less sensitive to calcium ions as compared with adults (Drahota et al., *Physiol Res* **61**(S1): S165, 2012). All these results support the hypothesis that cardiac mitochondria are deeply involved in the regulation of cardiac tolerance to oxygen deprivation during ontogenetic development.

Human epidemiological studies have shown a clear association between adverse perinatal environment and an increased risk of ischaemic heart disease in later adult life. One of the most common insults during perinatal development is hypoxaemia due to congenital cyanotic heart defects or pulmonary disease secondary to prematurity. We have observed in the rat model that the late myocardial effects of chronic hypoxia, experienced in early life, may be sex-dependent (Netuka et al., *Physiol Res* **59**: 127, 2010). Perinatal exposure to chronic hypoxia significantly increased cardiac tolerance to acute ischaemic injury in adult females, expressed as the lower incidence of ischaemic ventricular arrhythmias; the effect on arrhythmias in males was the opposite. These results (reviewed in Ošťádal et al., in: Dhalla, Nagano, Ošťádal, eds. *Molecular Defects in Cardiovascular Disease*, Dordrecht: Springer, pp. 55-67, 2011) would have important clinical implications, since cardiac sensitivity in adult patients may be significantly affected by perinatal hypoxia in a sex-dependent manner. These studies were performed almost exclusively by the team members.

Cardiac ischaemic tolerance in diseased states

It has been shown that cytochrome P450-dependent metabolites of arachidonic acid, epoxiecosatrienoic acids (EETs), play an important role in the regulation of cardiovascular functions. Previous studies demonstrated that the inhibition of EETs conversion into biologically inactive dihydroxyeicosatrienoic acids by soluble epoxide hydrolase (sEH) decreased myocardial ischaemia/reperfusion (I/R) injury and reduced the development of hypertension. We showed that chronic inhibition of sEH reduced arterial blood pressure and improved cardiac ischaemic tolerance in hypertensive Ren-2 renin transgenic rats, i.e. strain which represents a unique angiotensin II (Ang II)-dependent model of hypertension. The cardioprotective action of sEH inhibition was completely prevented by acute administration of a selective EETs antagonist supporting the notion that the improved cardiac ischaemic tolerance conferred by sEH inhibition is mediated by EETs (Neckář et al., *Clin Sci* **122**: 513, 2012). This study was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (grant #IAAX01110901, PI: Jan Neckář). The contribution of team members to these results was predominant.

We have also tested the vasodilatory activity of novel EET analogues using the different rat models of hypertension. Our experiments identified orally active EET analogues and delineated their possible antihypertensive and cardioprotective mechanism (Khan et al., *Hypertension* **62**: 905, 2013; Khan et al., *Clin Sci* **127**: 463, 2014). The contribution of the collaborating team from the Medical College of Wisconsin to these results was essential.

It is well known that Ang II type 1 receptor blockers (ARBs) are widely used in treating hypertension. In another project we tested the hypothesis that a novel ARB, azilsartan

medoxomil (AZL-M) prevents cardiovascular and renal injury in the spontaneously hypertensive obese rat (SHROB), a model of cardio-metabolic syndrome. AZL-M treatment improved left ventricular function, attenuated development of left ventricular hypertrophy, and reduced cardiac fibrosis in SHROB. It was accompanied by blood pressure reduction, improved vascular endothelial function and strong kidney protective effects. Similarly, AZL-M attenuated hypertension and protected kidney in Zucker diabetic fatty rats. These findings demonstrated that AZL-M has therapeutic potential to ameliorate cardiovascular complications in cardio-metabolic syndrome (Khan et al., *Cardiovasc Drug Ther* **28**: 313, 2014; Khan et al., *Am J Hypertension* **27**: 1087, 2014). These studies were partially supported by the Czech Science Foundation (grant #13-10267S, PI: Jan Neckář). The team members contributed to these results by heart analyses.

Spontaneously hypertensive rat (SHR) is the most widely studied animal model of human essential hypertension characterised by increased cardiac mass, impaired cardiac ischaemic tolerance and susceptibility to ventricular arrhythmias. Previous studies showed that SHR harbours a deletion variant of Cd36 gene that results in reduced transport of long-chain fatty acids into cardiomyocytes and predisposes SHR to cardiac hypertrophy. We analysed I/R-induced arrhythmogenesis and myocardial infarction in SHR with mutant Cd36 and in SHR-Cd36 transgenic rat with wild-type of Cd36. In open-chest rats, transgenic expression of Cd36 reduced myocardial infarct size but it markedly increased the susceptibility to ventricular arrhythmias induced by I/R. The depletion of catecholamines by reserpine completely eliminated the increased I/R-induced arrhythmogenesis in isolated hearts of SHR-Cd36 rats, which suggests that the pro-arrhythmic effect of Cd36 transgene appears to be dependent on adrenergic stimulation (Neckář et al., *Physiol Genomics* **44**: 173, 2012). Indeed, we determined later that SHR-Cd36 transgenic rats have higher levels of β-adrenergic receptors, increased amount of dominant myocardial isoforms of adenylyl cyclase and its higher stimulated specific activity, and increased levels of protein kinase A. Changes at the molecular level of adrenergic signalling were reflected by higher contractile response to stimulation by the adrenergic agonist dobutamin (Klevstig et al., *Pflügers Arch* **465**: 1477, 2013). This project was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (grant # IAAX01110901, PI: Jan Neckář). Contributions of team members and the collaborating group from Faculty of Science, Charles University in Prague to these results were equal.

We also participated on the project analysing heart functions in SHR and conplastic strain SHR-mt^{F433} characterised by the selective replacement of mitochondrial genome SHR with mitochondrial genome from inbred normotensive strain Fischer 433. SHR-mt^{F433} exhibited impaired cardiac contractile functions at baseline and also after stimulation with dobutamine. These data suggest that inherited alteration in mitochondrial genome, in the absence of variation in nuclear genome, predisposes to systolic dysfunction in rats with essential hypertension (Houštěk et al., *Physiol Genomics* **46**: 671, 2014). This project was partially supported by the Czech Science Foundation (grant #13-10267S, PI: Jan Neckář). The team members contributed to these results by performing echocardiography analysis of heart functions.

