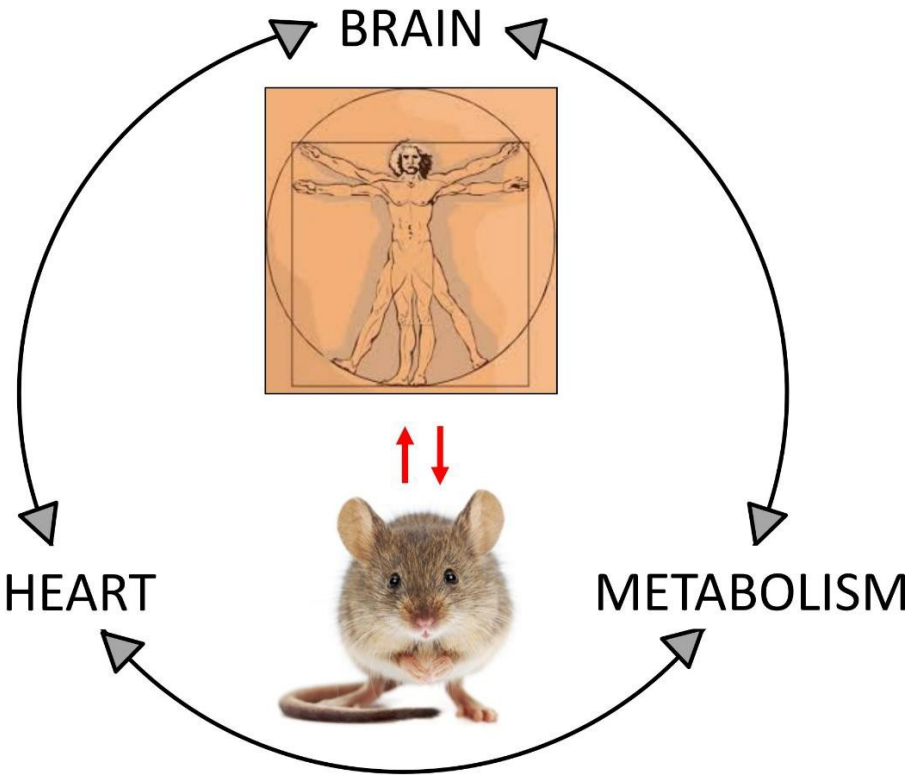
Description of main research directions investigated by the institute

The main results published by IPHYS scientists during the evaluated period (2015–2019) in three major fields of research are listed below.



1. Metabolic research

One of the research fields pursued at IPHYS since its early days is metabolism and its regulation. While some research teams concentrate on specific aspects of metabolism, their studies have implications for cardiovascular and neurophysiological research. The IPHYS Laboratories studying metabolism can be clustered into several groups, depending on the level of complexity. Specifically, this includes research into the activity and regulation of **selected protein complexes and transport proteins**, the function of **mitochondria** and theimpact of mitochondrial dysfunction on health, the mechanisms underlying **obesity- associated metabolic disorders** (the metabolic syndrome), including their prevention and treatment by nutritional interventions and pathophysiology of the **gastrointestinal tract**.

The array of methods employed in metabolic research ranges from a cell biology level to advanced **whole-body phenotyping** in rodents, including behavioural testing and the use of sophisticated **animal models** of human biology. For all of our studies, animal models represent an essential tool. The Institute has always been recognised for its research on laboratory **rats** (namely the SHR model) and the development of unique recombinant inbred strains, and congenic, transgenic, and knockout models (gene editing, ZFN and TALEN nucleases, Sleeping Beauty transposon system). Thus within several years, on-demand rat models could be used in mitochondrial (e.g. TMEM70 or DAPIT knockout) and metabolic studies of human genetic diseases and other pathologies. In recent years, increasing use of **mice** and new facilities on Krč campus of biological institutes of the CAS accelerated the development of transgenic murine models (e.g. tissue-specific and conditionalknockouts).These models are essential for the elucidation of molecular mechanisms and genetic determinants of both rare and common diseases, such as the metabolic syndrome associated with aberrant lipid and glucose metabolism and hypertension.

Phenotyping “omics” methods are also important for our metabolic research. We have systematically developed **proteomics analysis** and **metabolomics** for targeted and untargeted LC-MS characterisation and quantification of complex lipids including anti-

inflammatory and antidiabetic lipokines (Paluchova et al., *Diabetes* **69**: 300, 2020), polar metabolites, and various food components and drugs in diverse sample types.

The characterisation of 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 the regulation of selected signalling proteins whose functions are controlled through protein-protein and phosphorylation-dependent interactions. We elucidated the molecular basis of 14-3-3 protein-dependent activation of yeast neutral trehalase Nth1, in which 14-3-3 activates Nth1 by stabilizing the flexible part of its active site, and we highlighted its ability to function as an allosteric effector (Alblova et al., *Proc Natl Acad Sci U S A* **114**: E9811, 2017). Our structural studies also characterised 14-3-3 regulation of caspase 2 or kinases ASK1and CaMKK2**.** In the case of the forkhead transcription factor FOXO3 involved in cancer cell regulation, we have identified small molecular compounds (S9 and its oxalate salt) that interact with FOXO3. We showed that they are able to inhibit its activity in cancer cells (Hagenbuchner et al., *Elife* **8**: 2019). The expertise in protein structure analysis is further used in collaboration with other cellular physiology groups.

Membrane transporters represent a broad and diverse group of proteins involved in the transport of solutes across cytoplasmic or organellar membranes. The yeast cell serves as an important experimental model in studies of cell transport systems at the molecular level and the protein level. Our studies on cell cation and pH homeostasis focused on Na^+/H^+ antiporters and described a hydrophobic filter determining cation selectivity and transport activity (Kinclova-Zimmermannova et al., *J Mol Biol* **427**: 1681, 2015) and characterised cargo receptors of the secretory pathway ERV14 (Zimmermannova et al., *Biochim Biophys Acta Mol Cell Res* **1866**: 1376, 2019), in both yeast and plants (Rosas-Santiago et al., *Biochim Biophys Acta Mol Cell Res* **1864**: 1809, 2017). We were first to show that yeast Na^+/H^+ antiporter is negatively regulated via interaction with 14-3-3 proteins (Smidova et al., *Biochim Biophys Acta Mol Cell Res* **1866**: 118534, 2019). The characterszation of antifungal activity of antimicrobial peptides and the role of plasma membrane lipids in multidrug resistance are important outcomes of our transporter studies (in collaboration with IOCB) (Kodedova et al., *Cell Microbiol* **21**: e13093, 2019).

Mitochondria are key players in cellular energy provision and harbour a number of metabolic pathways, therefore mitochondrial (dys)function has impact on health. Our research into the molecular basis of mitochondrial structure, function and biogenesis was based on the development of numerous cellular and animal genetic models, and it covered several specific areas, all with an increasing impact on human diseases:

(i) Inherited disorders of the mitochondrial oxidative phosphorylation system, focused on ATP synthase depletion due to dysfunction of the TMEM70 biogenetic factor and its role in enzyme biogenesis (Vrbacky et al., *Hum Mol Genet* **25**: 4674, 2016; Kovalcikova et al., *FASEB J* **33**: 14103, 2019); Cytochrome *c* oxidase dysfunction due to the SURF1 factor mutation (Kovarova et al., *Biochim Biophys Acta* **1862**: 705, 2016); and complex I deficiencies caused by altered assembly factors (Hartmannova et al., *Hum Mol Genet* **25**: 4062, 2016; Alston et al., *Am J Hum Genet* **103**: 592, 2018)**.**

(ii) Redox signalling, ROS, and uncoupling proteins in mitochondrial physiology, focused on the essential role of mitochondrial phospholipase iPLA2γ in fatty acid stimulated insulin secretion (Jezek et al., *Antioxid Redox Signal* **23**: 958, 2015); targeting of oxidative stress in pancreatic beta cells by mitochondrial antioxidants (Plecita-Hlavata et al., *Oxid Med Cell Longev* **2019**: 1826303, 2019); and pleiotropic effects of biguanides on mitochondrial ROS production (Pecinova et al., *Oxid Med Cell Longev* **2017**: 7038603, 2017).

(iii) Mitochondrial metabolism in cancer cells, namely pharmacological inhibition of fatty-acid oxidation synergistically enhancing the effect of L-asparaginase in childhood ALL cells (Hermanova et al., *Leukemia* **30**: 209, 2016)**;** hypoxic widening of mitochondrial cristae in cancer cells (Plecita-Hlavata et al., *FASEB J* **30**: 1941, 2016); and the role of mitochondrial complex II in cell death initiation (Kluckova et al., *Cell Death Dis* **6**: e1749, 2015).

(iv) Mitochondrial functions specifically involved in heart failure (Melenovsky et al., *Eur J Heart Fail* **19**: 522, 2017)**,** angiogenesis (Wang et al., *Nat Commun* **7**: 12061, 2016) or pulmonary hypertension (Zhang et al., *Circulation* **136**: 2468, 2017).

The major focus is on the adipose tissue function and importance of this tissue for whole-

body metabolism, obesity and cancer. We apply dietary (omega-3 fatty acids), environmental (cold exposure), and pharmacological (antidiabetic drugs pioglitazone and MSDC-0160) manipulations to induce the *healthy adipocyte* phenotype. Primarily we use a model of diet-induced obesity in mice and various advanced techniques such as hyperinsulinemic-euglycemic clamps, FACS, and metabolipidomics analyses. We found that changes in adipose tissue metabolism (e.g. triacylglycerol/fatty acid cycling, de novo lipogenesis) during cold exposure reflect susceptibility to obesity (Flachs et al., *Int J Obes (Lond)* **41**: 372, 2017). We also demonstrated that omega-3 supplementation induces novel anti-inflammatory lipid mediators from the FAHFA family in adipose tissue of obese mice and diabetic patients (Kuda et al., *Diabetes* **65**: 2580, 2016).Omega-3 supplementation also differentially regulates the levels of endocannabinoids in adipose tissue and circulation in mice and patients (Rossmeisl et al., *Biochim Biophys Acta Mol Cell Biol Lipids* **1863**: 712, 2018). In older sedentary individuals, omega-3 wax esters (Calanus oil) potentiates insulin- sensitizing effects of exercise training, which itself induces anti-inflammatory and insulin- sensitizing PAHSA lipid mediators in adipose tissue and circulation (Brezinova et al., *BiochimBiophys Acta Mol Cell Biol Lipids* **1865**: 158576, 2020). We also demonstrated that Nrf2- mediated antioxidant defence and peroxiredoxin 6 are linked to the biosynthesis of 9-PAHSA regioisomer (Kuda et al., *Diabetes* **67**: 1190, 2018) and that 5-PAHSA is regulated by adipose triglyceride lipase and primes adipocytes for glucose metabolism (Paluchova et al., *Diabetes* **69**: 300, 2020). With regard to cancer cachexia, we showed that it may reflect aberrant metabolism of adipose tissue itself by inhibiting AMPK activity by high levels of CIDEA in adipocytes, leading to increased lipolysis and “burning” of released fatty acids in skeletal muscles and other tissues (Rohm et al., *Nat Med* **22**: 1120, 2016).

Gastrointestinal research was focused on the intestinal transport system, endocrine regulation and intestinal pathologies (colitis, inflammatory bowel disease). Our studies characterised psychophysiological and inflammatory stress-induced modulation of glucocorticoid (the HPA axis) signalling, glucocorticoid metabolism, and cardiovascular response. We elucidated the role of gut microbiota in shaping the chronic response at the periphery of the HPA axis (Vodicka et al., *Brain Behav Immun* **73**: 615, 2018) and in peripheral tissue (Vagnerova et al., *Front Immunol* **10**: 2655, 2019). We also characterised the role of corticosteroid hormones in the regulation of the peripheral circadian clock in the intestine.

2. Neurophysiological research

Neurophysiology research carried out at IPHYS covers a selected spectrum of topics ranging from the molecular mechanisms of actions of transmitters on their receptors and the effects of cellular context on neurotransmission to highly integrative functions of the central nervous system, such as memory and temporal regulation of physiological processes (circadian rhythms). We also studied pathological mechanisms, namely those of neuropathic pain, cerebral ischemia, neurological diseases (such as epilepsy), and psychiatric conditions (such as Alzheimer’s disease (AD), autism spectrum disorder (ASD), obsessive-compulsive disorder (OCD), and schizophrenia). We correlated some experimental approaches from experimental animals with healthy volunteers andcomplement our neurophysiology research with computational methods.

At the molecular level, we investigated mechanisms of operation of fast-acting ion channels (glutamate NMDA receptors, transient receptor potential (TRP) ion channels, purinergic (PTX) channels), and slow-acting metabotropic G-protein coupled receptors (muscarinic acetylcholine receptors) in both peripheral and central nervous systems. In the research of NMDA receptors, we identified residues that are key to extracellular channel gate function (Ladislav et al., *Front Mol Neurosci* **11**: 113, 2018). Further, we found that polymorphism of NMDA receptors of schizophrenia patients and individuals diagnosed with ASD leads to enhanced sensitivity to steroids (Vyklicky et al., *Front Mol Neurosci* **11**: 110, 2018). In the research of TRP channels, we investigated activation of the channel by heat and cold (Sinica et al., *Cells* **9**: 2019; Macikova et al., *Int J Mol Sci* **20**: 2019) and allosteric mechanism of channel gating (Zimova et al., *Sci Signal* **11**: 2018). In the research of P2X channels, we identified transmembrane residues that regulate receptor conductivity and agonist sensitivity (Jindrichova et al., *J Neurochem* **133**: 815, 2015). Further, we identified new allosteric modulators of P2X receptors (Sivcev et al., *J Neurochem* **150**: 28, 2019) and

analysed allosteric modulation of P2X receptors (Zemkova et al., *Pflugers Arch* **467**: 713, 2015; Mackay et al., *PLoS Comput Biol* **13**: e1005643, 2017). Concerning muscarinic receptors, we investigated molecular mechanisms of receptor activation and signalling bias (Randakova et al., *Pharmacol Res* **97**: 27, 2015; Randakova et al., *Neuropharmacology* **133**: 129, 2018; Randakova et al., *Br J Pharmacol* 2020). Further, we studied structure-activity relationships underlying the selectivity and long-lasting action of muscarinic antagonists(Jakubik et al., *J Mol Model* **21**: 284, 2015; Boulos et al., *Chem Biol Drug Des* **91**: 93, 2018; Randakova et al., *Br J Pharmacol* **175**: 1731, 2018). We also studied common mechanisms of allosteric modulation of muscarinic receptors (Jakubik et al., *Sci Rep* **7**: 40381, 2017; Jakubik et al., *Sci Rep* **9**: 4637, 2019; Jakubik et al., *PLoS One* **14**: e0214255, 2019).

At the level of the cellular context, we investigated the modulation of neurotransmission by various cellular and membrane components, especially membrane cholesterol and neurosteroids. We also investigated receptor regulation and trafficking at the cellular level. We found that membrane cholesterol and neurosteroids modulate the function of both NMDA receptors (Korinek et al., *J Physiol* **593**: 2279, 2015; Vyklicky et al., *Sci Rep* **5**: 10935, 2015; Vyklicky et al., *J Neurosci* **36**: 2161, 2016) and muscarinic acetylcholine receptors (Randakova et al., *Neuropharmacology* **133**: 129, 2018). We identified several mechanisms of TRP channels regulation including phosphoinositide binding (Macikova et al., *FEBS J* **286**: 3664, 2019), and phosphorylation and N-glycosylation (Hynkova et al., *Sci Rep* **6**: 28700, 2016; Marsakova et al., *Front Mol Neurosci* **10**: 16, 2017). At the cellular level, we investigated expression and trafficking of glutamate receptors (Petrovic et al., *Nat Neurosci* **20**: 529, 2017; Skrenkova et al., *Front Mol Neurosci* **11**: 188, 2018; Skrenkova et al., *Sci Rep* **9**: 12303, 2019), P2X receptors (Ivetic et al., *Front Cell Neurosci* **13**: 284, 2019). and the involvement of these mechanisms in the regulation of neurotransmission.

Within the tissue context, we focused on functional remodelling of neural tissues during development or various physiological and pathological processes. We identified and analysed action of ion channels involved in electrical activity and calcium signalling in pituitary corticotrophs (Zemkova et al., *Endocrinology* **157**: 1576, 2016; Fletcher et al., *J Neurophysiol* **117**: 2298, 2017). We identified cellular mechanisms responsible for the modulation of nociceptive synaptic transmission that involve TRP receptors (Li et al., *J Neurosci* **35**: 13487, 2015). Further, we investigated how various intracellular and extracellular signals mediate mechanical allodynia and enhanced responses to TRPV1 agonist capsaicin (Nerandzic et al., *Br J Pharmacol* **175**: 2322, 2018; Adamek et al., *Neuropharmacology* **146**: 163, 2019). At the tissue/organ level, we studied various aspects ofneurodevelopment. Namely, we studied the role of signalling proteins (Sema3F, Pin1, CRMP2) in axon guidance, axon pruning, and dendritic spine remodelling. We demonstrated that CRMP2 mediates Sema3F-dependent synapse pruning and that its dysfunction shares histological and behavioural features of ASD (Ziak et al., *EMBO Rep* **21**: e48512, 2020).

At the system level, we studied neuroinflammatory changes in the central nervous system, circadian rhythms, epilepsy, and learning, memory, and cognitive functions related to schizophrenia, AD, OCD, and narcolepsy. In the research of ***neuroinflammation***, we found that losartan treatment attenuates the development of neuropathic thermal hyperalgesia (Kalynovska et al., *Life Sci* **220**: 147, 2019) and that the sodium channel blocker protoxin II reduces burn injury-induced spinal nociceptive processing (Torres-Perez et al., *J Mol Med (Berl)* **96**: 75, 2018).