Other major results

Our team participated in other studies dealing with various cardioprotective interventions beyond the main areas outlined above. They include demonstrations of infarct size-limiting and/or anti-arrhythmic effects induced by iron chelator dexrazoxane (Neckář et al., *Can J Physiol Pharmacol* **90**: 1303, 2012), short-term fasting (Šnorek et al., *Physiol Res* **61**: 567, 2012), inhalational anaesthetic isoflurane (Říha et al., *Physiol Res* **60**: 709, 2011) or prolonged morphine exposure (Škrabalová et al., *Pharmacol Rep* **64**: 351, 2012; Drastichová et al., *PLoS ONE* **7**: e47167, 2012). These studies were accomplished in cooperation with the teams from the Charles University in Prague and Institute of Clinical and Experimental Medicine. Our team was responsible primarily for the assessment of cardiac ischaemic tolerance.

We also participated in a complex cooperative study which uncovered a novel role for endonuclease G in maladaptive cardiac hypertrophy associated with impaired heart function

(McDermott-Roe et al., *Nature* **478**: 114, 2011). Our team performed dobutamine stress echocardiography and data analysis.

Last but not least, we contributed (by the measurement of pulmonary blood pressure) to the study performed at the University of Milano Bicocca indicating that the development of pulmonary hypertension in chronic hypoxia is, at least partly, attributed to heterogeneity in local lung vascular response (Rivolta et al., *Eur Respir J* **37**: 943, 2011).

INTERNAL COLLABORATION (within the Institute)

Departments:

Cardiovascular Morphogenesis
Genetics of Model Diseases
Adipose Tissue Biology
Experimental Hypertension
Analysis of Biologically Important Compounds
Bioenergetics
Epithelial Physiology

Department of Developmental Cardiology and Department of Experimental Hypertension coordinated the program of the Center for Cardiovascular Research supported by the Ministry of Education, Youth and Sports till the end of 2011.

DOMESTIC COLLABORATION (within the country)

Department of Physiology and Department of Cell Biology, Faculty of Science, Charles University in Prague
Department of Physiology, 2nd Faculty of Medicine, Charles University in Prague
Institute of Clinical and Experimental Medicine in Prague
Institute of Biotechnology, The Czech Academy of Sciences, Prague
Department of Pharmacology, Faculty of Medicine in Hradec Králové, Charles University in Prague
Department of Biochemical Sciences, Faculty of Pharmacy in Hradec Králové, Charles University in Prague
National Institute of Public Health, Prague
Department of Pharmacology, Toxicology and Immunotherapy, Veterinary Research Institute, Brno

INTERNATIONAL COLLABORATION

Institute for Heart Research Slovak Academy of Sciences, Bratislava
Laboratory of Experimental Cardiology, Institute of Cardiology, Tomsk, Russia
Medical College of Wisconsin, Milwaukee, USA
Department of Experimental Medicine, University of Milano Bicocca, Italy
Cardiovascular Research Centre, University of Manitoba, Winnipeg, Canada
Department of Anesthesiology, Washington University School of Medicine, St. Louis, USA
Institute for Cardiovascular Research, Free University of Amsterdam, The Netherlands
Walter-Brendel-Centre of Experimental Medicine, Ludwig-Maximilians-University, Munich, Germany

KEY METHODOLOGY AND CORE FACILITIES

In our research, we use mostly laboratory rats and mice including suitable transgenic models. Cardiac tolerance to injury caused by temporal coronary artery occlusion and reperfusion is studied in anaesthetized open-chest animals, isolated perfused hearts are used to assess tolerance to injury due to global or regional ischaemia/reperfusion, and isolated ventricular

myocytes serve to analyse the effects of simulated anoxia/re-oxygenation. As major manifestations of injury we evaluate the size of myocardial infarction, the incidence and severity of ventricular arrhythmias, the post-ischaemic recovery of cardiac contractile function, and the viability of myocytes. Trans-thoracic echocardiography and catheterisation techniques are used to assess heart functions and haemodynamics. To study cardioprotective mechanisms, we employ suitable pharmacological tools and biochemical analyses.

INVOLVEMENT IN SIGNIFICANT PROJECTS

Centre for Cardiovascular Research was founded as the project of the Czech Ministry of Education, Youth and Sports. The main goal was to study the mechanisms of the ischaemic heart disease with a special attention to the risk factors, such as systemic and pulmonary hypertension and atherosclerosis, particularly from the developmental and sex-dependent standpoints. Department of Developmental Cardiology of the Institute of Physiology of the Czech Academy of Sciences represented one of the founding research teams; Bohuslav Ošťádal served as the principle investigator. The scientific activity of the Centre was based on the collaboration of more than 90 basic and clinical cardiologists (including more than 30 PhD students) from the Prague region (2nd Faculty of Medicine and Faculty of Science of the Charles University, Institute of Physiology of the Czech Academy of Sciences and research institutes of the Czech Ministry of Health). The scientific activity of the Centre terminated in 2011 after the major changes in the financial support of the Czech science after 12 years of the very fruitful collaboration.

SUMMARY AND RESEARCH IMPACT

Our results obtained in the past five years significantly contributed to the advancement of knowledge in specific fields of myocardial I/R injury and cardioprotection as outlined above. In particular, the following results can be highlighted as novel and most significant.

Concerning the sustainable protective mechanism conferred by chronic hypoxia, we have provided the first experimental evidence for the essential involvement of mitochondrial voltage-dependent calcium-activated potassium channels and its dependence on signalling via reactive oxygen species. The absence of protective cardiac phenotype due to a brief periodic interruption of hypoxic exposure can be attributed to the inactivity of these channels.