In the research of **circadian rhythms,** we focused on the changes of the circadian clock during the lifespan, the role of hormones and neuromodulators in the circadian regulation, the circadian clock in brain function and memory, and the human circadian system in neuropsychiatric disorders. We found that although the adult master clock in suprachiasmatic nuclei (SCN) is resilient to glucocorticoids, these hormones can entrain the fetal and neonatal master clock in SCN. The clock located in the maternal placenta serves as a glucocorticoid sensitive gatekeeper to control and deliver proper levels of glucocorticoids to the fetal SCN (Cecmanova et al., *J Biol Rhythms* **34**: 307, 2019). Proper maternal care may protect offspring from the development of pathological symptoms even if they are genetically programmed (Olejnikova et al., *Chronobiol Int* **32**: 531, 2015; Olejnikova et al., *J Physiol* **596**: 5757, 2018; Olejnikova et al., *Acta Physiol (Oxf)* **223**: e13020, 2018). Ageing does not impair the ability of the circadian clock in the SCN and the pancreas to generate a rhythmic signal, but it impairs

their ability to control output rhythms (Polidarova et al., *Chronobiol Int* **34**: 1, 2017; Novosadová Z. et al., *Sci. Rep.* **8**:11668, 2018). Thefunctional state of the circadian system in patients with bipolar disorder may vary depending on arousal state as accompanied with the episodes of mania and depression (Novakova et al., *Bipolar Disord* **17**: 303, 2015).

In the research of **epilepsy**, we focused on the mechanisms of epileptogenesis, epilepsy-related comorbidities, and the long-term impact of early pharmacological intervention on brain development. We developed new diagnostic techniques for epilepsy, and we searched for new potential anti-seizure drugs. To this end, we found that theactivation of endothelin B receptors results in the development of non-ischemia related seizures associated with an inflammatory process (Vondrakova et al., *Exp Neurol* **328**: 113255, 2020). We found that hyperthermia aggravates long-term outcome of status epilepticus (Suchomelova et al., *Neuroscience* **305**: 209, 2015), and we identified the keyrole of oxidative stress in the pathogenesis of epilepsy (Folbergrova et al., *Front Cell Neurosci* **10**: 136, 2016; Folbergrova et al., *Mol Neurobiol* **55**: 7512, 2018). In the research of the long-term impact of early brain insults on brain development, we found that early exposure to benzodiazepines has long-term impacts (Kubova et al., *Front Mol Neurosci* **11**: 382, 2018) and that long-term neonatal stress affects habituation to the experimental environment and impairs an ability to sustain attention to stimuli in adulthood (Holubova et al., *Behav Processes* **149**: 59, 2018). In our research of epilepsy-related pharmacology, we identified two possible therapeutic targets in early-life seizures, the adenosine A_1 and glutamate NMDA receptors (Fabera et al., *Front Pharmacol* **10**: 656, 2019; Szczurowska et al., *Epilepsy Res* **109**: 106, 2015). Moreover, $GABA_A$ antagonists have anti-convulsive effectsat certain stages of brain development (Mares, *Eur J Pharmacol* **818**: 26, 2018). In the development of new antiepileptics, we focused on neurosteroids and NMDA antagonists. In the development of new diagnostic methods, we focused on biomarkers of epileptogenesis, such as the integrity of the blood-brain barrier (Svoboda et al., *Physiol Res* **68**: 37, 2019), and monitoring of brief interictal epileptiform discharges. We found that the transition to seizure is not a sudden phenomenon, but it is instead a slow process characterised by the progressive loss of neuronal network resilience (Chang et al., *Nat Neurosci* **21**: 1742, 2018; Chvojka et al., *Epilepsy Behav* 106591, 2019).

In the research of **learning, memory, and cognitive functions**, we focused on brain areas and neurotransmitter systems (cholinergic, glutamatergic) related to cognitive symptoms in AD and schizophrenia. We showed that specific brain areas, namely the hippocampus, anterior cingulate and retrosplenial cortices, and neuronal circuits and cell populations, are involved in spatially- and task-oriented behavioural processes (Brozka et al., *Neurobiol Learn Mem* **141**: 93, 2017; Levcik et al., *Neurobiol Learn Mem* **155**: 127, 2018; Svoboda et al., *Front Psychiatry* **8**: 215, 2017). We thoroughly examined multiple biological levels in search of a common neurophysiological mechanism of cognitive symptoms in schizophrenia (Buchtova et al., *Hippocampus* **27**: 134, 2017; Krajcovic et al., *Acta Physiol (Oxf)* **226**: e13282, 2019; Uttl et al., *Front Pharmacol* **9**: 42, 2018; Szczurowska et al., *Prog Neuropsychopharmacol Biol Psychiatry* **81**: 275, 2018) and AD *(Petrasek et al., Front Aging Neurosci **10**: 250, 2018; Petrasek et al., Front Aging Neurosci **8**: 83, 2016; Horak et al., Prog Neuropsychopharmacol Biol Psychiatry **75**: 54, 2017)*. We showed that 7-methoxy derivative of tacrine is the open-channel blocker of the NMDA receptor and has neuroprotective and pro-cognitive effects that may be beneficial in the treatment of AD (Rambousek et al., *Neuropharmacology* **105**: 594, 2016; Kaniakova et al., *Neuropharmacology* **140**: 217, 2018; Wesierska et al., *Neurobiol Learn Mem* **162**: 59, 2019; Laczo et al., *Neurobiol Aging* **51**: 67, 2017). In two mouse models of AD, we have showed that lipid-based diets improve cholinergic neurotransmission in the hippocampus (Janickova et al., *Curr Alzheimer Res* **12**: 923, 2015; Dolejsi et al., *J Neurochem* **136**: 503, 2016). These results demonstrate that lipid-based diets represent a viable complement to the pharmacological treatment of AD.

In healthy volunteers, we focused on the translation of rodent model tasks tohumans and basic research into the electrophysiology of human spatial orientation concerning AD and schizophrenia. Our methods testing visual and non-visual spatial navigation exert high discrimination accuracy among AD, mild cognitive impairment, and healthy control groups (Laczo et al., *Neurobiol Aging* **51**: 67, 2017; Mokrisova et al., *Behav Brain Res* **307**: 150, 2016). In the research related to schizophrenia, we focused on visuospatial abilities and how they influence patients' quality of life (Rodriguez et al., *Front Behav Neurosci* **9**: 322, 2015). In our

current research, we describe extensive networks of the spatial-scene or object selective brain areas consisting of the occipital place area, the parahippocampal area, and retrosplenial complex and object processing in the lateral occipital complex area.

In the **computational approach**, we employed theoretical methods to describe and understand particular processes in neural systems on the level of single cells or populations. The focus was mainly on the neural coding problem, mathematical models of neuronal activity, and biophysical modelling of axon growth and circuit formation. Namely, we showed how olfactory receptor neurons adjust their encoding to pheromone fluctuations (Levakova etal., *PLoS Comput Biol* **14**: e1006586, 2018). We described mechanisms of axon fasciculationand network formation (Smit et al., *Elife* **6**: 2017), the rate coding capabilities of neurons (Barta et al., PLoS Comput Biol 15: e1007545, 2019) and established a critical size of the neural population for reliable information transmission (Kostal et al., Phys Rev E 100: 050401, 2019). We studied the entropy factor for randomness quantification in neuronal data (Rajdl et al., *Neural Netw* **95**: 57, 2017) andthe instantaneous firing rate of neurons (Kostal et al., *Chaos* **28**: 106305, 2018).

3. Cardiovascular research

The IPHYS research into physiology and pathophysiology of the cardiovascular system may be divided into four main closely related directions: (i) systemic hypertension, ii) myocardial ischemia and heart failure, (iii) cardiac development, and (iv) vascular replacement.

In the research of **systemic hypertension**, we mainly studied the role of principal vasoactive systems in the mechanisms of blood pressure regulation in various hypertensive rat models. We showed that augmented sympathetic vasoconstriction plays a major role in the development and maintenance of hypertension in spontaneously hypertensive rats (SHR) (Behuliak et al., *Hypertension* **72**: 676, 2018), Ren-2 transgenic rats (TGR) with angiotensin II-dependent hypertension (Vaneckova et al., *J Hypertens* **33**: 161, 2015), and Dahl rats with salt hypertension (Zicha et al., *Physiol Res* **68**: 873, 2019). We also demonstrated (i) different changes of the sympathetic nervous system in prehypertensive SHR and in SHR with established hypertension (Vavrinova et al., *Hypertens Res* **42**: 949, 2019; Vavrinova et al., *Hypertens Res* **42**: 1872, 2019), (ii) the hypotensive effect of gabapentin mediated by voltage-dependent calcium channels (Behuliak et al., *Hypertension* **72**: 676, 2018), and (iii) the diverse contribution of calcium influx and calcium sensitization to blood pressure regulation in various forms of hypertension (Behuliak et al., *Biomed Res Int* **2017**: 8029728, 2017). In hypertensive TGR, the enhanced blood pressure reduction induced by either the calcium channel blocker nifedipine or the Rho-kinase inhibitor fasudil is related to impaired baroreflex efficiency (Vaneckova et al., *Hypertens Res* **42**: 145, 2019). Regarding the involvement of the endothelin vasoconstrictor system, we showed that the reduced calcium influx is responsible for the antihypertensive action of a selective ETA receptor blocker in TGR (Vaneckova et al., *J Hypertens* **33**: 161, 2015). Importantly, a substantial reduction of blood pressure was achieved by sodium nitrate or beetroot supplementation in salt-sensitive hypertensive Dahl rats (Morris et al., *Hypertension* **73**: 1042, 2019). Our results also provide evidence for a new role of Plzf (promyelocytic leukaemia zinc finger) in the regulation of blood pressure, cardiac hypertrophy, and fibrosis (Liska et al., *Hypertension* **69**: 1084, 2017).

Regarding **myocardial ischemia and heart failure**, we focused on beneficial effects of a therapy based on synthetic analogues of epoxyeicosatrienoic acids (EETs) and inhibitorsof soluble epoxide hydrolase, an enzyme responsible for conversion of EETs to inactive metabolites. We showed that cardioprotection induced by EETs analogue is mediated by stabilisation of hypoxia-inducible factor-1α (Hif-1α) due to downregulation of its degrading enzyme prolyl hydroxylase-3 at reperfusion (Neckar et al., *Am J Physiol Heart Circ Physiol* **315**: H1148, 2018). We also demonstrated that EETs analogue attenuates post-infarction heart failure progression in normotensive rats (Hrdlicka et al., *Front Pharmacol* **10**: 159, 2019) and in SHR without affecting blood pressure (Neckar et al., *Clin Sci (Lond)* **133**: 939, 2019). EET-based therapy in the setting of myocardial infarction was ineffective in hypertensive Ren-2 transgenic rats, except for reducing blood pressure and life-threatening ventricular arrhythmias (Cervenka et al., *J Hypertens* **36**: 1326, 2018). In rats with chronic heart failure induced by volume overload, we showed that severity of morphological phenotype (hypertrophy) correlated with a drop of connexin 43 phosphorylation and electrophysiological changes

related to arrhythmogenesis (Sedmera et al., *Front Physiol* **7**: 367, 2016).

Research into the molecular mechanism of **myocardial protection** induced by chronic hypoxia revealed a crucial role of antioxidant defence activated by tumour necrosis factor-α via its type-2 receptor signalling (Chytilova et al., *Acta Physiol (Oxf)* **214**: 97, 2015). Using the conplastic SHR strain harbouring mitochondrial genome of normotensive rats, we showed that mitochondrial DNA modulates the cardioprotective effects of chronic hypoxia (Neckar et al., *Clin Sci (Lond)* **131**: 865, 2017). Regular exercise during hypoxia does not further augment the improved ischemic tolerance, suggesting a common protective mechanism dependent on persisting antioxidant response (Alanova et al., *J Appl Physiol (1995)* **122**: 1452, 2017).

In the research of **cardiac development**, we participated in a study which showed that conditional deletion of Hif-1α suppresses the embryonic development of cardiac sympathetic innervation and results in coronary artery anomalies and decreased cardiac contractility (Bohuslavova et al., *Proc Natl Acad Sci U S A* **116**: 13414, 2019). Globalreduction of Hif-1α gene dosage increases the predisposition of mouse offspring exposed to maternal diabetes to cardiac dysfunction (Cerychova et al., *Cardiovasc Diabetol* **17**: 68, 2018). These results underscore Hif-1α as a critical transcription factor in the fetal programming of adult cardiovascular disease. We also revealed the novel role of mitochondrial tryptophanyl-tRNA synthetase WARS2 as a determinant of angiogenesis in theheart and other tissue (Wang et al., *Nat Commun* **7**: 12061, 2016). In studies on developing hearts, we showed that the presence of a specialised conduction system in the vertebrate ventricle is linked to the ventricular septum rather than to homeothermy, as was believed previously (Hanemaaijer et al., *Development* **146**: 2019).

Regarding **vascular replacement**, we succeeded in reconstructing a continuous endothelial cell layer on the inner surface of synthetic polymeric vascular prostheses used in current clinical practice, i.e. prostheses made of expanded polytetrafluoroethylene or polyethylene terephthalate. Their inner surface is coated with fibrin layers of nanofibrous morphology, which allowes for the development of a confluent, mature endothelial cell layer. These vascular prostheses are not suitable for reconstruction of tunica media containing vascular smooth muscle cells (VSMC). Therefore, we used decellularized porcine matrices, which maintain a similar structure and composition as the original tissue *in vivo*. For recellularisation of decellularised blood vessels or pericardium, we used human adipose tissue-derived stem cells obtained by liposuction and expanded *in vitro* in our dynamic bioreactors for planar and tubular tissue (Musilkova et al., *Biomed Mater* **15**: 015008, 2019). These cells can easily differentiate towards VSMC as demonstrated in our laboratory. Three main factors for the differentiation were used: (i) scaffolds with appropriate mechanical properties for soft tissue engineering, i.e. decellularised matrices or fibrin gel, (ii) appropriate composition of the cell culture media containing transforming growth factor-β and bone morphogenetic protein-4, and (iii) appropriate mechanical stress generated in the bioreactor. Under these conditions, the ASCs synthesise alpha-actin, an early marker of VSMC differentiation, and calponin, an intermediate marker of VSMC differentiation (Bacakova et al., *Muscle Cell and Tissue: Current Status of Research Field*, **book chapter**, 2018). We alsoused porcine ASCs for recellularisation of porcine blood vessels and started implantations of these constructs into pigs in collaboration with the Institute for Clinical and Experimental Medicine (**IKEM**).

Research activity and characterization of the main scientific results

The main results obtained in the field of cardiovascular tissue engineering

In this field, we have attempted (1) to reconstruct a continuous endothelial cell layer on the inner surface of synthetic polymeric vascular prostheses used in current clinical practice, i.e. prostheses made of expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (PET), (2) to prepare our own tissue-engineered small-diameter vascular replacements and, (3) to use human or porcine pericardium for constructing replacements of heart valves.

The inner surface of synthetic polymeric vascular prostheses is usually bioinert, e.g. too hydrophobic, and in the case of knitted vascular replacements, it is rough and of irregular morphology, and thus it is not suitable for direct adhesion and growth of endothelial cells. Therefore, the inner surface was coated with fibrin layers of nanofibrous morphology, which were prepared by an *in vitro* simulation of a part of physiological hemocoagulation, in collaboration with the Institute of Macromolecular Chemistry of the Czech Academy of Sciences (Dr. E. Brynda, Dr. T. Riedel). These nanolayers then allowed the development of a confluent, mature endothelial cell layer (**Fig. 1**).

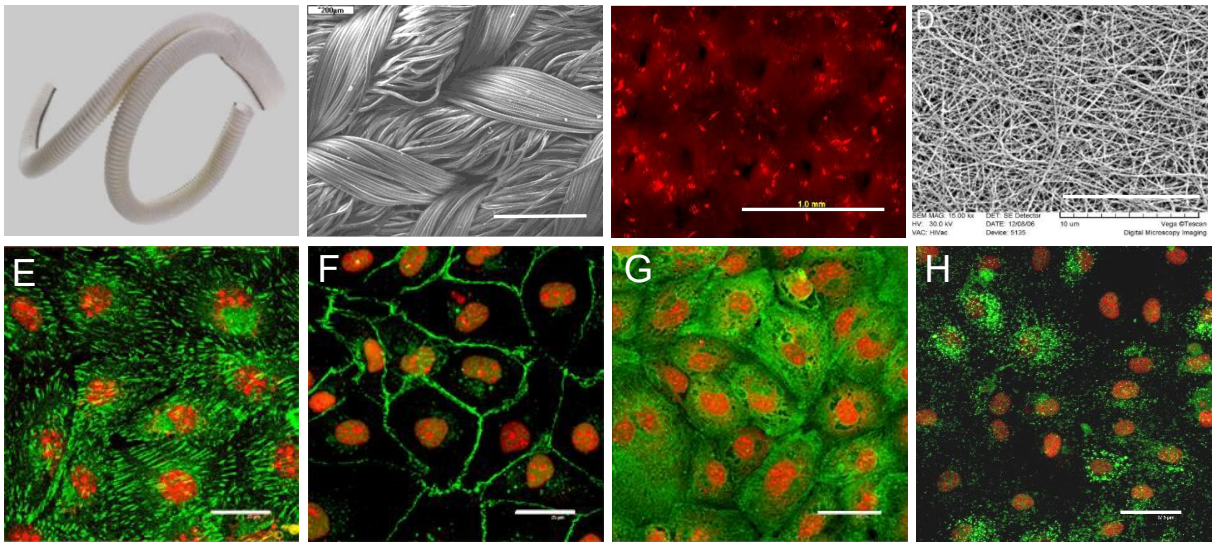


Figure 1. *Example of innovation of a synthetic polymeric vascular prosthesis by endothelialization.* ***A****: A knitted vascular prosthesis made of PET in the VUP Joint-Stock Company, Brno, Czech Republic;* ***B****: The inner surface of the prosthesis;* ***C****: poor adhesion of vascular endothelial cells on the inner surface (sparse and non-spread cells);* ***D****: coating of the inner surface with a fine nanofibrous fibrin layer;* ***E-F****: a confluent endothelial cell layer on fibrin; immunofluorescence staining for talin, a protein of focal adhesion plaques and a marker of cell-material adhesion (****E****), for VE-cadherin, a marker of cell-cell adhesion (****F****), cytoskeletal protein β-actin, a marker of cell spreading (****G****) and for von Willebrand factor, a marker of phenotypic maturation of endothelial cells (****H****). Scale bar 400 μm (B), 1 mm (C), 10 μm (D), 25 μm (E-F).*

However, currently-used synthetic polymeric vascular prostheses are not suitable for reconstruction of *tunica media*, another important layer of the blood vessel containing vascular smooth muscle cells (VSMC), which perform the contractile function. While endothelial cells form a monolayer, i.e. a 2D structure on the inner surface of a vascular replacement, VSMCs need to grow in a 3D environment similarly as in a physiological blood vessel *in situ*. Matrices prepared by decellularization of porcine blood vessels were used as 3D scaffolds in our experiments. It has been reported that after decellularization, the tissues lose a major part - up to 90% - of their immunogenic potential. From this point of view, decellularized matrices could

be used in xenogeneous form, e.g. porcine matrices for reconstruction of blood vessels in human patients. At the same time, decellularized matrices maintain a structure and composition similar to that of the original tissue *in vivo*. In addition, it is believed that decellularized blood vessels, subsequently recellularized with a desired cell type, would have more suitable mechanical properties than synthetic polymeric vascular replacements. We also intended to use decellularized and recellularized pericardium in cardiovascular tissue engineering, e.g. as vascular patches or for constructing heart valve replacements. Porcine blood vessels and pericardium for decellularization and recellularization were obtained within joint grant-funded projects with the Institute of Clinical and Experimental Medicine (IKEM; Prof. J. Pirk, Dr. J. Chlupac).

Another challenge in advanced tissue engineering is the use of an appropriate cell type for recellularizing a decellularized matrix. For reconstruction of the *tunica media*, we usedhuman adipose tissue-derived stem cells (ASCs). These cells can be obtained in a relatively high quantities and by a less invasive method, i.e. by liposuction. The ASCs obtained within the framework of a joint grant-funded project with the Bulovka Hospital in Prague (under ethical approval and with informed consent of the patients, in collaboration with Dr. M. Molitor) were expanded *in vitro* and were used for recellularizing decellularized blood vessels or pericardium (**Fig. 2**)., Dynamic bioreactors for planar and tubular tissues, constructed in our lab, were used for decellularizing and recellularizing these matrices [1].

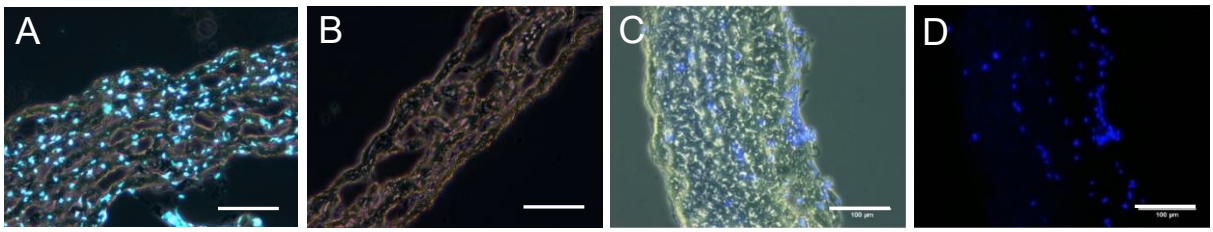


***Figure 2**. Decellularization of porcine pericardium in an automatic lab-made decellularization system. **A**: native pericardium, **B**: decellularized pericardium after treatment with sodium dodecyl sulphate (SDS), DNAse and final washing. **C, D**: pericardium recellularized for 10 days with adipose tissue-derived stem cells in a lab-made pulsatile pressure bioreactor (physiological pressure 120/80 mmHg, 1.2 Hz pulse rate). Cryogenic section 50 µm thick, DAPI-stained cell nuclei. Native and fluorescence-stained images merged (C), fluorescence image of cells only (D). Scale bar 100 µm.*

ASCs can be relatively easily differentiated towards VSMC, as proven in our laboratory. Three main factors for the differentiation were used: (1) scaffolds with appropriate mechanical properties for soft tissue engineering, i.e. decellularized matrices or a fibrin gel, (2) appropriate composition of the cell culture media, e.g. with the addition of transforming growth factor beta (TGF-β) and bone morphogenetic protein-4 (BMP-4), and appropriate mechanical stress, e.g. pulsatile pressure stress generated in our lab-made bioreactor. Under these conditions, the ASCs synthesized alpha-actin, an early marker of VSMC differentiation, and calponin, an intermediate marker of VSMC differentiation [2] (**Fig. 3**).

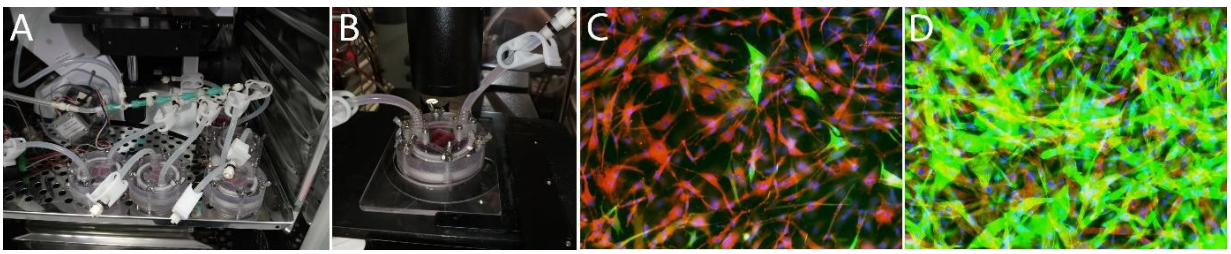


***Figure 3**. **A**: a lab-made dynamic cell culture system generating pulse pressure stress used for mechanical stimulating adipose tissue-derived stem cells (ASCs). **B**: detail of a chamber*

of this system with microscopic live-cell imaging. ***C, D****: Immunofluorescence staining of SM α-actin (red) and calponin (green), i.e. markers of differentiation towards vascular smooth muscle cells, in ASCs cultured for 7 days in a fibrin gel deposited on glass in a medium with TGF-β1 and BMP-4 under static conditions (C) and under dynamic conditions (D).*

Similar experiments were also performed with porcine ASCs (PrASC), which were isolated from pigs within joint grant-funded projects with IKEM. We used these cells for recellularizing decellularized porcine blood vessels and pericardium in dynamic cell culture systems. We also started implantating these constructs recellularized with autologous PrASCs into laboratory pigs, in collaboration with IKEM.

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The main results obtained in the field bone tissue engineering

Engineering of bone replacements in our laboratory was carried out in two main directions: (1) development of novel surface modifications of metallic bone implants currently used in orthopaedic practice in order to achieve better osseointegration, secondary stability and long-term durability of the implants, and (2) bone tissue engineering based on 3D matrices colonized with cells.

The metallic bone implants in our studies usually involved replacements of big joints, such as hip, knee and shoulder joints. We concentrated on the parts of these prostheses which are integrated into the bone tissue, e.g. stems anchored to the femur and cups anchored to the iliac bone, in the case of the total hip replacements. We participated in the development of various coatings of these integral parts of joint prostheses by investigating the behaviour of osteoblast and bone marrow stromal cells on these coatings, such as the cell adhesion, proliferation, osteogenic cell differentiation, bone matrix deposition and mineralization, and also potential cell damage and immune activation, in order to elaborate the most suitable coatings for firm, long-term osseointegration of the implants. The main investigated coatings included a diamond-like carbon (DLC) layer doped with Cr of Ti (in collaboration with the Institute of Physics of the Czech Acad. Sci., Prof. M. Jelínek), nanocrystalline diamond films terminated by various atoms, chemical functional groups and biomolecules, including BMP-7 (Inst. Physics, Dr. A. Kromka), films of ferroelectric ceramics (Inst. Physics, Dr. P. Vaněk), layers of TiO_2 nanotubes (University of Chemistry and Technology, Dr. Joska), and also zeolite films, particularly novel aluminium-free silicalite-1 films (Heyrovsky Institute of Physical Chemistry of the Czech Acad. Sci., Dr. I. Jirka) (**Fig. 4**).

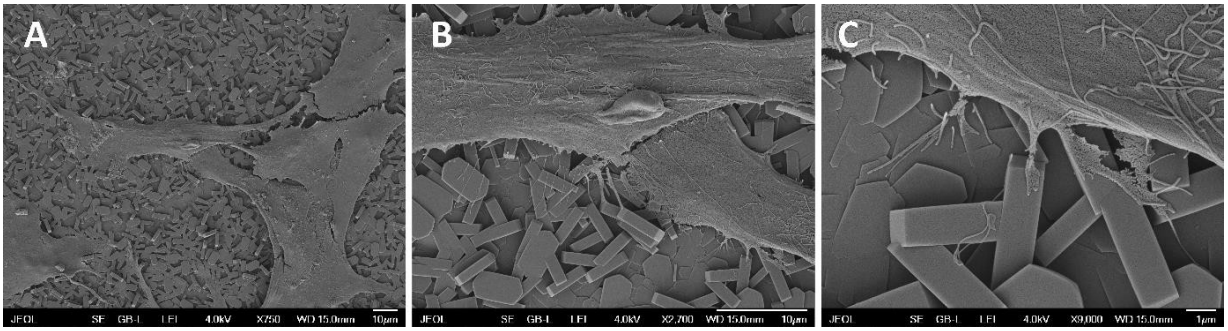

Figure 4. *Human osteoblast-like MG 63 cells on day 1 after seeding on silicalite-1 films for potential bioactive coating of metallic bone implants. Scanning electron microscopy, original magnification 750x (**A**), 2700x (**B**) and 9000x (**C**).*

In the field of bone tissue engineering based on 3D matrices colonized with cells, we investigated the performance of bone-derived cells within various scaffolds based on synthetic and natural polymers, such as degradable electrospun synthetic polymeric nanofibrous scaffolds, made of polylactide (PLA) or polylactide-*co*-glycolide (PLGA), reinforced with ceramic or diamond nanoparticles (in collaboration with the Inst. Physics of the Acad. Sci. CR, Dr. A. Kromka), degradable porous PLGA scaffolds enriched with hydroxyapatite (AGH University of Chemistry and Technology, Cracow, Poland, Prof. E. Pamula), scaffolds based on cellulose/collagen/hydroxyapatite composites (CEITEC – Central European Institute of Technology, Brno University of Technology; Dr. L. Vojtová),and also novel scaffolds based on heat-treated polyacrylonitrile (HT-PAN), also known as Black Orlon, which were obtained in collaboration with the Institute of Macromolecular Chemistry of the Czech Acad. Sci. (Dr. M. Hrubý). Orlon was previously used in fabricating vascular prostheses (in the 1950s), but after heat treatment (i.e., partial carbonization), it emerged as a promising material for bone tissue engineering, due to its mechanical resistance and its specific weight similar to that of the bone tissue. Using succinonitrile,which served as porogen, it was possible to prepare this material in the form of porous sponges, which can be penetrated and colonized with human bone-derived cells (**Fig. 5**).

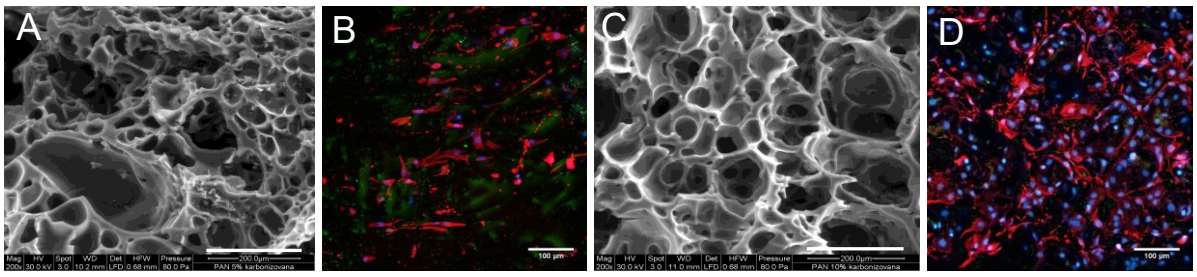

Figure 5*. Morphology of HT-PAN scaffolds prepared from a mixture with an initial content of 5% PAN (**A**) or 10% PAN (**C**) in succinonitrile, and human osteoblast-like MG 63 cells on day 7 after seeding on these scaffolds (B, D). Cells were stained with Texas Red C_2-maleimide (red fluorescence) and Hoechst #33342 (blue fluorescence). Green fluorescence represents autofluorescence of the material. **A, C**: scanning electron microscopy; **B, D**: Leica TCS SPE DH 2500 confocal microscope, summarization of horizontal optical sections (**C**: 20 sections of 4.7 µm in distance, total depth of 95 µm; **D**: 42 sections of 4.9 µm in distance, total depth of 205 µm). Scale bar is 200 µm (A, C) or 100 µm (B, D).*

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The main results obtained in the field of skin tissue engineering

Our work in the field of skin tissue engineering was based on electrospun degradable synthetic polymeric nanofibrous membranes made of polylactide (PLA), polylactide-*co*- glycolide (PLGA) and polycaprolactone (PCL), obtained mainly in collaboration with theTechnical University of Liberec, CR. In order to make these membranes more attractive for skin cells, they were coated with additional fine nanofibrous meshes, which were developedin our laboratory in two forms: (1) meshes coating each individual fiber in the electrospun membrane, and (2) meshes coating each individual fiber and also forming an additional continuous nanofibrous mesh over the electrospun membrane. This second type of nanocoating proved to be more suitable for the adhesion and growth of dermal fibroblasts [1] (**Fig. 6**).

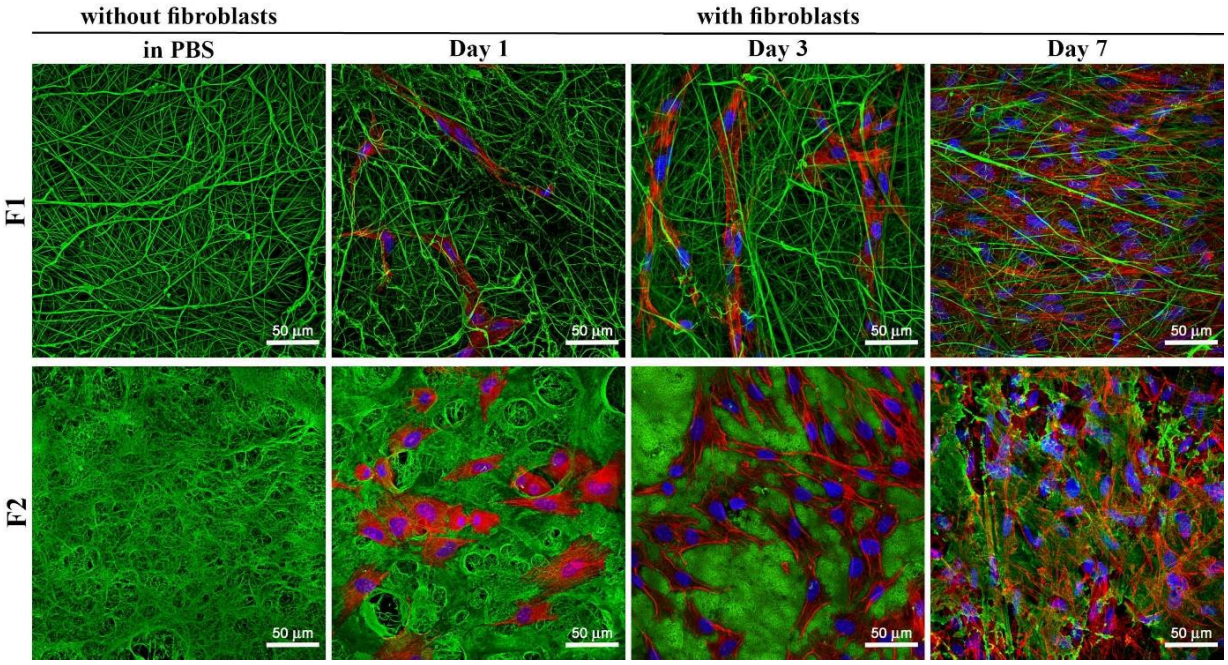

Figure 7*. Immunofluorescence staining of two different types of fibrin nanocoatings on polylactic acid membrane (column 1), and the shape of human dermal fibroblasts on the fibrin nanocoatings (columns 2–4) on days 1, 3, and 7 after cell seeding. F1: the membrane with the fibrin nanocoating covering only individual fibers, F2: the membrane with the fibrin covering individual fibers and forming a mesh on the surface of the membrane The cellswere stained with phalloidin–tetramethylrhodamine (actin cytoskeleton – red) and with Hoechst #33258 (cell nuclei – blue). Leica TCS SPE DM2500 confocal microscope, magnification 40×/1.15 numerical aperture oil.*

In addition, we developed coatings with electrospun nanofibrous meshes with type I collagen, which proved to be more suitable for epidermal keratinocytes. Based on these findings, we

developed a bilayer dermal-epidermal skin construct. First, an electrospun nanofibrous membrane was coated with a type (2) fibrin nanocoating, and was seeded with human dermal fibroblasts. After reaching confluence, the fibroblasts were covered with a hydrogel gel, and were allowed to immigrate and proliferate within this hydrogel. After colonization of the hydrogel with fibroblasts, this construct, mimicking the dermis, was seeded with human epidermal keratinocytes in order to reconstruct the epidermis [2]. The preparation and the morphology of the dermal-epidermal construct is depicted in **Fig. 7**.