Concerning ontogenetic aspects of myocardial tolerance to I/R injury, we have revealed that the high tolerance of immature hearts reflects a decreased sensitivity of the mitochondrial permeability transition pore to calcium-induced opening. Another key observation in this area points to the late sex-dependent effects of chronic hypoxia, experienced in early life, on cardiac tolerance to I/R injury in adulthood. This finding deserves to be further explored as it may have important clinical implications.

Out of the novel data characterising myocardial ischaemic tolerance in various forms of systemic hypertension, we can emphasize our demonstration of cardioprotective and antihypertensive effects of epoxiecosatrienoic acids in angiotensin II-dependent hypertension. We have also shown that transgenic expression of fatty acid transporter Cd36 in spontaneously hypertensive rats markedly increased the susceptibility to ventricular arrhythmias induced by I/R due to the sensitisation of β-adrenergic signalling pathway.

Other major findings are listed in the section “Key results”. Altogether, in 2010 – 2014, the results of our team have been published in 49 articles in international journals with impact factor and in 3 book chapters.

Research Report of the team in the period 2010–2014

Institute	Institute of Physiology of the CAS, v. v. i.

Scientific team	Biochemistry of Membrane Receptors

Report on the scientific activity of the team in 2010–2014

1) **TEAM DESCRIPTION**

RESEARCH FOCUS

In 2010-2014 our research topics were oriented to the three areas:

1)

Cellular and molecular mechanisms of desensitisation of hormone action mediated by G-protein-coupled receptors (GPCR) and trimeric G proteins. Mechanisms of desensitization were studied in (**1a**) opioid receptor (OR) and (**1b**) thyreotropin-releasing hormone receptor signaling cascades.

2)

Introduction of methods of fluorescence spectroscopy and confocal fluorescence microscopy for analysis of structural organization of G protein-coupled receptors, hydrophobic matrix of plasma membrane and membrane-water interface. This work was carried out in collaboration with Department of Biophysics from Heyrovsky Institute of Physical Chemistry (Prof. M. Hof, Dr. J. Sýkora and Dr. P. Jurkiewitz).

3)

Postnatal development of $GABA_B$-R-signalling cascade (**3a**) and lipofuscin-like pigments (**3b**) (LFPs) in the forebrain cortex of rats.

(1a) Opioid receptors, OR

This topic was represented by studies of desensitization of μ-OR, δ-OR and κ-OR signaling cascades in forebrain cortex of rats exposed to increasing doses of morphine (10-50 mg/kg) for prolonged period of time, 10 days (Bourova et al., *Med Sci Monit* **16**: BR260, 2010; Ujcikova et al., *Biochim Biophys Acta* **1810**: 1220, 2011; Ujcikova et al., *Physiol Res* **63 Suppl 1**: S165, 2014; Ujcikova et al., *Proteome Sci* **12**: 11, 2014). Model HEK293 cell line stably expressing the fusion protein between δ-OR and G_i1 ($C^{351}I$) was used in studies of the role of membrane domains and cholesterol in δ-OR signaling (Brejchova et al., *Biochim Biophys Acta* **1808**: 2819, 2011) and of the functional significance of monovalent cations for δ-OR activity (Vosahlikova et al., *Physiol Res* **60**: 541, 2011; Vosahlikova et al., *Naunyn Schmiedebergs Arch Pharmacol* **387**: 487, 2014;).

Functional activity of trimeric Gα and Gβ subunits of G_i/G_o family represents the crucial step in signal transfer and regulation of μ-, δ- and κ-OR-initiated signaling cascades. Simultaneously, the theoretical basis and molecular mechanisms of opioid tolerance and addiction proceeding in central nervous system (CNS) are still not understood. Trimeric G-protein activity was measured by high-affinity [^{35}S]GTPγS binding assay in Percoll^R-purified plasma membranes isolated from forebrain cortex of rats exposed to increasing doses of morphine (10-50 mg/kg) for 10 days (Bourova et al., *Med Sci Monit* **16**: BR260, 2010).

DAMGO (μ-OR agonist)- and DADLE (δ-OR agonist)-stimulated [^{35}S]GTPγS binding was significantly *desensitized* in PM isolated from morphine-treated rats. The U-23554 (κ-OR)- and baclofen ($GABA_B$-R)-stimulated [^{35}S]GTPγS binding was unchanged. The *order of efficacy* DADLE > DAMGO > U-69593 was the same in control and morphine-treated PM. The amount of G protein alpha subunits in PM was unchanged by morphine treatment. Behavioral tests

performed under in vivo conditions indicated that morphine-treated animals were fully drug dependent and developed tolerance.

Our data supported the view that the ***primary mechanism of the long-term adaptation of brain to morphine is based on desensitization G protein response to stimulation by μ-OR and δ-OR agonists.*** *This work was supported by the Centre of Neurosciences and Centre of Fluorescence Spectroscopy (projects LC554 and LC 06063 of Ministry of Education of the Czech Republic), GACR (GA309/06/0121), Grant Agency of AS CR (IAA500110606), MSM0021620858 and Academy of Sciences of the Czech Republic (AV0Z50110509).*

Further analysis of PM isolated from forebrain cortex of control and morphine-treated rats (Ujcikova et al., *Biochim Biophys Acta* **1810**: 1220, 2011) indicated that **adenylyl cyclase I (ACI) and II (ACII) were increased 8x and 2.5x**, respectively, in PM prepared from rats exposed to increasing doses of morphine (10-50 mg/kg) for 10 days (group +M10) when compared with control animals (group −M10). Increase of ACI and II by morphine represented the specific effect as the amount of ACIII-ACIX, of sodium plus potassium activated ouabain-sensitive adenosine triphophatase (Na, K-ATPase) and of trimeric G protein α and β subunits was unchanged. Increase of AC I and II was not detected in PM isolated from animals which were exposed to morphine for 10 days and subsequently nurtured for 20 days in the absence of this drug (group +M10/−M20). Thus, the **marked increase of ACI and ACII faded away 20 days since the last dose of morphine**. *These results may be regarded as* ***"good message"*** *for drug addicts, because the dramatic morphine-induced change of the crucial component of opioid receptor cascade represented by hyper-sensitization or up-regulation of AC, is fully reversible after withdrawal of the drug.*

Our studies of PM prepared from fore brain cortex of control and morphine-treated rats also included the analysis of the effect of sodium cations on δ-OR. Sodium cations, which represent the crucial allosteric modulator of OR, inhibited binding of δ-OR agonist [^{3}H]DADLE to PM prepared from brain cortex of control rats but had no effect on [^{3}H]DADLE binding to PM isolated from morphine-treated rats. This result was confirmed by saturation binding study using more specific ligand [^{3}H]DPDPE. The maximum number of receptor sites (B_{max}) was increased by morphine (Vosahlikova in Ujcikova et al., *Biochim Biophys Acta* **1810**: 1220, 2011). For more details, see the results obtained in studies of model HEK293 cell line stably expressing δ-OR- $G_i1α$ ($C^{351}I$) (Vosahlikova et al., *Physiol Res* **60**: 541, 2011; Vosahlikova et al., *Naunyn Schmiedebergs Arch Pharmacol* **387**: 487, 2014).

We could conclude that ***prolonged exposure of brain to morphine, results in alteration of sodium interaction with the ligand binding site of δ-OR.*** *This is an important area for further research performed in species more closely related to humans than rats, because allosteric modulations of OR represent the up-to-day topic and morphine-induced change of sodium or lithium effectiveness were not studied in drug-addicted animals nor humans.* This work was supported by projects LC554 and LC06063 of MSMT, GACR (305/08/H037) and Academy of Sciences of the Czech Republic (AV0Z50110509).

Studies of specific signaling molecules (opioid receptors, G proteins and AC isoforms) in forebrain cortex were extended by proteomic analysis of the over-all protein composition in post-nuclear supernatant (**PNS**) and Percoll-purified membranes (**PM**) prepared from rats exposed to increasing doses of morphine (10-50 mg/kg) for 10 days, i.e. in groups +M10 and −M10 (Ujcikova et al., *Physiol Res* **63 Suppl 1**: S165, 2014; Ujcikova et al., *Proteome Sci* **12**: 11, 2014).

In PNS, the 10 up- and down-regulated proteins exhibiting the *largest morphine-induced change* were selected, excised manually from the gel and identified by MALDI-TOF MS/MS. The identified proteins were of cytoplasmic (4), cell membrane (2), endoplasmic reticulum (1) and of mitochondrial (3) origin and 9 of them were significantly increased. The 4 out of 9 up-regulated proteins were described as functionally related to *oxidative stress*; the 2 proteins participate in genesis of *apoptotic cell death*.

In PM, the long-term morphine exposure resulted in up or down-regulation of 18 proteins which were identified by LC-MS/MS. These proteins were of *plasma membrane* (2), *myelin membrane* (1), *cytoplasmic* (11) and *mitochondrial* (4) origin. The immunoblot analysis of the same PM resolved by 2D-ELFO indicated the "active", morphine-altered pool of trimeric Gβ subunits. This poll of Gβ represented just a small fraction of the total immunoblot signal of Gβ subunits. The total signal / amount of Gβ subunits was unchanged by morphine.

We could therefore conclude that brain cortex of rats exposed to increasing doses of morphine cannot be regarded as fully adapted to steadily increasing concentrations of this drug. ***Significant up-regulation of proteins functionally related to oxidative stress and apoptosis suggests a change of energy metabolism resulting in the state of "brain cell discomfort" or even death.*** This work was supported by the GACR (P207/12/0919), Centrum of Neurosciences (P304/12/G069) and by Academy of Sciences of the Czech Republic (RVO: 67985823).

Our research oriented to opioid receptors was also carried out in **model cell lines** with the aim to understand the functional role of specific compartments of plasma membrane (PM), denominated as membrane domains (MD). These compartments are enriched in cholesterol and concentrate signaling molecules (Brejchova et al., *Biochim Biophys Acta* **1808**: 2819, 2011). Decrease of PM cholesterol level results in degradation of MD. Biophysical studies of fluorescence anisotropy of DPH and Laurdan generalized polarization were performed in plasma membranes (PM) isolated from control and cholesterol-depleted HEK293 cells stably expressing pertussis toxin (PTX)-insensitive δ-OR-$G_i1α$ ($C^{351}I$) fusion protein. PM isolated from control, PTX-untreated, cells were compared with PM isolated from PTX-treated cells. Results from both types of PM indicated that i) hydrophobic membrane interior was more accessible to water molecules and more chaotically organized in cholesterol-depleted samples, ii) cholesterol depletion resulted in an overall increase of surface area, fluidity and mobility of plasma membrane constituents.

Analysis of δ-OR-G protein coupling in PM isolated from PTX-treated and PTX-untreated cells indicated that cholesterol depletion did not alter the agonist binding site of δ-OR (B_{max} and K_d) but the ability of δ-OR agonist DADLE to activate G proteins was markedly impaired. In PTX-untreated membranes, EC_{50} of DADLE-stimulated [^{35}S]GTPγS binding was shifted from 4.3×10^{-9} M (in control PM) to 2.2×10^{-8} M in cholesterol-depleted PM samples. In PTX-treated membranes, EC_{50} was shifted from 4.5×10^{-9} M to 2.8×10^{-8} M.