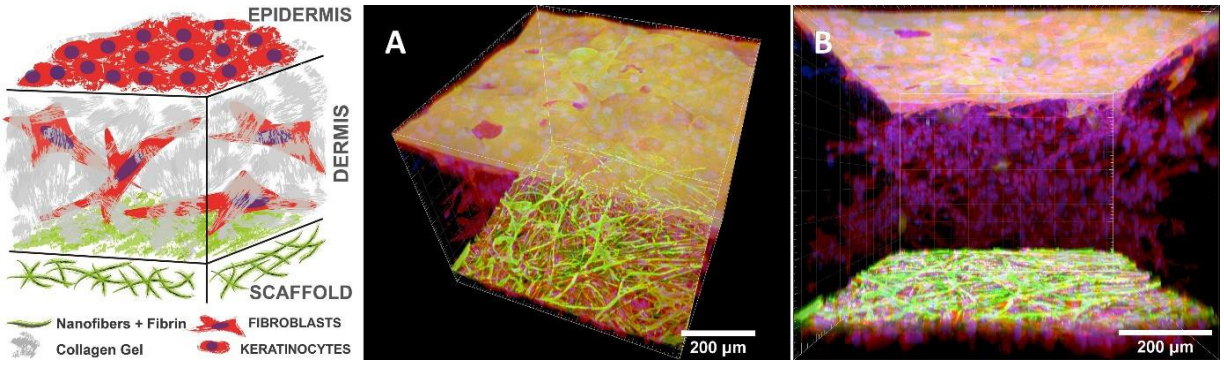

Fig. 7. *Developing a bilayer construct of keratinocytes and fibroblasts on a PLLA nanofibrous membrane modified with fibrin and collagen gel.* ***Left****: schematic design;* ***right*** *(A, B): various views of the real construct.*

References

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Research activity and characterisation of the main scientific results

OUTSTANDING RESULTS

I. **Molecular basis of 14-3-3 protein-dependent activation of yeast neutral trehalase Nth1. In the study published by PNAS USA (**Alblova *et al., Proc Natl Acad Sci USA.* **114**:E9811-E9820, 2017**) we presented the crystal structures of yeast Nth1 (enzyme playing a crucial role in the energy metabolism of yeast) and its complex with the 14-3-3 protein and proposed a molecular mechanism in which 14-3-3 activates Nth1 by stabilizing the flexible part of its active site.**

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 this work we reported the crystal structure of the yeast enzyme Nth1 and its complex with 14-3-3 protein, which, together with mutational and fluorescence studies, indicated that the binding of Nth1 by 14-3-3 triggers Nth1's activity by enabling the proper 3D configuration of Nth1's catalytic and calcium-binding domains relative to each other, thus stabilizing the flexible part of the active site required for catalysis. The activation of the enzyme Nth1 happens during the G1/S transition phases of the cell cycle through phosphorylation and 14-3-3 protein binding. The active enzyme starts to cleave trehalose to glucose, which is used as the energy source of the biosynthetic processes in the laterphases of cell cycle.

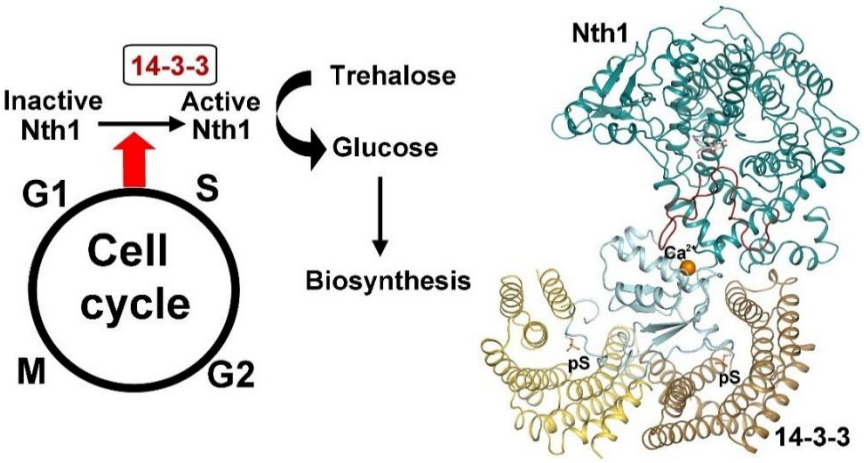


Fig. 1. Detail view on the structure of enzyme Nth1 and its complex with 14-3-3 protein.Nth1 is activated by PKA phosphorylation and subsequent 14-3-3 protein binding in G1/S phase of cell cycle. The active Nth1 triggers the hydrolysis of trehalose to glucose which subsequently serves as the energy source in later phases of cell cycle (Alblova *et al., Proc Natl Acad Sci USA.* **114**:E9811-E9820, 2017).

The reported structure of the 14-3-3:Nth1 complex revealed that the binding of Nth1 by 14-3-3 triggers Nth1's activity by enabling the proper 3D configuration of Nth1's catalytic and calcium-binding domains relative to each other, thus stabilizing the flexible part of the active site required for catalysis. The presented structure of the Bmh1:Nth1 complex highlighted the ability of 14-3-3 to modulate the structure of a multidomain binding partner and to function as an allosteric effector. The reported structure was the second structure of the complex between 14-3-3 and a fully active enzyme reported so far (in 2017) and provided new structural insight into how 14-3-3 proteins regulate bound enzymes. Results of this study are relevant for many other complexes between 14-3-3 and multi/domain enzymes (Alblova *et*

al., Proc Natl Acad Sci USA. **114**:E9811-E9820, 2017). This result was the best publication of IPHYS in 2017.

Supported by Czech Science Foundation (Project 17-00726S, PI: Veronika Obšilová and project 16-02739S, PI: Tomáš Obšil) with the major involvement of group of V. Obšilová/T. Obšil and their postdoc MA and PhD student AS. In collaboration with the group from Organic Chemistry Dpt., Faculty of Science (VD and JV) that contributed by synthesis of dansyl trehalose and Institute of Physics, Charles University (PH) that contributed by time- resolved fluorescence measurements.

II. Innovative method of the Forhkead transcription factor FOXO3 regulation. The study published in prestigious journal eLife (Hagenbuchner *et al., eLife* **8**:e48876, 2019**) identified small molecule compounds that interact with the Forkhead box O3 transcription factor (FOXO3) and modulate its activity.**

FOXO3, with the characteristic fork head DNA-binding domain is part of the O subclass of the forkhead family of transcription factors. These transcription factors have important roles in mammalian cells in regards to regulating cell homeostasis, differentiation, longevity and steer cell death. The activity of FOXO3 in particular contributes to therapy-resistance programs that protect cancer cells during chemo and radiotherapy. Recent studies have also found the DNA-binding domain (DBD) of FOXO aid protein-protein interactions with other keyregulators of longevity and death, and drug resistance. A reversible inhibition of FOXO3 activity by small compounds thereby might boost anti-tumor immune responses.

In order to inhibit the FOXO3 activity, it was first necessary to identify small molecule compounds that could block the interaction between FOXO3 and DNA. Using the structural data of FOXO3 DBD and FOXO4 DBD, we developed six different pharmacophore models that were used for *in silico* screening of small molecule compound databases. Selected compounds were then tested for their ability to inhibit FOXO3 function both *in vitro*, and in cancer cell lines. The interactions of these compounds with FOXO3 DBD were assessed using NMR spectroscopy and docking studies. Our data revealed that the compounds S9 and its oxalate salt S9OX directly interact with FOXO3 DBD and inhibit the FOXO3 activity in cancer cells. In addition, obtained data also suggested that these compounds may interfere with protein-protein interactions of FOXO3. The advantage of these compounds is the strict control of application-dose and -time and the fact that they are not immunogenic allowing repeated applications – so dose- and application time can be adjusted to damage cancercells or boost anti-cancer immunity, but also limit unwanted side effects of FOXO-inhibition on stem cells and other somatic tissues (Hagenbuchner *et al., eLife* **8**:e48876, 2019). This result was listed among the important achievements of IPHYS in 2019.

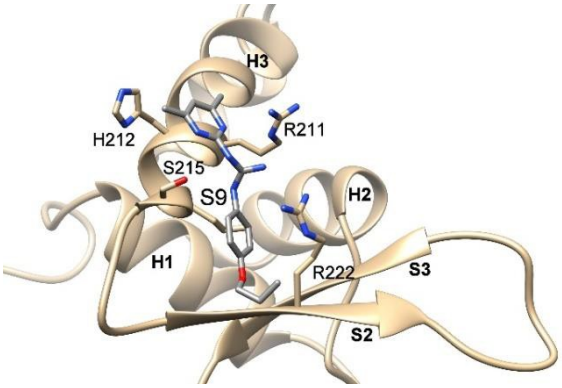


Fig. 2. Compounds S9 blocks the DNA binding surface of Forkhead transcription factor FOXO3. The figure shows the structural model of the DNA-binding domain of FOXO3 with bound compound S9 based on data from NMR measurements and docking simulations (Hagenbuchner *et al., eLife* **8**:e48876, 2019).

The development of specific FOXO inhibitors requires a detailed understanding of structural differences between individual FOXO DNA-binding domains. Therefore, within this project we also reported the high-resolution structure of the DNA-binding domain of FOXO1 and its comparison with structures of other FOXO proteins. This revealed differences in both their conformation and flexibility, which are encoded by variations in protein sequences and account for the distinct functions of FOXO proteins. The availability of apo structures of DNA-binding domains of all three major FOXO proteins will support the development of FOXO- type-specific inhibitors (Psenakova K, *et al., Cells* **8**:pii: E966, 2019).

Supported by Czech Science Foundation (Project 17-33854L and Austrian Science Fund I3089-B28). The whole project was based on a tight collaboration between the groups of prof. T. Obsil and prof. J. Vesely (Faculty of Science), dr. V. Obsilova (IPHYS) and prof. M.J. Ausserlechner (Medical University Innsbruck, Austria). Small molecule compounds were synthesized by group of prof. J. Vesely (member of the research team), the interactions of prepared compounds with FOXO1-DBD, FOXO3-DBD and FOXO4-DBD proteins were investigated by groups of prof. T. Obsil and dr. V. Obsilova. These two groups were also responsible for the preparation of all recombinant proteins, structural study of FOXO1-DBD and all docking simulations. The potency of prepared compounds to interfere with FOXO3 target promoter binding, gene transcription and modulate the physiologic program activated by FOXO3 in cancer cells was investigated by the group of prof. M.J. Ausserlechner. In collaboration with IOCB (VV) which contributed NMR expertise.

KEY RESULTS

14-3-3 protein dependent regulation of caspase-2

The overall goal of this project was to understand the structural basis of the 14-3-3- protein dependent regulation of caspase-2 and to characterize the molecular determinants of this interaction. The results of this project significantly broadened the knowledge about the mechanism of negative regulation of key apoptotic caspase-2 (C2) through the interaction with the adaptor 14-3-3 protein. C2 is activated through dimerization and autoproteolytic cleavage and inhibited through phosphorylation followed by association with the adaptor protein 14-3-3, which maintains C2 in its immature form procaspase (proC2). We showed that human proC2 interaction with 14-3-3 is governed by phosphorylation at two serine residues (139 and 164). Furthermore, we showed that the binding of 14-3-3 to proC2 masks both the nuclear localization sequence (NLS) and the C-terminal region of the p12 domain which is involved in C2 dimerization. Thus, these results indicated that the 14-3-3 binding is an important regulatory element of caspase-2 activation through interference with C2 oligomerization and/or its nuclear localization (Kalabova *et al., Biochem Biophys Res Commun.* **493**:940-945, 2017; Smidova *et al*., *FEBS J.* **285**:4196-4213, 2018).

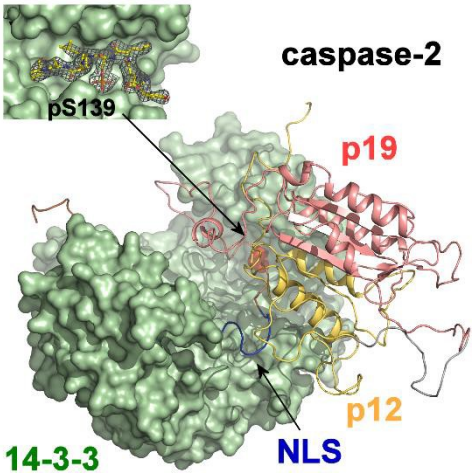


Fig. 3. Caspase-2 is an apical protease responsible for the proteolysis of cellular substrates directlyinvolved in mediating apoptotic signaling cascades.Caspase-2 activation is inhibited by phosphorylation followed by binding to the scaffolding protein 14-3-3. Here we report the structural analysis of the 14-3-3ζ:caspase-2 complex which suggested that 14-3-3 binding masks both the nuclear localization sequence (NLS in blue) and the dimerization interface of caspase-2 formed by p12 domain (in yellow) (Smidova *et al*., *FEBS J.* **285**:4196-4213, 2018).

Supported by Czech Science Foundation (Project 17-00726S, PI: Veronika Obšilová) with the major involvement of group of V. Obšilová/T. Obšil and their postdoc MA and PhD students AS, DK, KP. In collaboration with Institute of Microbiology that contributed chemical cross-linking (MR) and Institute of Physics, Charles University (PH) that contributed by time-resolved fluorescence measurements.

14-3-3-protein-dependent regulation of the activity of Na^+, K^+/H^+ antiporter Nha1

In this work, we identified the residues through which Nha1 interacts with the 14-3-3 protein. Biophysical characterization of the interaction between the C-terminal polypeptide of Nha1 and Bmh proteins *in vitro* revealed that the 14-3-3 protein binds to phosphorylated Ser481 of Nha1, and the crystal structure of the phosphopeptide containing Ser481 bound to Bmh2 provided the structural basis of this interaction. Our data indicated that 14-3-3 binding induces a disorder-to-order transition of the C-terminus of Nha1, and *in vivo* experiments showed that the lack of Ser481 significantly increases cation efflux activity via Nha1, which renders cells sensitive to low K^+ concentrations. Hence, 14-3-3 binding is apparently essential for the negative regulation of Nha1 activity, which should be low under standard growth conditions, when low amounts of toxic salts are present and yeast cells need to accumulate high amounts of K^+ (Smidova *et al., Biochim Biophys Acta Mol Cell Res.* **1866**:118534, 2019).

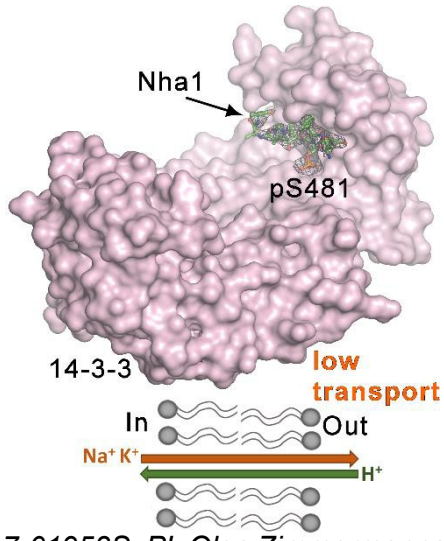


Fig. 4. Yeast housekeeping Na^+, K^+/H^+ antiporter Nha1 interacts with 14-3-3 proteins through the C-terminus at serine 481. This interaction induces a disorder-to-order transition of the Nha1 C-terminus and results in lower cation efflux activity via Nha1. 14-3-3 binding is essential for the negative regulation of Nha1 upon K^+ deficiency.

Supported by Czech Science Foundation (Project 17-01953S, PI: Olga Zimmermannová, Department of Membrane transport). The wholeproject was based on tight collaboration of group of V. Obšilová, her postdoc OP and PhD student AS and group of O. Zimmermannová and her graduate student KS. In collaboration with Institute of Microbiology that contributed two-photon polarization microscopy (JL).

Role of the 14-3-3 proteins in the regulation of kinases ASK1 and CaMKK2

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed mitogen-activated protein kinase kinase kinase 5, which mediates various stress signals including oxidative stress. The catalytic activity of ASK1 is tightly controlled by oligomerization and binding of several cofactors. Among these cofactors, the 14-3-3 and thioredoxin stands outas the most important ASK1 inhibitors. In the first part of this project we contributed significantly to the characterization of the inhibitory mechanism of protein kinase ASK1 through interaction with regulatory proteins 14-3-3 and thioredoxin (TRX1). We performed structural analysis of the complex between the ASK1 kinase domain phosphorylated at Ser966 (pASK1-CD) and the 14-3-3 protein and showed that pASK1-CD:14-3-3 complex is dynamic and conformationally heterogeneous. In addition, we showed that the 14-3-3 proteinbinding inhibits the enzyme activity of ASK1 through several mechanisms including structural modulation of its active site, the steric blocking of Thr838 phosphorylation, and/or the blocking of interaction between ASK1 and its substrates (Petrvalska *et al., J Biol Chem.* **291**:20753-20765, 2016). In non-stress conditions, ASK1 is also inhibited by association with thioredoxin (TRX) which binds to the TRX binding domain (ASK1-TBD) at the N-terminus of ASK1. We showed that from the

two catalytic cysteines of TRX1 the residue Cys32 is responsible for the high-affinity binding of TRX1 to ASK1-TBD in reducing conditions. The disulfide bond formation between Cys32 and Cys35 within the active site of TRX1 is the main factor responsible for the TRX1 dissociation upon its oxidation. The oxidative stress induces intramolecular disulfide bonds formation within ASK1-TBD and affects its structure in regions directly involved and/or important for TRX1 binding (Kylarova *et al., FEBS J.* **283**:3821-3838, 2016; Psenakova *et al. FEBS J.* **doi:** 10.1111/febs.15101).

In the second part of this project we investigated the molecular basis of the 14-3-3-mediated inhibition of CaMKK2 activity. Calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) is a member of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family involved in adiposity regulation, glucose homeostasis and cancer. This upstream activator of CaMKI, CaMKIV and AMP-activated protein kinase is inhibited by phosphorylation, which also triggers an association with the scaffolding protein 14-3-3. We showed that the 14-3-3 gamma protein directly interacts with the kinase domain of CaMKK2 and the region containing the inhibitory phosphorylation site Thr145 within the N-terminal extension. In addition, our data suggested that CaMKK isoforms (CaMKK1 and CaMKK2) substantially differ in their 14-3-3-mediated regulations and that the interaction between 14-3-3 protein and the N-terminal 14-3-3-binding motif of CaMKK2 might be stabilized by small-molecule compounds, e.g. fusicoccins, a diterpenoid glycosides produced by the fungus *Fusicoccum amygdali* (Psenakova *et al., Biochim Biophys Acta Gen Subj.* **1862**:1612-1625, 2018). Moreover, we also showed that interactions between the CaMKK2 kinase domain and the autoinhibitory region differ from those of other CaMKs. In the absence of Ca^{2+}/CaM binding the autoinhibitory region inhibits CaMKK2 by both blocking access to the RP insert and by affecting the structure of the ATP-binding pocket. This corroborated the hypothesis that Ca^{2+}/CaM binding causes unique conformational changes in the CaMKKs relative to other CaMKs (Kylarova *et al., Biochim Biophys Acta Gen Subj.* **1862**:2304-2313, 2018).