These results indicated that perturbation of optimum PM organization by cholesterol depletion deteriorated the functional coupling of δ-OR to covalently bound $G_i1α$ as well as endogenously expressed PTX-sensitive G proteins of Gi/Go family. The ä-OR ligand binding site was unchanged by cholesterol depletion. ***The biophysical state of hydrophobic plasma (cell) membrane interior has to be regarded as one of regulatory factors of δ-OR-signaling cascade.***

This work was supported by the Grant Agency of AS CR (IAA500110606), Ministry of Education of the Czech Republic (LC 554), Grant Agency of Czech Republic (305/08/H037) and by Academy of Sciences of the Czech Republic (AV0Z50110509).

Analysis of HEK293 cells transiently expressing Flag-epitope tagged version of δ-OR indicated that cholesterol depletion alone induced transfer of receptor molecules into the cell interior. Incubation of cells with cholesterol depleting agent β-cyclodextrin (10 mM, 30 min) caused the significant increase of intracellular pool of δ-OR, while in control, β-CDX-untreated cells, the small intracellular signal of δ-OR, distributed among numerous faint fluorescent patches, was unchanged. Massive transfer of receptor molecules from the cell surface into the cell interior was detected after δ-OR stimulation by agonist DADLE. This transfer was decreased in β-CDX-treated cells. We could therefore conclude that alteration of PM integrity by degradation of membrane domains is associated with spontaneous transfer of a portion of δ-OR molecules into the cell interior. Massive internalization of δ-OR induced by stimulation with specific agonist is suppressed in cholesterol depleted cells. Therefore, the agonist-induced internalization of δ-OR, which has been traditionally attributed to clathrine-mediated

pathway, seems proceed at least partially via membrane domains / caveolae [Brejchová, J. (2014) The role of membrane cholesterol in δ-opioid receptor signaling, *correlation with plasma membrane structure.* PhD Thesis, Faculty of Natural Sciences, Charles University in Prague]

As already mentioned, **sodium cations** represent the crucial alllosteric modulators of opioid as well as other GPCRs. Sodium is bound to the inactive / resting state of receptor in the deep interior of ligand binding pocket with high affinity. Agonist binding induces removal of sodium from the binding site and transfer of receptor molecules from the resting state / conformation **R** to the active conformation, **R***. Because of this transfer, agonist binding to specific receptor sites is increased in parallel with decrease of antagonist binding. Antagonists are preferentially bound to the non-active conformation of receptor.

The effect of sodium, potassium and lithium on δ-opioid receptor agonist and antagonist binding and coupling with the cognate G proteins was studied in HEK293 cell line stably expressing PTX-insensitive δ-OR-G_i1α ($C^{351}I$) fusion protein (Vosahlikova et al., *Physiol Res* **60**: 541, 2011; Vosahlikova et al., *Naunyn Schmiedebergs Arch Pharmacol* **387**: 487, 2014). Agonist [^{3}H]DADLE binding was decreased in the order $Na^+ \gg Li^+ > K^+ >$ (+)NMDG. When plotted as a function of increasing NaCl concentrations, the binding was best-fitted with a two-phase exponential decay considering two Na^+-responsive sites ($r^2 = 0.99$). High-affinity Na^+-sites were characterized by K_d = 7.9 mM and represented 25% of the basal level determined in the absence of ions. Remaining 75% represented the low-affinity sites (K_d = 463 mM). Inhibition of [^{3}H]DADLE binding by lithium, potassium and (+)-NMDG proceeded in low-affinity manner only. Surprisingly, the *affinity/potency* of DADLE-stimulated [^{35}S]GTPγS binding was increased in a reverse order: $Na^+ < K^+ < Li^+$. This result was demonstrated in PTX-treated as well as PTX-untreated cells. Therefore, it is not restricted to G_i1α ($C^{351}I$) within the δ-OR-G_i1α fusion protein, but is also valid for stimulation of endogenous G proteins of G_i/G_o family in HEK293 cells.

Biophysical studies of interaction of monovalent ions with polar head-group region of lipids by Laurdan generalized polarization indicated the low-affinity type of interaction only. This low-affinity interaction proceeded in the order: $Cs^+ < K^+ < Na^+ < Li^+$. Results of this study were discussed in terms of interaction of Na^+, K^+ and Li^+ with the high- and low-affinity sites located in water-accessible part of δ-OR binding pocket. We have also considered the role of negatively charged Cl^-, Br^- and I^- counter ions. This work was supported by the Grant Agency of AS CR (IAA500110606), Ministry of Education of the Czech Republic (LC 554), Grant Agency of Czech Republic (305/08/H037), Academy of Science of CR (AV0Z50110509), Grant Agency of Czech Republic (P207/12/0919 and P304/12/G069) and Academy of Sciences of CR (RVO: 67985823).

Membranes prepared from HEK293 cells expressing δ-OR-Gi1α ($C^{351}I$) fusion protein were also used in functional assays of interaction of RGS3 and 14-3-3 protein with trimeric G proteins of Gi/Go family (Rezabkova et al., *J Struct Biol* **170**: 451, 2010). Our data indicated that inhibitory effect of 14-3-3 protein on RGS3-induced increase of G protein activity was exclusively oriented to δ-OR agonist DADLE-induced activation of G proteins. The basal, receptor independent activity of G proteins, was not effected (work done by LB and PS). It should be mentioned, in the context of our research oriented to brain, that Regulators of G protein signaling (**RGS proteins**) represent a very important group of signaling molecules involved in regulation of **brain function** because they increase the low, endogenous GTPase activity of trimeric Gα subunits and act, in this respect, as GTPase-activating proteins (**GAPs**). Results obtained with isolated RGS domain of RGS3 (performed in Department of protein structures FgU (L.R., E.B., P.H., J.V., M.S., V.O., T.O.) indicated that this domain alone can interact with 14-3-3 protein. Crystal structure of the RGS domain of RGS3 was solved at 2.3 angstrom resolution. Data derived from structure of RGS domain suggested that 14-3-3 protein-induced conformational change affects the specific region of RGS3 protein which interacts with G_iα subunits. This work was supported by Grant Agency of the Academy of Sciences of the Czech Republic Grant IAA501110801; Ministry of Education, Youth, and

Sports of the Czech Republic Research Projects MSM0021620857 and MSM0021620835, Center of Neurosciences LC554 and Academy of Sciences of Czech Republic Research (AV0Z50110509).