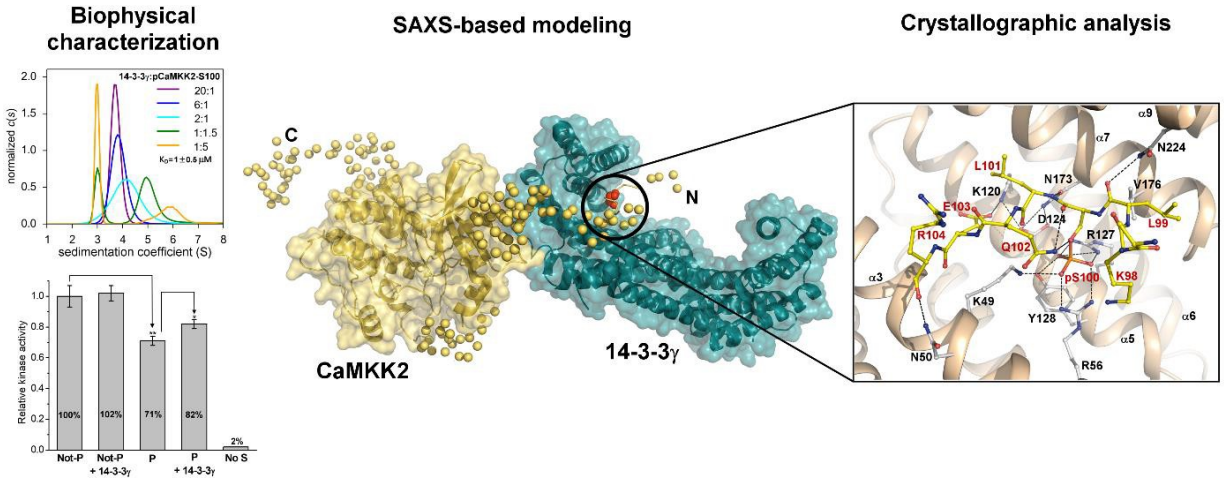


Fig. 5. Mechanistic insight onto the inhibition of CaMKK2 by 14-3-3 protein using the integrative approach of structural biology. The stoichiometry of the complex was elucidatedby analytical ultracentrifugation measurements (AUC) and correlated with the kinase activity measurements. The architecture of their complex, was studied using small-angle x-ray scattering (SAXS). The interaction was supported by crystal structure of the 14-3-3 binding motif of CaMKK2 bound to the 14-3-3.

Supported by Czech Science Foundation (Projects 16-02739S and 19-00121S, PI: Tomáš Obšil) with the major involvement of group of V. Obšilová/T. Obšil and their postdoc OP and PhD students SK, KP, LSD, DKa, DKo. In collaboration with Institute of Microbiology that

contributed H/D exchange and chemical crosslinking (PM, ZK) and Institute of Physics, Charles University (PH, JV) that contributed by time-resolved fluorescence measurements.

Structural basis for the 14-3-3 protein-dependent inhibition of phosducin

Phosducin (Pdc) is a conserved phosphoprotein that, when unphosphorylated, binds with high affinity to the complex of βγ-subunits of G protein transducin ($G_t\beta\gamma$). The ability of Pdc to bind to $G_t\beta\gamma$ is inhibited through its phosphorylation at Ser54 and Ser73 within the N-terminal domain (Pdc-ND) followed by association with the scaffolding protein 14-3-3. In this project we studied interaction between 14-3-3 and phosphorylated phosducin and showed that the 14-3-3 binding affects the structure and the accessibility of several regions within both domains of phosphorylated phosducin by sterically occluding the whole $G_t\beta\gamma$ binding interface of phosducin. Thus, we provided the mechanistic explanation for the 14-3-3- dependent inhibition of phosducin function (Kacirova *et al., J Biol Chem.* **290**:16246-16260, 2015). Next, we performed structural analysis of the complex between 14-3-3 and Pdc using small-angle x-ray scattering, high-resolution NMR spectroscopy, and limited proteolysis coupled with mass spectrometry. Obtained results revealed that phosphorylated Pdc and 14- 3-3 form a complex in which the Pdc-ND region 45-80, which forms a part of Pdc's $G_t\beta\gamma$ binding surface and contains both phosphorylation sites, is restrained within the central channel of the 14-3-3 dimer, with both 14-3-3 binding motifs simultaneously participating in protein association. The N-terminal part of Pdc-ND is likely located outside the centralchannel of the 14-3-3 dimer, but Pdc residues 20-30, which are also involved in $G_t\beta\gamma$ binding,are positioned close to the surface of the 14-3-3 dimer. The C-terminal domain of Pdc is located outside the central channel and its structure is unaffected by the complex formation. (Kacirova *et al., Biophys J.* **112**:1339-1349, 2017).

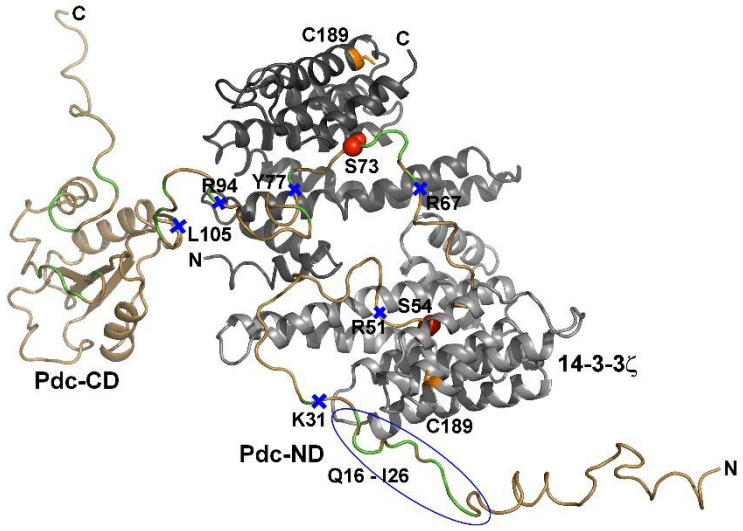


Fig. 6. The best-scoring model of the pPdc:14-3-3 complex calculated using the AllosMod-FoXS simulation. The proteolytic cleavage sites are labeled by blue crosses. The 14-3-3ζ residue Cys189 used as an attachment site for the IPSL spin label in PRE NMR measurements is shown in orange. Region where a significant relaxation enhancement was observed in the presence of the oxidized spin label is marked by blue ellipse.Residues directly involved in $G_t\beta\gamma$ binding are shown in green.

Supported by Czech Science Foundation (Projects 16-02739S, PI: Tomáš Obšil) with themajor involvement of group of V. Obšilová/T. Obšil and their PhD students MK, DKo. Incollaboration with Institute of Microbiology that contributed H/D exchange and chemical crosslinking (PM, AK), Institute of Physics, Charles University (PH, JV) that contributed by time-resolved fluorescence measurements and CEITEC, Brno (JN) that contributed NMR measurement.

Mapping of the intracellular domains of TRPV1 channel as a CaM/S100A1/PIP2 binding sites

The TRPV1 receptor is a member of the TRP channel family. This channel functions as a polymodal signal transducer of noxious stimuli. Ca^{2+} ions are described to participate in the regulation of TRP channels through the interaction with Ca^{2+}-binding proteins, such as calmodulin or S100A1. However, the role of the Ca^{2+}-binding protein S100A1 in the process of TRP channel regulation remains elusive. Here we identified a region on the TRPV1 C- terminus responsible for the interaction with S100A1 and found out that this region overlaps with previously identified calmodulin (CaM) and phosphatidylinositol-4,5-bisphosphate (PIP2) binding sites and that S100A1 competes with calmodulin and PIP2 for this binding site. Our data suggest a mechanism for the mutual regulation of PIP2 and the Ca^{2+}-binding proteins S100A1 and calmodulin to TRPV1 (Grycova *et al., ACS Chem Neurosci.* **18**:386-392, 2015).

The study was made with the major involvement of group of J. Teisinger and his postdoc LG and PhD students BH, MJ and KB. In collaboration with Institute of Microbiology that contributed SPR (LB), B Cube, Dresden (ZL) that contributed by fluorescence measurements.

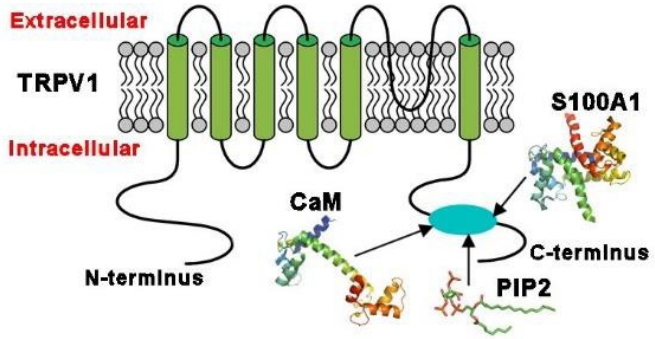


Fig. 7. Architecture of TRMV1 channel with depicted overlappingbinding sites for CaM, S100A1 and PIP2 in cyan oval.

Research activity and characterisation of the main scientific results

As can be seen from the focus of our team, our past, present and also planned research activities are centred around the function of mitochondrial oxidative phosphorylation (OXPHOS) apparatus. In the period 2015-2019 we studied various aspects of the assembly of individual OXPHOS complexes. This represented either pure basic research or it was driven by uncovered gene variants which may be responsible for mitochondrial disease. Such genetic discoveries required further biochemical characterisation and description of particular protein function on mitochondrial biogenesis. Ultimately, we also applied our expertise in mitochondrial metabolism to other clinical conditions in collaboration with various teams of clinicians either from the Czech Republic or international ones. Individual particular achievements are summarised in the following sections.

Mitochondrial ATP synthase

Function and biogenesis of mitochondrial ATP synthase was among the most studied topics in the laboratory of Bioenergetics in the period 2015-2019. It represents continuation of long-lasting research interest in this field. ATP synthase is the key enzyme of the mitochondrial oxidative phosphorylation (OXPHOS) system and hence of the cellular energy provision. The human enzyme is a multisubunit protein complex with stepwise biogenesis from individual subunits towards the whole complex. In mammals, the biogenesis of ATP synthase depends on at least four ancillary proteins. ATPAF1, ATPAF2 and c7orf55 (FMC1) are responsible for the formation of F_1. The last one, TMEM70, is specific for higher eukaryotes and its mutations were historically identified by our laboratory to cause isolated deficiency of ATP synthase. We described the first patient with an ATP synthase deficiency of nuclear origin in 1999 (Houstek et al., *Hum Mol Genet* **8**: 1967, 1999) and associated it with TMEM70 protein in 2008 (Cizkova et al., *Nat Genet* **40**: 1288, 2008). Since then, we try to uncover molecular mechanisms of TMEM70 function and decipher potential therapies in humans.

Animal model of whole body Tmem70 knockout. To study this ancillary factor *in vivo*, we generated Tmem70-deficient mice and found that the Tmem70 ablation results in profound growth retardation and embryonic lethality at 9.5 days post coitum. Studies in affected embryos demonstrated an isolated deficiency in fully assembled ATP synthase (80% decrease) and a marked accumulation of F_1 catalytic complexes indicative of impairment in ATP synthase biogenesis that was stalled at the early stage, following the formation of F_1 oligomer. Consequently, a decrease in ADP-stimulated respiration, respiratory control ratio and ATP/ADP ratios, revealed compromised mitochondrial ATP production. Tmem70$^{-/-}$ embryos exhibited delayed development of the cardiovascular system and a disturbed heart mitochondrial ultrastructure, with concentric or irregular cristae structures. Tmem70$^{+/-}$ heterozygous mice were fully viable and displayed normal postnatal growth and development of the mitochondrial OXPHOS system. Nevertheless, they presented with mild deterioration of heart function. Our results demonstrated that Tmem70 knockout in the mouse results in embryonic lethality due to the lack of ATP synthase and impairment of mitochondrial energy provision (Vrbacky et al., *Hum Mol Genet* **25**: 4674, 2016).

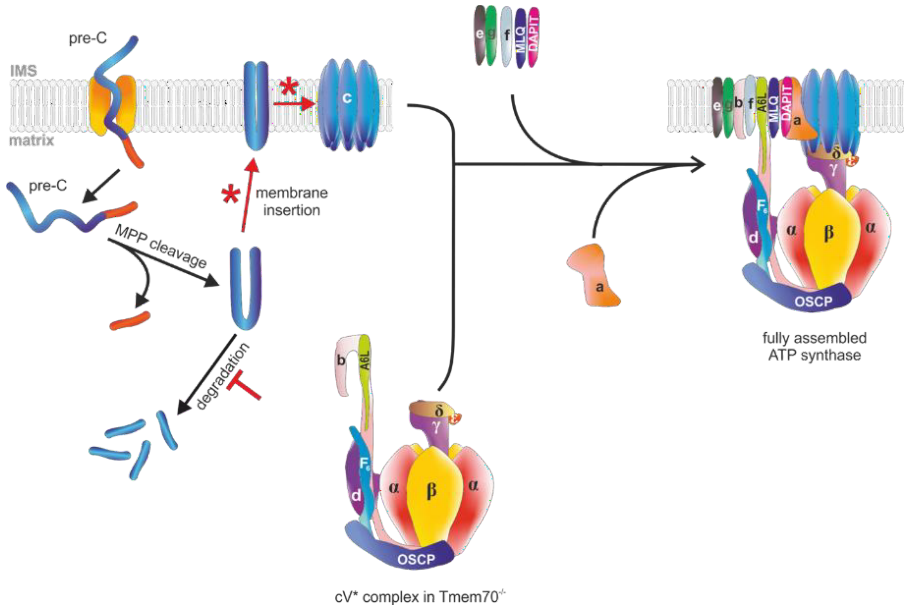


Fig. 1: Characterisation of molecular function for Tmem70 protein. Points of possible Tmem70 action are indicated by red asterisk.

Our studies aimed at understanding TMEM70 function were further based on generation of the model of the tamoxifen inducible knockout, by crossing of Tmem70$^{tm1c(KOMP)Wtsi}$ mice with mice harbouring inducible whole-body Cre recombinase. This approach overcome the embryonic lethality in *Tmem70-/-* mice and enabled TMEM70 excision in adult animals. Using this model, we could finally underpin the molecular nature of TMEM70 function. We demonstrated, that absence of TMEM70 impairs the early stage of enzyme biogenesis by preventing incorporation of hydrophobic subunit c into rotor structure of the enzyme. This results in the formation of an incomplete, pathologic enzyme complex consisting of F_1 domain and peripheral stalk but lacking F_o proton channel composed of subunits c and a.We demonstrated direct interaction between TMEM70 and subunit c and showed that overexpression of subunit c in TMEM70$^{-/-}$ cells partially rescued TMEM70 defect. Accordingly, TMEM70 knockdown prevented subunit c accumulation, which can otherwisebe observed in F_1-deficient cells. From these studies we could conclude that TMEM70 is specific ancillary factor for subunit c. The biologic role of TMEM70 is to increase the low efficacy of spontaneous assembly of subunit c oligomer, the key and rate-limiting step of ATP-synthase biogenesis, and thus to reach an adequately high physiologic level of ATP synthase in mammalian tissues. (Kovalcikova et al., *FASEB J* **33**: 14103, 2019)

ATP synthase central stalk subunits in the assembly of the mammalian enzyme. We followed our previous research into the central stalk assembly (Havlickova et al., *Biochim Biophys Acta* **1797**: 1124, 2010; Mayr et al., *Hum Mol Genet* **19**: 3430, 2010) and this time we focused on the role of the central stalk subunits γ and δ in enzyme assembly, using shRNA knockdown in the human HEK293 cell line. We have found that when the protein levels of γ and δ were decreased to 10-30% of control levels, the content of the fully assembled ATP synthase was decreased in accordance with the levels of the silenced subunits. This was also the case for most structural ATP synthase subunits. In contrast, the hydrophobic c subunit was increased to 130-180%, respectively and unused subunit caggregates were detected by 2D PAGE. In addition, the IF1 protein was upregulated to195% - 300% of control levels.

Cell lines silenced for either γ or δ subunits displayed typical features of decreased ATP synthase functional capacity - low respiratory rate in ADP- stimulated respiration, increased sensitivity of respiration to ATP synthase inhibitor oligomycin, and impaired utilization of mitochondrial membrane potential for ADP phosphorylation. Overall, similar biochemical and structural phenotype of γ, δ and ε subunit deficiencies points to a uniform requirement for assembled central stalk as driver of the c- oligomer attachment in the assembly process of mammalian ATP synthase (Pecina et al., *Biochim Biophys Acta Bioenerg* **1859**: 374, 2018).
We took advantage of the fact, that central stalk subunits knockdown leads to uniform decrease in assembled ATP synthase content without any potentially pathological subassemblies and on the range of knockdown clones analysed pathophysiological adaptations to varying residual content of ATP synthase. We identified, that the threshold for manifestation of the ATP synthase defect equals to 10-30% of residual ATP synthase activity and is associated with metabolic rewiring towards glycolysis. This also helps to explain, why we observe pathological phenotypes in TMEM70 patients, where ATP synthase content drops below 30%. On the other hand, it opens possibility for potential treatment, since only small increase above this threshold may prevent pathogenesis. (Nuskova et al., *Biochem Biophys Res Commun* **521**: 1036, 2020).
All studies into function of ATP synthase or specifically Tmem70 protein were initiated in our laboratory and our team members are first as well as senior authors on them. Collaborations typically involved some in vivo phenotypization techniques, such as echocardiography or embryo histology.

Cytochrome c oxidase

Cytochrome c oxidase (COX), the terminal enzyme of mitochondrial electron transport chain, couples electron transport to oxygen with generation of proton gradient indispensable for the production of vast majority of ATP molecules in mammalian cells. In addition to ATP synthase, COX represents yet another OXPHOS complex, which is historically studied in ourlaboratory. Again, our original interest is usually sparkled by subunits or assembly factors identified as disease-causing genes in patients with mitochondrial myopathies. We build on that foundation and develop further model systems to understand pathological mechanisms. Summary of major outcomes is given below.