(**1b**) **Thyrotropin-releasing hormone receptor, TRH-R**

Numerous our previous studies of HEK293 cells stably expressing TRH-R and $G_{11}\alpha$ protein (clone E2M11) or TRH-R alone (clone E2) indicated that the long term stimulation of TRH-R by agonist (TRH) results in desensitization, internalization and down-regulation of exogenous $G_{11}\alpha$ (murine) as well as endogenous $G_q\alpha/G_{11}\alpha$ (human) proteins. *Agonist-induced internalization of $G_q\alpha/G_{11}\alpha$ proceeded within the much longer time-scale (hours) than internalization of TRH-R (minutes).*

Results published in 2010 (Drastichová et al., *J. Cell. Biochem.* **109**: 255, 2010) were aimed at analysis of the over-all plasma membrane protein composition in E2M11-cells exposed to TRH for long period of time, 16 hours. Under such conditions, the significant down-regulation of $G_q\alpha/G_{11}\alpha$ proteins occurred. Purified plasma membrane fraction was prepared by Percoll gradient centrifugation, proteins resolved by 2D electrophoresis and stained with SYPRO Ruby gel stain. The high enrichment in PM proteins in isolated PM was confirmed by a multifold increase of the number of TRH receptors and TRH-stimulated G protein activity, compared to post-nuclear supernatant fraction, PNS. By a combination of these approaches we were able to determine a number of clearly discernible protein changes in PM isolated from cells treated with TRH for 16 hours: **4** proteins disappeared, the level of **18** proteins was decreased and the level of **39** proteins was increased. Our concomitant immunochemical determinations indicated a clear down-regulation of $G_{11}\alpha$/ $G_q\alpha$ proteins in preparations from hormone-stimulated cells. In parallel, we observed the decrease of caspase 3 and alterations of some other ***apoptotic marker proteins***, which were in line with the presumed anti-apoptotic effect of TRH. This work was made jointly in 1:1 proportion in Institute of Physiology (Z.D., L.H., J.N., P.S.) and Charles University in Prague (Z.D., L.B., P.S., J.N.) and was supported by Ministry of Education of Czech Republic (MSM0021620858 and LC554), GACR (309/06/0121, 305/08/H037) and AV0Z50110509.

(2)
Introduction of methods of fluorescence spectroscopy and confocal fluorescence microscopy for analysis of structural organization of G protein-coupled receptors in membrane domains and of hydrophobic matrix of plasma membrane and membrane-water interface.

HEK293 cells stably expressing δ-opioid receptor (Ostasov et al., *Chem Phys Lipids* **167**: 62, 2013) were labeled first with fluorescent analog of cholesterol, 22-NBD-cholesterol, exposed to cholesterol-depleting agent β-cyclodextrin (β-CDX) and analyzed by fluorescence lifetime imaging microscopy (FLIM). In accordance with chemical analysis of cholesterol level, the total cellular signal of this probe was decreased to half. Distribution of lifetime (τ_{tot}) values of 22-NBD-cholesterol, however, when screened over the whole cell area indicated no significant difference between control (τ_{tot} = 4.9 ± 0.1 ns) and β-CDX-treated (τ_{tot} = 4.8 ± 0.1 ns) cells.

On the contrary, comparison of control (τ_{tot} = 5.1 ± 0.1 ns) and β-CDX-treated (τ_{tot} = 4.4 ± 0.1 ns) cells by FLIM analysis of 25-NBD-cholesterol fluorescence, indicated the highly significant decrease of lifetime values of this probe. The observation that 22-NBD-cholesterol appears to be indifferent to the changes of membrane packing in living cells is in agreement with previous studies of model membranes. Our data also indicated that the alternation of plasma membrane structure by decrease of cholesterol level makes the membrane environment of NBD moiety of 25-NBD-cholesterol probe a significantly more hydrated. This finding encourages using 25-NBD-cholesterol in living cells and tissues, but also demonstrates that previously drawn discouraging conclusions on the use of 25-NBD-cholesterol in model membranes are not valid

for living cells (work done by P.O., J.B. and PS). This work was supported by GACR (P207/12/0919) and Academy of Sciences of the Czech Republic (AV0Z50110509 and RVO: 67985823) and due to decision of Prof. Hof may be attributed fully to Institute of Physiology.

(3a)

Postnatal development of $GABA_B$-R-signalling cascade in the forebrain cortex of rats.

Our data indicated the significant intrinsic efficacy of $GABA_B$-receptors in rat forebrain cortex already in 2-days-old animals (PD2) (Kagan et al., *Physiol Res* **61**: 629, 2012). Subsequently, baclofen- and SKF97541-stimulated G-protein activity, measured as agonist-stimulated, high-affinity [^{35}S]GTPγS binding, was increased. The highest level of both baclofen and SKF97541-stimulated [^{35}S]GTPγS binding was detected between postnatal-day-10 (PD10) and PD15. In older rats, baclofen- and SKF97541-stimulated [^{35}S]GTPγS binding was continuously decreased so, that the level in adult, 90-days-old animals, was not different from that in newborn animals. The potency of G-protein response to baclofen (characterized by EC_{50} values) was also high at birth but unchanged by further development.