SURF1 protein and mechanism of cytochrome c oxidase (COX) deficiency. Surf1 is an important ancillary factor of COX biogenesis and its mutations are the most frequent cause of COX defect in humans. There is mouse SURF1-/- knockout model of the disease, but surprisingly, it presents with much milder phenotype than patients. In our work, we focussed on interspecies differences in COX biogenesis, and compared SURF1-/- mouse tissues and fibroblasts with patient fibroblasts lacking Surf1 protein. Studying the kinetics of COX recovery after depletion, we revealed rather stable proportion between COX monomer and supercomplexes in human control cells, while in SURF1 patient cells COX monomer markedly decreased and assembled COX was preferentially localised into supercomplexes. In mouse cells, however, the recovery mostly proceeded just to the level of COX monomer, without formation of supercomplexes. Pulse-chase metabolic labelling clearly showed higher stability of COX monomer and faster degradation of accumulated COX assembly intermediates in SURF1-/- mouse fibroblasts, while more persistent COX assembly intermediates prevailed over the gradually decreasing COX monomer in patient cells. Our experiments clearly demonstrated crucial importance of the Surf1 protein for effective COX biogenesis in human cells and indicated that its absence is much better tolerated in mouse cells and tissues due to the faster COX turnover. This was collaboration with the group of Prof M. Zeviani who provided

mouse tissue samples and fibroblast cultures (Kovarova et al., *Biochim Biophys Acta* **1862**: 705, 2016).

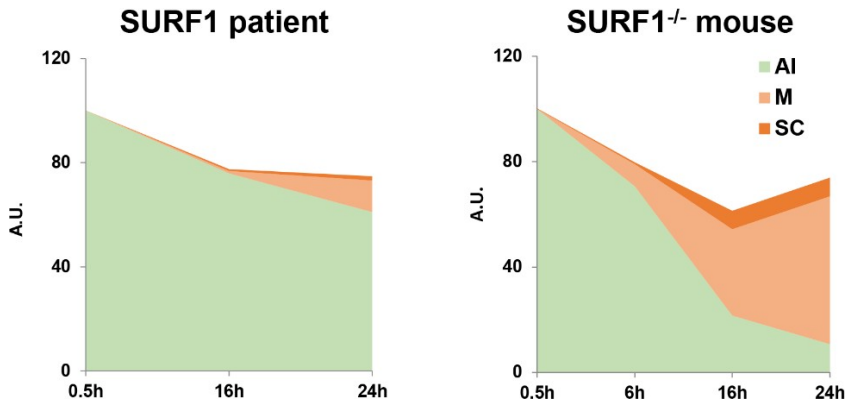


Fig. 2: Differences between patients and mouse SURF1-/- model in the dynamics of COX assembly. AI – assembly intermediate, M – monomer, SC – supercomplexes.

9205delTA microdeletion in mtDNA removes the stop codon of the MT-ATP6 gene and affects the cleavage site in the MT-ATP8/MT-ATP6/MT-CO3 polycistronic transcript. This interferes with the processing of mRNAs for Atp6 and Cox3. To gain more insight into the pathogenic mechanism, we prepared 9205delTA cybrids with mutation load ranging between52 and 99% and investigated changes in the structure and function of ATP synthase and theCOX. The biochemical effects caused by the 9205delTA microdeletion displayed a pronounced threshold effect above approximately 90% mutation heteroplasmy. We observeda linear relationship between the decrease in subunit F_o-a or Cox3 content and the functionalpresentation of the defect. Therefore, we conclude that the threshold effect originated from agene-protein level. (Hejzlarova et al., *Biochem J* **466**: 601, 2015)

Yet another study looking at the role of **isoform switch in Cox IV** subunit was published past the end of the evaluation period but represents culmination of our efforts from theperiod 2016-2019 to decipher regulatory role of Cox IV subunit. In that study, we unanimously demonstrated, that Cox IV-2 isoform increases p_{50} (partial pressure of oxygenat half-maximal respiration), indicating decreased oxygen affinity of COX IV-2 containing enzyme. Our finding thus supported key role of the COX IV-2 containing enzyme in hypoxia sensing pathways of energy metabolism (Pajuelo Reguera et al., *Cells* **9**: 2020).

Functional validation of disease-causing genes

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. Identification of novel disease-causing genes has been eased by the advent of next generation sequencing. However, functional validation of potentially pathogenic gene variants still requires close cooperation between clinical and research laboratories. We managed to established such consortia in Prague through collaborations between clinical researchers from General University Hospital in Prague (groups of Prof J. Zeman and Assoc Prof T. Honzík), molecular geneticists from Institute of Inherited Metabolic Diseases, 1st Faculty of Medicine (group of Prof S. Kmoch) to biochemists in our laboratory. Most importantly, this research also secured substantial funding for the whole period 2015-2019 inthe form of Centre of Excellence for study of mitochondrial biology (MITOCENTRE), provided by Czech Science Foundation.

Since many of studied diseases are ultra-rare, to pool several cases together requires establishment of international consortia for every gene studied. Here the fruitful collaboration emerged with the group of Prof R. Taylor (Wellcome Centre for Mitochondrial Research, Newcastle UK), who helped to establish collaborations in case of NDUFA6 and RMND1 mutations and with whom we also currently collaborate on further cases. During the period 2015-2019 we were involved in description of the following disease-causing genes:

NDUFAF6. Here we characterized the genetic cause and molecular mechanism of dysfunction for Acadian variant of Fanconi syndrome, presenting with generalized proximal tubular dysfunction from birth, slowly progressive chronic kidney disease and pulmonary interstitial fibrosis. Based on exome and genome sequencing in affected families, we identified pathogenic mutation in noncoding region of NDUFAF6 gene encoding assembly factor of respiratory chain complex I. Analysis of pathogenic transcripts indicated that mutation leads to a loss of mitochondrial isoform of NDUFAF6 factor and subsequently to alteration of biogenesis and function of complex I. Pathogenicity of mutation was finally confirmed by complementation – rescue of enzyme defect in patient fibroblasts by transfection with wild-type NDUFAF6. Elucidation of molecular pathogenesis of Acadian variant of Fanconi syndrome will improve diagnostics and prevention of the disease in affected families and broadens the spectrum of the clinical presentation of mitochondrial diseases associated with complex I dysfunction. In this project, our team was responsible for biochemical characterization of samples, analyses of complex I assembly and genetic complementation. One of team members is shared first author (Hartmannova et al., *HumMol Genet* **25**: 4062, 2016).

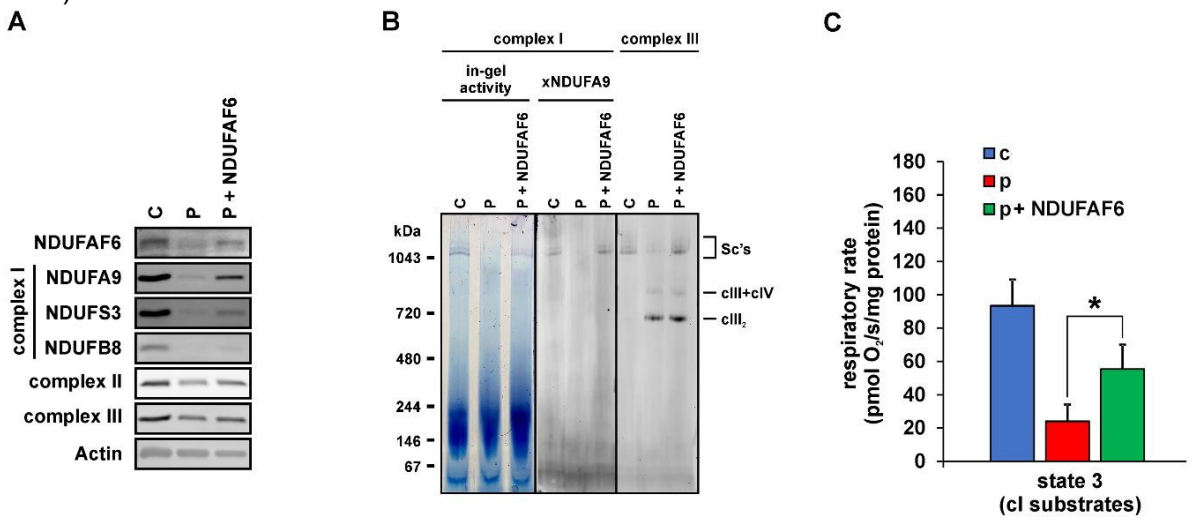


Fig. 3: Complex I defect in fibroblasts of Fanconi syndrome patient is complemented by wtNDUFAF6. A – content of NDUFAF6 protein and of representative subunits from complexes I-III in fibroblasts from. B – Content of native complexes I, III and IV C –Complementation of ADP stimulated respiration by transfection with wtNDUFAF6. (C) control, (P) patient, (P+NDUFAF6) patient transfected with wtNDUFAF6.

NDUFA6. In this case we managed to uncover disease causing gene in four unrelated children who presented with neuroradiological findings and elevated lactate levels, both of which are suggestive of an underlying mitochondrial disease. By NGS sequencing, we identified bi-allelic variants in NDUFA6, which encodes a 15 kDa LYR-motif-containing complex I subunit that forms part of the Q-module. Functional investigations using subjects' fibroblast cell lines demonstrated complex I assembly defects. Complexome profiling confirmed a marked reduction in incorporated NDUFA6 and a concomitant reduction in otherQ-module subunits.

Complementation with wt NDUFA6 normalized complex I levels. These data also support supercomplex formation, where the ~830 kDa complex I intermediate is in complex with assembled complexes III and IV, despite the lack of N-module. Our team was responsible for initial biochemical characterization of one of the subjects. We reclassified itas complex I defect and thus allowed match with exome sequencing data. We also contributed to the write-up of the manuscript (Alston et al., *Am J Hum Genet* **103**: 592, 2018).

RMND1. Ultimately, we were also involved in the characterization of clinical symptoms of RMND1 mutations in a large multicentric study that identified 14 new cases from 11 pedigrees. RMND1 plays an important role in stabilization of the mitochondrial ribosomenear the site of mRNA maturation and its dysfunction can cause multiple mitochondrial respiratory chain deficiencies. In our laboratory we performed analyses on patients from one pedigree. (Ng et al., *J Med Genet* **53**: 768, 2016).

Targeting mitochondria for anticancer therapies

Mitochondria are important players in metabolic adaptations during cancerogenesis. While aerobic glycolysis is often preferred way of ATP production in cancer cells, they do require mitochondria for numerous biosynthetic processes and targeting them may prove as perspective treatment strategy. In many of the studies in this area we joined forces with established teams working in cancer field (e.g. team of Prof J. Trka form 2nd Faculty of medicine, Charles University working on acute leukaemia) and performed metabolic arms of respective studies.

Inhibition of fatty acid oxidation enhances anticancer effects of asparaginase. This study represents good example of such cooperation. L-asparaginase (ASNase), is used for the treatment of childhood acute lymphoblastic leukaemia (ALL). It hydrolyses asparagine and glutamine, which leads to an amino acid deprivation and consequently affects mainly bioenergetics. Treating leukemic cells with ASNase increased mitochondrial fatty acid oxidation (FAO) and cell respiration and concomitantly inhibited glycolysis. FAO was regulated through inhibition of the RagB-mTORC1 pathway, whereas the effect on glycolysiswas RagB-mTORC1 independent. Pharmacological inhibition of FAO significantly increased the sensitivity of ALL cells to ASNase. Therefore, we uncovered novel therapeutic option based on the combination of ASNase and FAO inhibitors. Our part started with proposition of possible metabolic changes in ASNase treated cells and continued with all metabolic analyses. To the contrary, partner initiated project by asking the question, whether ASNase role can go beyond asparagine hydrolysis and also did all pharmacological studies (Hermanova et al., *Leukemia* **30**: 209, 2016).

ROS production by Succinate dehydrogenase (SDH). SDH inhibition can induce cell death, but the mechanistic details need clarification. To elucidate the role of reactive oxygen species (ROS) formation upon the ubiquinone-binding (Qp) site blockade, we substituted CII subunit C (SDHC) residues lining the Qp site by site-directed mutagenesis. I56F and S68A variants did not inhibit SDH, but differed in ROS production and death induction. R72C variant inhibited SDH activity, did not induce ROS nor ROS mediated cell death. These results demonstrated that cell death initiation upon CII inhibition depends on ROS and that the extent of cell death correlates with the potency of inhibition at the Qp site unless intracellular succinate is high. In addition, this validates the Qp site of CII as a target for cell death induction with relevance to cancer therapy. Together with the MitoMet studies (next paragraph), this was result of

collaboration with the team of Prof J. Neuzil from IBT CAS, Prague, who works on potential mitochondrially targeted cancer therapeutics. Our laboratory was involved in analyses of SDH assembly for individual mutant cell lines as well as in measurement of ROS production (Kluckova et al., *Cell Death Dis* **6**: e1749, 2015).

Metformin and MitoMet in mitochondrial physiology. Metformin is a widely prescribed drug against type 2 diabetes mellitus, but also trialled for other conditions, including pancreatic cancer. While molecular target of metformin still remains elusive, one of the prominent candidates is mitochondrial respiratory complex I (CI). Team of Prof J. Neuzil prepared mitochondrially targeted form of metformin (MitoMet) with the idea of improving metformin efficacy for cancer. MitoMet was found to kill a panel of pancreatic cancer cells three to four orders of magnitude more efficiently than metformin and respiration assessment documented CI as the molecular target for MitoMet. We were collaborating on that study and performed respiratory measurements (Boukalova et al., *Mol Cancer Ther* **15**: 2875, 2016). As a follow-up on the MitoMet studies and again within a collaborative project, we demonstrated role of metformin on modulation of oxidative stress in ischemia-reperfusion setting (Cahova et al., *Am J Physiol Gastrointest Liver Physiol* **309**: G100, 2015). All these studies brought us to the conclusion, that it is necessary to dissect in detail the direct role of biguanides on OXPHOS complexes. We initiated our own study and demonstrated that biguanides non-specifically target the activities of all respiratory chain dehydrogenases (mitochondrial NADH, succinate, and glycerophosphate dehydrogenases), but only at very high concentrations (10^{-2} - 10^{-1} M) that highly exceed cellular concentrations observed in patients. We concluded that the beneficial effect of biguanides is probably associated with a subtler mechanism, different from the generalized inhibition of the respiratory chain (Pecinova et al., *Oxid Med Cell Longev* **2017**: 7038603, 2017). This was also further emphasised in our review regarding mitochondrial targets of metformin (Pecinova et al., *Biofactors* **45**: 703, 2019).

Mitochondrial function in cardiovascular physiology

In this field we contributed our expertise in mitochondrial physiology towards collaborative studies with other partners. Notable is our long-lasting cooperation with the Institute of Clinical and Experimental Medicine (IKEM) in Prague on mitochondrial function in failing heart, which was so far supported by two grants from the Czech medical research council. The second project was major collaborative study headed by Prof S. Cook (Duke-NUS Medical School, Singapore), which also involved several laboratories from IPHYS CAS. Details of these outputs are given below.

Role of iron in mitochondrial dysfunction in human heart failure. The myocardial iron deficiency leads to mitochondrial respiratory chain dysfunction in human heart failure. Iron replacement improves clinical status in iron-deficient patients with heart failure (HF), but the pathophysiology is poorly understood. Iron is essential not only for erythropoiesis, but also for cellular bioenergetics. The impact of myocardial iron deficiency (MID) on mitochondrial function, measured directly in the failing human heart, is unknown. LV samples were obtained from 91 consecutive HF patients undergoing transplantation and 38 HF-free organ donors (controls). Myocardial iron content was lower in HF compared to controls and this was independent of anaemia. MID (the lowest iron tercile in HF) was associated with more extensive coronary disease and less beta-blocker usage compared to non-MID HF patients. Compared to controls, HF patients displayed reduced myocardial O_2 respiration and reduced activity of all examined mitochondrial enzymes. MID in HF was associated with preserved

activity of respiratory chain enzymes but reduced activity of aconitase and citrate synthase (by -26% and -15%) and reduced expression of catalase, glutathione peroxidase and superoxide dismutase 2. In conclusion, we demonstrated that myocardial iron content is decreased and mitochondrial functions are impaired in advanced HF. MID in HF is associated with diminished citric acid cycle enzyme activities and decreased ROS-protecting enzymes. MID may contribute to altered myocardial substrate use and to worsening of mitochondrial dysfunction that exists in HF (Melenovsky et al., *Eur J Heart Fail* **19**: 522, 2017).

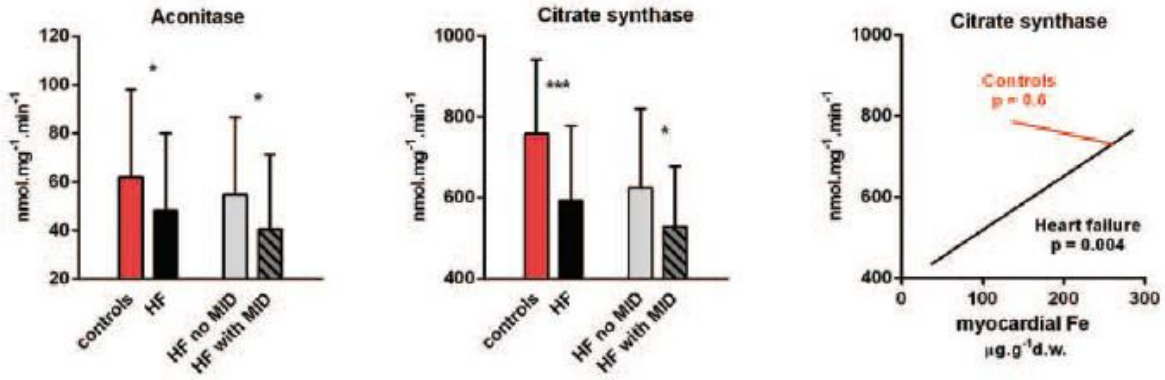


Fig. 4: Aconitase and citrate synthase activities in failing hearts, correlation between citrate synthase activity and myocardial iron content. HF – heart failure, MID – myocardial iron deficiency.

Wars2 as a determinant of angiogenesis Impaired coronary blood flow causes heart disease, affecting millions of people worldwide. In the absence of coronary artery disease, coronary flow (CF) is largely determined by capillary vessel density. Mitochondrial tRNA synthetases are nuclear encoded essential components of intra-mitochondrial protein synthesis of mtDNA encoded subunits of oxidative phosphorylation enzymes. We identified that mitochondrial tryptophanyl-tRNA synthetase (Wars2) is also a determinant of angiogenesis. Coronary flow (CF) measured ex vivo is largely determined by capillarydensity that reflects angiogenic vessel formation in the heart in vivo. By exploiting this relationship, we show that CF in the rat is influenced by a locus on rat chromosome 2 that is also associated with cardiac capillary density. In SHR rat strain the mitochondrial Wars2 encodes an L53F protein variant within the ATP-binding 'HXGH motif', that was prioritized asthe candidate at the locus by integrating genomic data sets. WARS2 (L53F) has low enzymeactivity and inhibition of WARS2 in endothelial cells reduces angiogenesis. In the zebrafish, inhibition of wars2 results in trunk vessel deficiencies and disordered endocardial-myocardialcontact. Inhibition of Wars2 in the rat causes angiogenesis defects and diminished cardiac capillary density. Our data demonstrate a pro-angiogenic function for Wars2 both within and outside the heart that may have translational relevance given the association of WARS2 withcommon human diseases. In this large-scale study, our team was responsible for analysis of mitochondrial phenotypes in congenic and transgenic rat strains and also for analysis of mitochondrial proteosynthesis in derived cells (Wang et al., *Nat Commun* **7**: 12061, 2016). In our subsequent work we also demonstrated, that Wars2 L53F variant in SHR rats also influences the function of brown adipose tissue and can predispose to obesity (Pravenec et al., *Physiol Res* **66**: 917, 2017).

Research activity and characterisation of the main scientific results

Cholinergic mechanisms at the system level

The cholinergic system is involved in many physiological processes. In our laboratory, we have focused on the role of the cholinergic system in behaviour and its changes in Alzheimer's disease. Cholinergic mechanisms at system level we study at transgenic-mouse models. We studied alteration in cholinergic mechanisms at two transgenic-mouse models of Alzheimer's disease (AD).

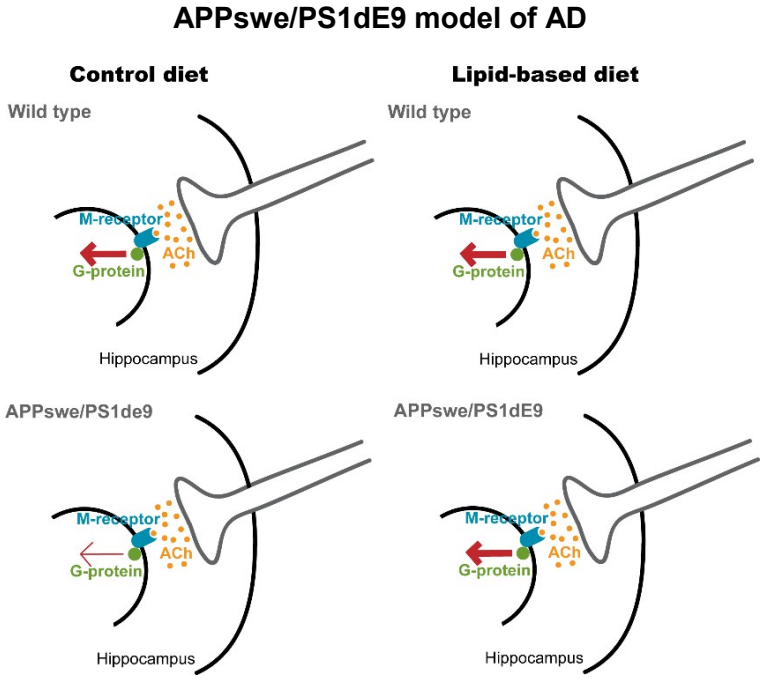
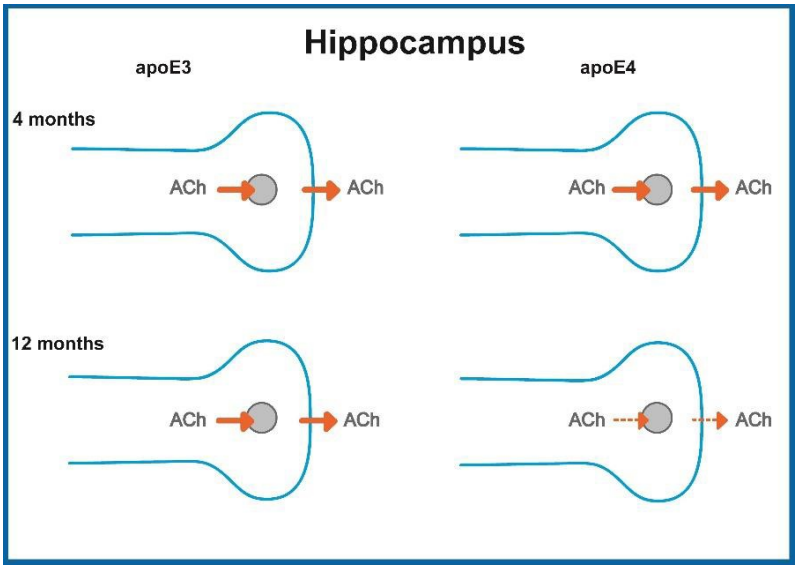


Figure 1 - Lipid-Based Diets Improve Muscarinic Neurotransmission in the Hippocampus of Transgenic APPswe/PS1dE9 Mice

The classical mouse model of AD is APPswe/PS1dE9 which overexpress the Swedish mutation of amyloid precursor protein (APP) together with presenilin-1 (PS1) deleted in exon 9. Transgenic APPswe/PS1dE9 mice demonstrate accumulation of β-amyloid fragments resulting in the formation of amyloid plaques, the hallmark of AD. Short-term feeding of APPswe/PS1dE9 mice with three different experimental diets showed the positive effects of special lipid-based diet on markers of cholinergic neurotransmission in transgenic mice. These results demonstrate that lipid-based diets represent a viable complement to the pharmacological treatment of AD (*Janickova et al. 2015*). This study also points to the possible role of cholesterol in the progress of AD. These and other results led us to investigate the effects of cholesterol on cholinergic transmission at the cellular and molecularlevel (see below).



Apolipoprotein E4 model of AD

Figure 2 – In contrast to apolipoprotein E3, apolipoprotein E4 reduces evoked hippocampal acetylcholine release in adult mice

Apolipoprotein E4 (apoE4) is the most prevalent genetic risk factor for Alzheimer's disease. In our study (*Dolejší E et al. 2016*), we investigated whether cholinergic dysfunction, which increases during ageing and is a hallmark of Alzheimer's disease, is accentuated by apoE4. We show that, in contrast to apoE3, the evoked release of ACh from hippocampal nerve terminals is impaired age dependently in apoE4-targeted replacement mice in which the endogenous mouse apoE4 was replaced by human variant. These mice serve as an attractive and needed model for studying mechanisms underlying the pathological effects of apoE4 on the cholinergic system in neurodegenerative diseases.

Mechanisms behind cholinergic control of striatal-based behaviour

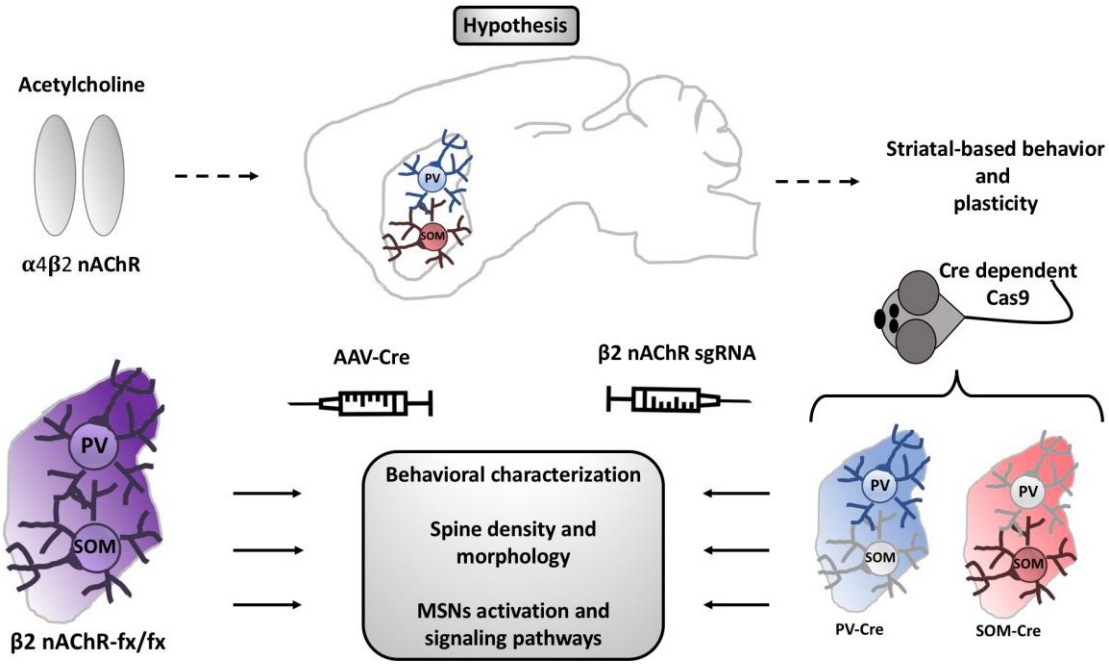


Figure 3 – Cholinergic signalling plays an important role in both cortical and striatal regions involved in control of the goal-directed behaviour (GDB). The cholinergic

system modulates synaptic plasticity by activation of nicotinic and muscarinic acetylcholine receptors. Among many other cell types, the nicotinic receptors are expressed by striatal GABAergic interneurons which importance for the control of circuits' plasticity has recently become recognized.

Dr Janickova started her research of mechanisms behind cholinergic control of GDB in striatum. For this purpose, she developed a new animal model using the CRISPR/Cas9 system deleting α4β2 nAChRs specifically from the striatal GABAergic interneurons. Transgenic mice are tested in different behavioural paradigms to determine alterations of synaptic plasticity in the striatum.

Xanomeline and cholesterol

Activation of muscarinic receptors by xanomeline

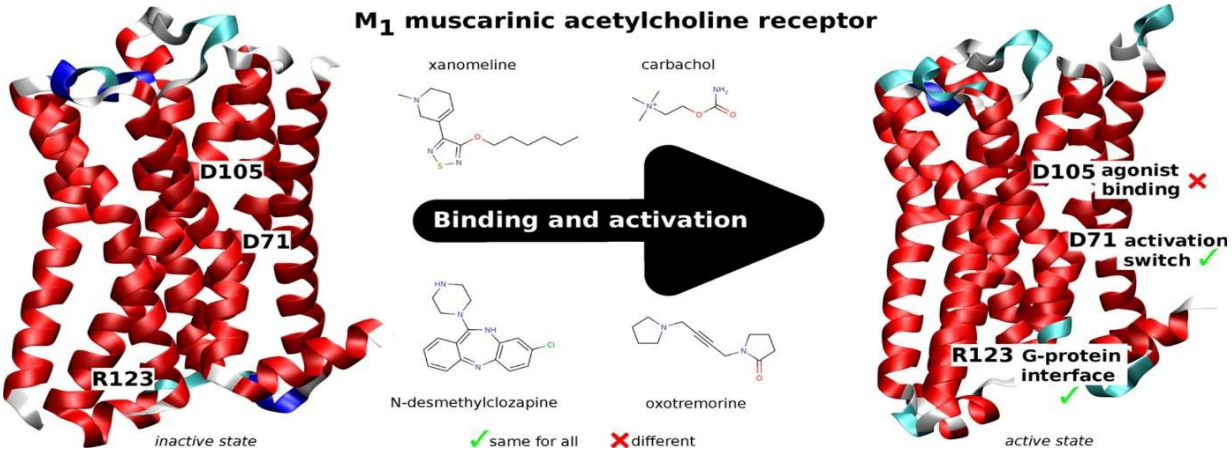
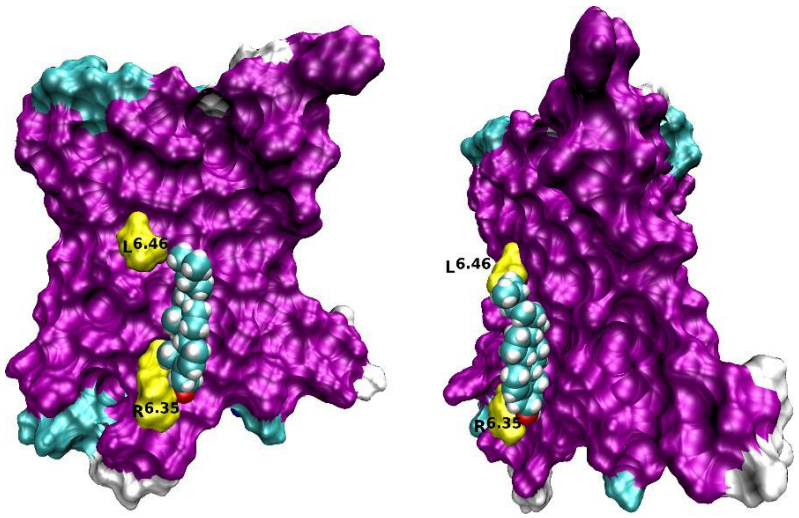


Figure 4 – Classical and atypical agonists activate M_1 muscarinic acetylcholine receptors through common mechanisms but they differ in the way they interact with the orthosteric binding site

Xanomeline is prototypic M_1/M_4 preferring agonist developed for the treatment of Alzheimer's disease and is one of the first functionally selective muscarinic agonists. Xanomeline exerts the same binding affinity for all subtypes of muscarinic receptors. Thus, its apparent selectivity is surprising. Molecular basis of xanomeline functional selectivity remained enigmatic for decades. In our studies, we have shown that the principal difference between classical agonists and xanomeline is in the way they interact with the orthosteric binding site. Further, we showed that the orthosteric binding site also plays a key role in long-term receptor activation by xanomeline (*Randáková et al. 2015*). These findings are essential for delineation of the molecular basis of xanomeline functional selectivity.



Allosteric modulation by cholesterol

Figure 5 – Molecular model of cholesterol bound at TM6 of M_1 muscarinic receptor. Yellow – key residues $L^{6.46}$ and $R^{6.35}$

Several lines of evidence, including our data, suggest that membrane cholesterol modulates the function of muscarinic receptors. Crystallographic data of various GPCRs show co-crystallized cholesterol in the vicinity of the orthosteric binding site that we proposed as the site of xanomeline wash-resistant binding. In our study (*Randáková et al.2018*), we show that membrane cholesterol indeed specifically interacts with muscarinic receptors. We located its binding site at the sixth transmembrane α-helix in the inner leaflet on the membrane. Further, we show that apparent functional selectivity of xanomeline stems from subtype differences in cholesterol binding to the muscarinic receptors. The possibility to achieve pharmacological selectivity based on receptor-membrane interactions changes our view on the molecular basis of pharmacological selectivity. We continue the detailed study of allosteric modulation of muscarinic receptors by cholesterol and cholesterol derivates like steroid hormones and neurosteroids.

Muscarinic agonists

Operational model of agonism

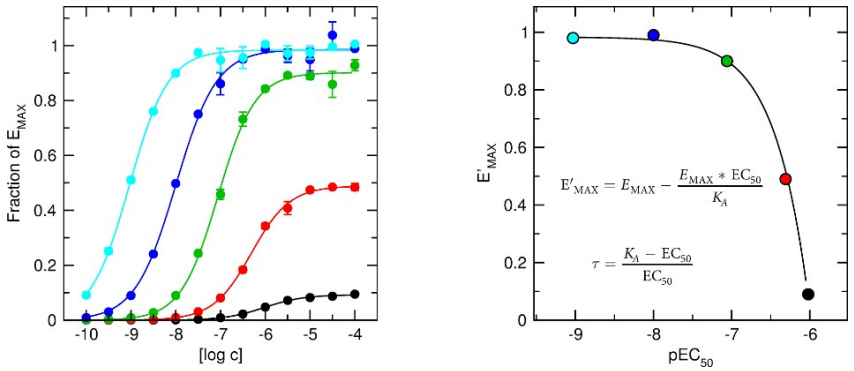


Figure 6 – two-step procedure of fitting of operational model of agonism to to experimental data

Commonly, efficacy is rather described in relative terms, e.g. an agonist that produces a more robust maximal response than another is considered more efficacious. In this case, the latter agent is considered a partial agonist. However, a mere comparison of the magnitude of the maximal response may be misleading. Importantly, the proper determination of agonist efficacy is essential in the assessment of agonist selectivity and signalling bias.Determination

of agonist activity must be system-independent. Based on these premises the operational model (OM) of pharmacological agonism was formulated by Black and Leff in 1983. Over time, the OM became a golden standard in the pharmacological analysis. Extended and modified versions of this model are widely applied also to allosteric activation and allosteric modulation or to analyse agonist signalling bias. However, the functionalefficacy (τ) is inter-dependent on two other parameters of OM; agonist's affinity (K_A) and the highest response that could be evoked in the system by any stimulus (E_{MAX}). In our work (*Jakubík et al. 2019*), we propose the way to avoid fitting inter-dependent parameters. We developed a two-step analysis of functional response as the robust way of fitting OM to experimental data for the proper ranking of agonists' efficacies in various systems. The procedure applies to any receptor-effector system as well as to all possible variants of the operational model.