The highest plasma membrane level of $GABA_B$-R (determined by saturation binding assay with antagonist [^{3}H]CGP54566A) was detected in 1-day-old animals (2.27 pmol × mg^{-1}). The further development was reflected in a decrease of $GABA_B$-R. The B_{max} values of [^{3}H]CGP54566A binding sites were decreased to 1.38 and 0.93 pmol × mg^{-1} in PM isolated from 13- and 90-days-old rats, respectively. This work was supported by GACR (P207/12/0919 and P304/12/G069) and by Academy of Sciences of Czech Republic (RVO: 67985823).

With the aim to understand the onset of expression and developmental profile of plasma membrane (PM) content of the crucial components of $GABA_B$-R signaling cascade in detailed manner, $GABA_B$-R1a, $GABA_B$-R1b, $GABA_B$-R2, $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ subunit proteins were determined by quantitative immunoblotting and compared in PM isolated from brain cortex of rats of different ages: between postnatal-day-1 (PD1) and postnatal-day-90 (PD90) (Dlouhá et al., *Physiol Res* **62**: 547, 2013). PM content of $GABA_B$-R1a, $GABA_B$-R2, $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ was high already at birth and further development was reflected in parallel decrease of both $GABA_B$-R1a and $GABA_B$-R2 subunits. The major decrease of $GABA_B$-R1a and $GABA_B$-R2 subunits occurred between the birth and PD15. Contrarily, PM level of the cognate G-proteins $G_i1/G_i2\alpha$, $G_i3\alpha$, $G_o\alpha$, $G_z\alpha$ and Gβ was unchanged in the course of the whole postnatal period.

Maturation of $GABA_B$-R signaling cascade was substantially different from ontogenetic development of prototypical plasma membrane marker, Na, K-ATPase, which was low at birth and further development was reflected in continuous increase of PM density of this enzyme. The major increase of PM content of Na, K-ATPase molecules occurred between the birth and postnatal-day-25 (PD25). In adult rats, membrane content of Na, K-ATPase was 3-times higher than around the birth. This work was supported by GACR (P207/12/0919 and P304/12/G069) and by Academy of Sciences of Czech Republic (RVO: 67985823).

(3b)

Postnatal development of lipofuscin-like pigments (LFPs) in the forebrain cortex of rats.

Fluorescent pigments (Lipofuscin like pigments, **LFPs**) are the end-products of free radical damage of biolological membranes and their constituents, lipid and protein molecules. For example, LFPs are formed by lipid peroxidation, which results mainly in production of unsaturated aldehydes with free amino groups. The characteristic emission maxima of LFPs are found in wide range of wavelengths, between 420 and 470 nm, when these fluorescent species are excited at 340-390 nm. Owing to their intrinsic fluorescent properties and molecular stability, these products are easily measured by means of spectrofluorometry and may be used as markers of ***oxidative stress*** and ***membrane protein degradation by free radicals***.

We have observed (Wilhelm et al., *Mol Cell Biochem* **347**: 157, 2011), that the high level of LFPs is generated immediately after the birth, during the first five days of postnatal life. The

PM fraction was prepared from forebrain cortex of rats representing the 10 groups of animals of different ages: 7-days before birth, 1-day before the birth and 1-, 2-, 5-, 10-, 15-, 25-, 35- and 90-days after the birth.

Maximum LFP concentration was measured on the postnatal-day-2 (PD2). Starting from the postnatal-day-10, LFP concentration returned to the prenatal level. A new rise of LFP concentration in PM was observed after 3 months of life, i.e. in 90-days-old rats. It may be assumed that this rise is associated with the beginning of the aging process. LFPs were characterized by fluorescence spectroscopy using 3-dimensional excitation spectra, synchronous spectra and HPLC with fluorescence detection. We managed to discern the several tens of fluorescent compounds of unknown structure that were generated by oxidative damage of brain and metabolized to different degrees during its early development. It might be assumed that at least part of these fluorescent substances is formed after respiratory burst of microglia phagocytosing the apoptotic cells shortly after the birth.

2) PERSONNEL

Senior scientists (3):

Petr Svoboda, Doc., RNDr, DSc (team leader), experience in physiology, biochemistry and molecular pharmacology, age 65, H-index 22

Lenka Roubalová, RNDr, PhD, expert in analysis of G-protein coupled receptors and trimeric G protein in brain and other mammalian tissues and cells, age 49, H-index 9

Jiří Wilhelm, Prof., RNDr, PhD, since 2012, biochemist, expert in bioenergetics and free radical damage, H-index 17

Junior scientists (4):

Mgr. Pavel Ostašov, PhD (2012-2013)
Ing. Miroslava Vošahlíková, PhD,
RNDr Hana Ujčíková, PhD
RNDr Jana Brejchová, PhD

Postdoctoral fellow(s): 0

Laboratory technicians: 0

PhD students (7)

Mgr. Pavel Ostašov (PhD defense in 2012)
Ing. Miroslava Vošahlíková (PhD in 2014)
RNDr Hana Ujčíková (PhD in 2014)
RNDr Jana Brejchová (PhD in 2014)
Mgr. Dmytro Kagan
Ing. Kateřina Dlouhá-Stolařová
Mgr. Michaela Czerneková, since 2013

MS students (6): Alexandra Rejhová, MS degree in 2011; Lenka Ulrychová, in 2011; Kateřina Višněvská in 2013; Karolina Kettnerová in 2014; Ladislav Merta in 2014, Kristina Cechova, since 2014.

Bc students (5) Lenka Ulrychová, Kateřina Višněvská, Ladislav Merta, Kristina Cechová, Adéla Provazníková

3) KEY RESULTS were specified in detailed manner in sections 1-3 of RESEARCH FOCUS

4) **INTERNAL COLLABORATION (within the Institute)**

Deparment of Biomathematics, Dr. Jiří Janáček, CSc; application of modern methods of confocal fluorescence microscopy, FRAP and RICS for analysis of mobility and structural organization of G protein-coupled receptors in plasma membrane of living cells.

5) **DOMESTIC COLLABORATION**

Department of Biophysics, Heyrovsky Institute of Physical Chemistry, ASCR, M. Hof, J. Sýkora and P. Jurkiewitz (see the published results)

6) **INTERNATIONAL COLLABORATION**

M. Parenti, Department of Pharmacology, University of Milano-Biccoca, Italy, gift of plasmids containing δ-OR-eCFP, δ-eYFP, μ-OR-eCFP and μ-OR-eYFP); Tae-Weon Lee, Amgen, Palo Alto, USA; methodological advises, technical help; M. Buhneman, Department of Pharmacology and Clinical Pharmacy, Marburg, Germany, gift of plasmids containing Gα-eCFP and Gβ-eYFP; Alexander Faussner (Institute for Cardiovascular Prevention, Munich, Germany), experience with preparation of stable cell lines using Flp-In™ T-REx™ technology; V. Hruby (Department of Chemistry and Biochemistry; University of Arizona, Tucson, USA), gift of lipophilic μ- and δ-opioid agonists exhibiting the mixed μ-OR/δ-OR potency, help in planning the future experiments.

7) **KEY METHODOLOGY AND CORE FACILITIES**

Methodological approaches for analysis of GPCR-initiated signaling cascades in plasma membrane; subcellular fractionation of mammalian tissues and cells in culture, fluorescent spectroscopy and microsocopy.

8) **INVOLVEMENT IN SIGNIFICANT PROJECTS**

2005-2011, Centrum of Neurosciences (project of MSMT LC554), Dr L. Vyklicky, project leader.

2006-2011, Centrum of Fluorescence spectroscopy (project MSMT LC06063), Prof. M. Hof, project leader.

2012-2016, GA ČR P207/12/0919, The role of hydrophobic plasma membrane interior in regulation of functional activity of δ-opioid receptors.

2012-2018, Project of Excellence in Neurosciences, L. Vyklický, project leader (GA CR P304/12/G069)

8) **OTHER RELEVANT INFORMATION**

The personal conditions for performing the research in highly competitive area of analysis of opioid receptor function and drug addiction has dramatically deteriorated in our Institute since May 2013. The same applies to analysis of $GABA_B$-R initiated signaling cascades in brain. I hope that this unfortunate situation will be improved in 2015. We hope to be able to begin the new collaboration with Dr. Petr Popov and Prof. Michal Miovský, PhD from Clinic of Addictology, 1st Medical Faculty, Charles University in Prague. This collaboration will be oriented to analysis of μ-OR, δ-OR and κ-OR in control human blood lymphocytes and lymphocytes collected from drug addicts. We also hope that our methodological knowledge and experience collected over the years in area of fluorescence spectroscopy and microscopy will be brought to higher level by supervision carried out by Dr. J. Janáček from Department of Biomathematics, Institute of Physiology.

9) **SUMMARY AND RESEARCH IMPACT**

The functional significance of δ-OR and mechanism(s) of mutual interaction of μ-, δ- and κ-opioid receptor signaling cascades in **genesis of drug addicted state** are not clearly defined.

Our results (Bourova et al., *Med Sci Monit* **16**: BR260, 2010) support the view that the mechanism of addiction to morphine is primarily based on ***desensitization of μ- and δ-opioid receptor signaling cascades.*** Desensitization of μ-OR and δ-OR agonist response proceeds already at the level of G protein functional activity. The amount of G proteins is unchanged by the prolonged morphine exposure.

Adenylyl cyclase I and II were dramatically increased in plasma membranes (PM) isolated from forebrain cortex of rats exposed to increasing doses of morphine (10-50 mg/kg) for 10 days (Ujcikova et al., *Biochim Biophys Acta* **1810**: 1220, 2011). Membrane content of ACIII-ACIX, Na, K-ATPase and of trimeric G protein α and β subunits was unchanged. Increase of AC I and II was not detected in PM isolated from animals exposed to morphine for 10 days

and subsequently nurtured for 20 days in the absence of the drug. Thus, the marked increase of ACI and ACII faded away 20 days since the last dose of morphine. *These results may be regarded as "**good message for drug addicts**", because our data indicate that dramatic morphine-induced change of the crucial component of opioid receptor cascade is reversible after withdrawal of the drug.*

The long-term exposure of rats to morphine also results in change of sensitivity of δ-OR to sodium ions. Analysis of the inhibitory effect of different monovalent ions on agonist binding to δ-OR-$G_i1α$ ($C^{351}I$) fusion protein stably expressed in HEK293 cells (Vosahlikova et al., *Naunyn Schmiedebergs Arch Pharmacol* **387**: 487, 2014) confirmed the preferential sensitivity of δ-OR to sodium ions. We were able to distinguish the ***high- and low-affinity Na+ sites***. Sensitivity of high-affinity sites, expressed as Na+ concentration inducing the half-maximum decrease of agonist binding (≈7.9 mM), was close to the sensitivity of stereo-specific sodium binding site in high-resolution crystals of δ-OR (13 nM). *By revealing the relatively high sodium affinity, our studies demonstrate that at physiological sodium concentrations (140 mM) the sodium site is likely to be saturated, i.e. in the absence of agonist, most of δ-OR exist in the non-active state / conformation.*

Study of HEK293 cells stably expressing fusion protein between δ-OR and pertussis toxin-insensitive mutant of $G_i1α$ protein, δ-OR-$G_i1α$ ($C^{351}I$) (Brejchova et al., Biochim Biophys Acta **1808**: 2819, 2011) indicated that perturbation of optimum plasma membrane organization by cholesterol depletion deteriorated the functional coupling of δ-OR to covalently bound $G_i1α$ as well as endogenously expressed PTX-sensitive G proteins of Gi/Go family. The δ-OR ligand binding site was unchanged by cholesterol depletion. **The biophysical state of hydrophobic plasma (cell) membrane interior has to be regarded as one of regulatory factors of δ-OR-signaling cascade.**