Development of G_i-biased agonists

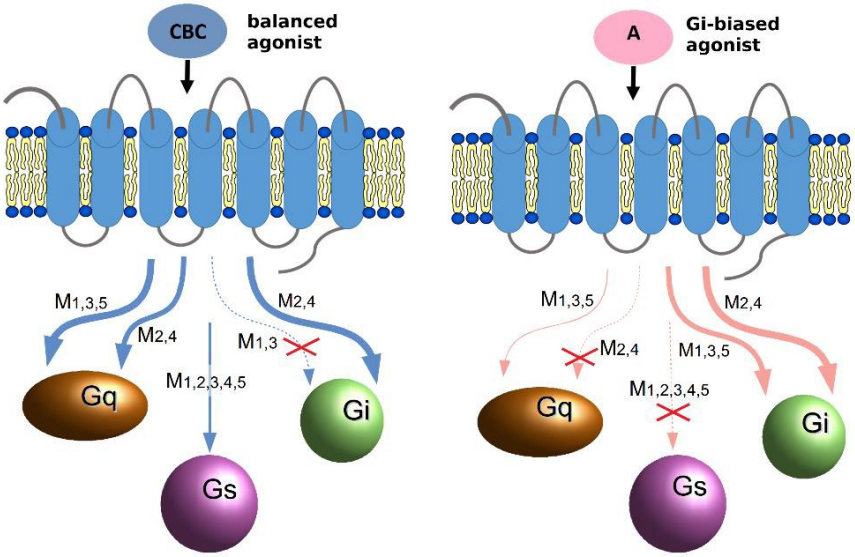


Figure 7 – Comparison of signalling of balanced agonist carbachol (CBC), left, and novel G_i-biased agonists (A), right.

Apparent selectivity of xanomeline led us to investigate xanomeline-based novel structural scaffold of N-substituted tetrahydropyridine salts for agonists. Structurally diverse agonists induce different changes in the conformation of GPCRs that can lead to non-uniform modulation of signalling pathways. This preferential orientation of signalling of a given GPCR towards a subset of its signal transducers is termed signalling bias. The property may be employed to develop drugs that selectively produce desired effects while avoiding side effects associated with activation of unwanted signalling pathways. Residues in the binding site that mediate activation of a specific signalling pathway are termed functional hot spots. Binding of an agonist to one or a subset of functional hot spots within the binding site results in activation of a subset of signalling pathways and thus in ligand-mediated signalling bias. An agonist relatively small has a better chance to bind to a smaller number of functional hot spots than a larger agonist. With this in mind, we have designed and synthesized small muscarinic agonists that solely inhibit the synthesis of cAMP (*Randakova et al. 2020*). These compounds may provide lead structures in the search for novel non-steroidal and non-opioid analgesics. Much research has been carried out on agonists biased towards either G-protein- or arrestin-mediated pathway. In this work, as a proof of concept, we demonstrate signalling bias among individual classes of G-proteins.

Muscarinic antagonists

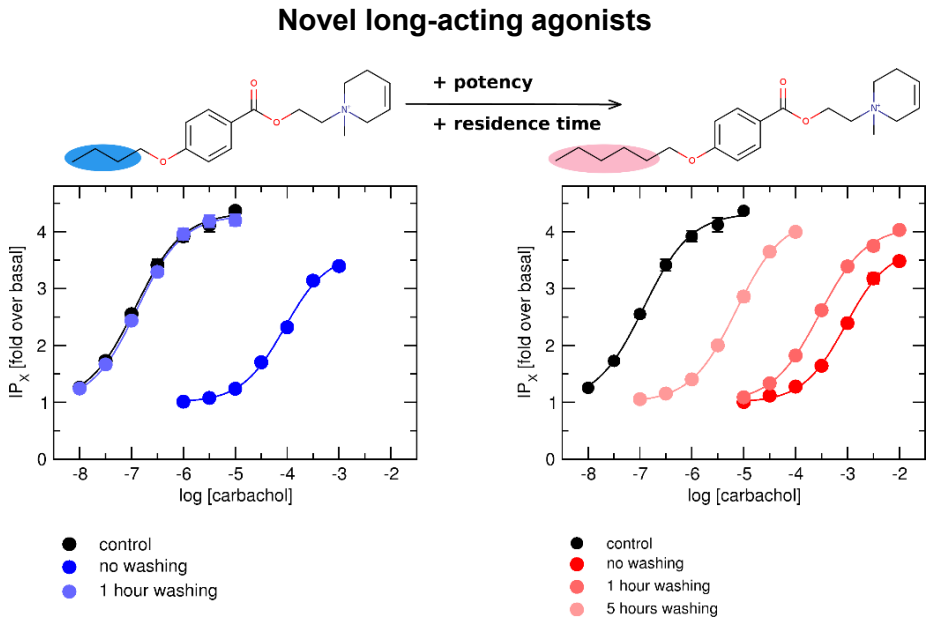


Figure 8 – Comparison of antagonism of carbachol induced accumulation of inositol phosphates by the lead compound (left) and analogue with increased potency and residence time (right)

Antagonists with long-residence time at receptors are longer acting, thus allowing to reach a maximum therapeutic effect at lower doses. Therefore, wash-resistant binding of xanomeline led us to investigate novel structural scaffold of N-substituted tetrahydropyridine salts also for antagonists. We have developed novel muscarinic antagonists exerting selectivity for M_2 receptors (*Boulos et al. 2018*). We have developed long-acting antagonists by substitution of hexyloxy moiety (that is responsible for xanomeline wash-resistant binding) in novel M_2 selective antagonists (*Randáková et al. 2018*). Besides improved residence time, these antagonists displayed improved potency except M_2 receptors. These antagonists became M_1 selective indicating that hexyloxy moiety is not only responsible for a long residence time of antagonists and xanomeline but also M_1 selectivity. These and other structurally related M_1 selective antagonists may have therapeutic potential in striatal cholinergic dystonia, delaying epileptic seizure after organophosphate intoxication or relieving depression. These compounds may also serve as a tool in research into cognitive deficits.

Allosteric modulations of muscarinic receptors

We have continued our long-going interest in allosteric modulation of muscarinic receptors. Besides allosteric modulation by cholesterol described above, we also investigated allosteric modulation of muscarinic receptors by small proteins. Weak toxin from Naja kaouthia (WTX) belongs to the group of nonconventional three-finger proteins. In our study (*Lyukmanova et al. 2015*), we show that conformational plasticity of the loop II could explain the WTX activity toward structurally unrelated targets, nicotinic and muscarinic receptors, and provide a structural framework for rationalization of target-specific positive/negative allosteric regulation. These findings are indispensable for the understanding of molecular mechanisms of action of three-finger proteins as well as a general mechanism of allosteric modulation of muscarinic receptors via the common allosteric binding site at the extracellular domain of the receptor.

Binding of orthosteric ligands to the allosteric binding site

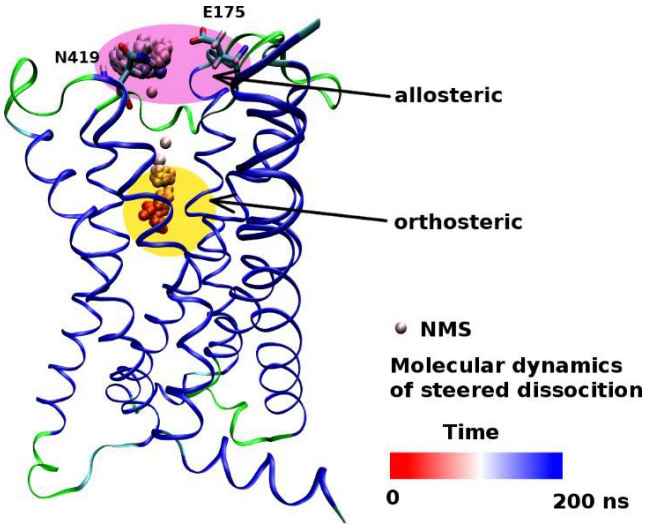
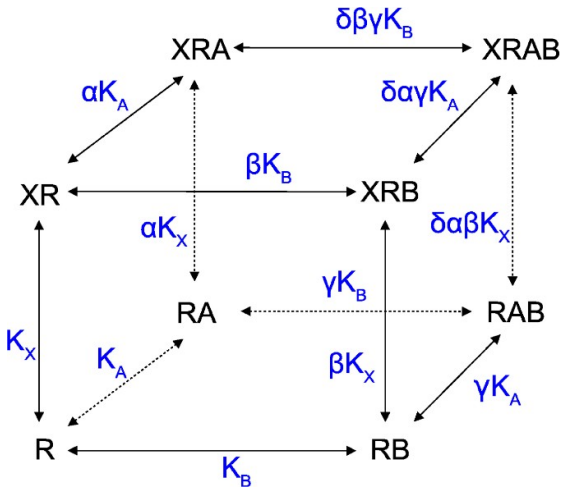


Figure 9 – The orthosteric antagonist N-metylscopolamine (NMS) binds to the allosteric binding site during simulation of molecular dynamics of dissociation

Further, we investigated the binding of the orthosteric ligands to the allosteric binding site. In our study (*Jakubík et al. 2017*), we show that orthosteric ligands also interact with the binding site located in the vestibule to the binding pocket of muscarinic receptors between the second and third extracellular loops on their way to and from the orthosteric binding site. Thissite is common for binding of some allosteric, ectopic and bitopic ligands. Transient bindingof orthosteric ligands to this extracellular site affects ligand affinity and kinetics and thus has pharmacologically important consequences. For example, current studies of bronchodilator tiotropium show that its binding to the secondary allosteric site on the M_3 receptor prolongs residence time and enhances its bronchoprotective effects.

Binding of an orthosteric tracer and two allosteric modulators



$$Y'/Y = \frac{[X]+K_X}{[X]+K_X\dfrac{1+\dfrac{[A]}{K_A}+\dfrac{[B]}{K_B}\left(1+\dfrac{[A]}{\gamma K_A}\right)}{1+\dfrac{[A]}{\alpha K_A}+\dfrac{[B]}{\beta K_B}\left(1+\dfrac{[A]}{\alpha\gamma\delta K_A}\right)}}$$

$$K'_A = K_A\frac{1+\dfrac{[B]}{K_B}+\dfrac{[X]}{K_X}\left(1+\dfrac{[B]}{\beta K_B}\right)}{1+\dfrac{[B]}{\gamma K_B}+\dfrac{[X]}{\alpha K_X}\left(1+\dfrac{[B]}{\beta\gamma\delta K_B}\right)}$$

Figure 10 – Derivation of equations describing equilibrium binding of an orthosteric tracer modulated by two allosteric modulators using their equilibrium dissociation constants and factors of cooperativity

Many allosteric modulators are unsuitable to serve as radio-labelled ligands. Under such circumstances, binding of allosteric modulators to the receptor is investigated indirectly using a radio-labelled orthosteric ligand as a tracer. Effects of increasing concentrations of an

allosteric modulator on the binding of a fixed concentration of a labelled orthosteric tracer allow determination of the equilibrium dissociation constant of an allosteric modulator and factor of cooperativity between an allosteric modulator and the tracer. In our work (*Jakubik et al. 2019*), we derive equations describing equilibrium binding of an orthosteric tracer modulated by two allosteric modulators using their equilibrium dissociation constants and factors of cooperativity. Then we analyse the model under various scenarios and explore its limits. We show that under equilibrium conditions, binding of two allosteric modulators to the same site can be distinguished from binding to two separate sites that is important in the location of allosteric sites on the receptor. Further, we show that in the model of binding of two molecules of one allosteric modulator to one receptor only the apparent dissociation constants can be determined.

Research activity and characterization of the main scientific results

Laboratory of Functional Morphology - from 1st January 2020 renamed to Laboratory of Pain Research

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, hyperalgesia) 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 lower quality of life. There are also significant costs associated with pain and chronic pain treatment for the society every year. Also most of the available analgesic therapies have significant unwanted side effects, including the lately highly visible opioid epidemic in the US. There is a high requirement and unmet medical need for new analgesic drugs with better side effect profile.

The cellular and physiological mechanisms underlying the development of chronic pain and associated phenomena are still not fully understood. This makes it difficult to designnew 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. Lately the role of neuroinflammation and glial cells activation was shown to have a significant impact on the development of different pathological pain states.

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 in different models of pain. 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. 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 under different pathological conditions, the sensitivity of spinal TRPV1 receptors is increased and they have a significant impact on synaptic nociceptive modulation in the spinal cord dorsal horn.

During this 5-year evaluated period we have concentrated and published papers on several projects. One of them was to study mechanisms of painful neuropathy development after chemotherapy treatment (Fig. 1). Paclitaxel is a basic cytostatic used for solid tumors therapy. The peripheral neuropathy and neuropathic pain are the major side effects and are dose limiting factors often leading to discontinuation of the cancer therapy. The associated neuropathy often becomes chronic and persists after the end of paclitaxel administration, is refractory to analgesic treatments and is often accompanied with deep burning pain.

In our original work we have shown that paclitaxel may activate peripheral and central neurons directly, induces increased sensitivity/expression of TRPV1 receptors and this is mediated by activation of Toll-like receptor 4 (TLR4). Direct functional interaction between the TLR4 and TRPV1 was shown in rat and human DRG neurons, in HEK293 cells expression system and in rat and mouse spinal cord slices. Our results suggest that targetingTLR4/TRPV1 receptors may be important for the prevention of chemotherapy induced peripheral neuropathy in patients. This work was done in collaboration with Prof. Doughertyat University of Texas M.D. Anderson Cancer Center under the Czech–American collaborative grant to JP (LH12058). The Czech team (PA, PM, JP) contributed to the paper with seeding the TRPV1 hypothesis, by recording the electrophysiological experiments on spinal cord slices and participating in writing the paper.

Li Y., Adámek P., Zhang H., Tatsui C. E., Rhines L. D., Mrózková, P., Li Q., Kosturakis A. K., Cassidy R. M., Harrison D. S., Cata J. P., Sapire K., Zhang H., Kennamer-Chapman R. M.,

Jawad A. B., Ghetti, A., Yan J., Paleček J., Dougherty P. M. The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4 . Journal of Neuroscience. 2015, Vol. 35, 39, p. 13487-13500 . IF = 5.924

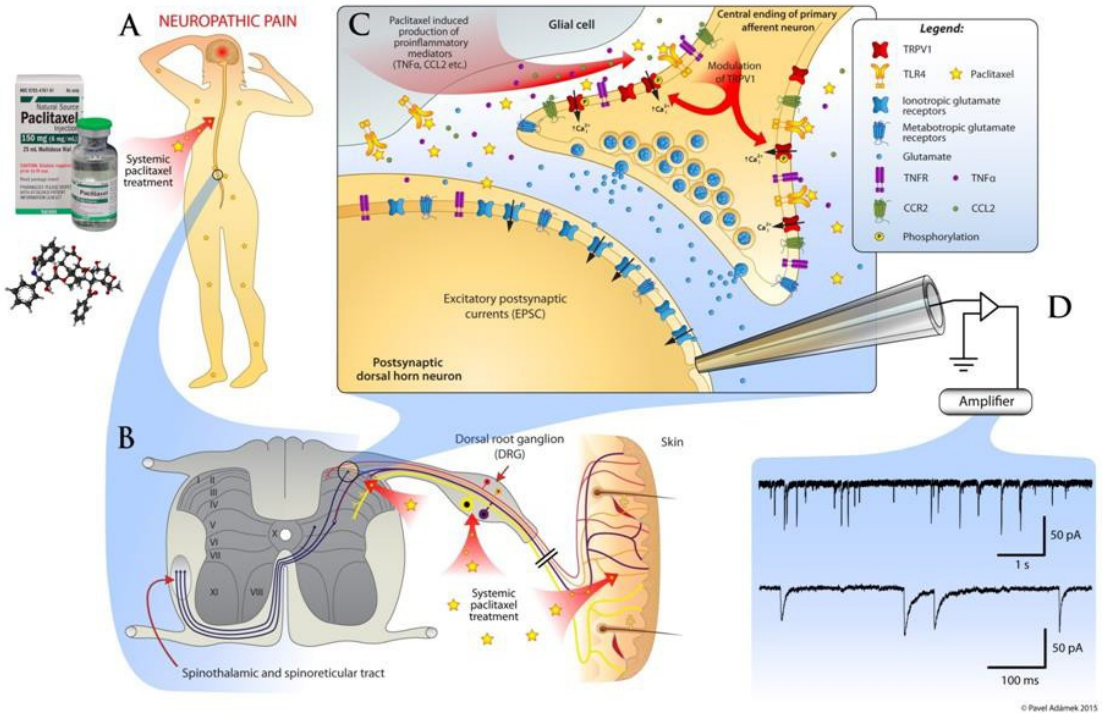


Fig. 1. Schematic drawing of paclitaxel effect at the spinal cord level. (A) Paclitaxel after systemic treatment may modulate properties of peripheral nerves. However, low concentration of paclitaxel penetrates through hematoencephalic-barrier to the central nervous system (B). In the spinal cord paclitaxel may activate TLR4 receptors and modulate release of neurotransmitters from presynaptic endings by modulation of TRPV1 receptors function (C). These changes are studied by whole-cell patch clamp recordings from spinal cord dorsal horn neurons, as changes in excitatory postsynaptic currents (D).

Our next effort concentrated on the possible mechanism of the TLR4 and TRPV1 receptors functional interaction after the paclitaxel treatment (Fig. 2). We have investigated the role of phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinases in this process. A single paclitaxel administration in mice induced robust mechanical allodynia and generated reduced tachyphylaxis of capsaicin-evoked responses for up to eight days. Paclitaxel application also induced increased Akt kinase phosphorylation in rat DRG neurons. All these paclitaxel-induced changes were prevented by the PI3K antagonist wortmannin *in vivo* pretreatment. Acute co-application of wortmannin or LY-294002 with paclitaxel in spinal cord slices also attenuated the paclitaxel effect on capsaicin-evoked responses. Our data suggest that the inhibition of PI3K signaling may help alleviate pathological pain syndromes in the paclitaxel-induced neuropathy. Our new unpublished data confirmed this by using clinically tested PI3K antagonists. This approach may prove to attenuate neuropathic pain in chemotherapy patients. This work was done entirely by the team.

Adámek P., Heleš, M., Paleček, J. Mechanical allodynia and enhanced responses to capsaicin are mediated by PI3K in a paclitaxel model of peripheral neuropathy. Neuropharmacology 2019, 146 (Mar 1), 163-174. IF = 4.367

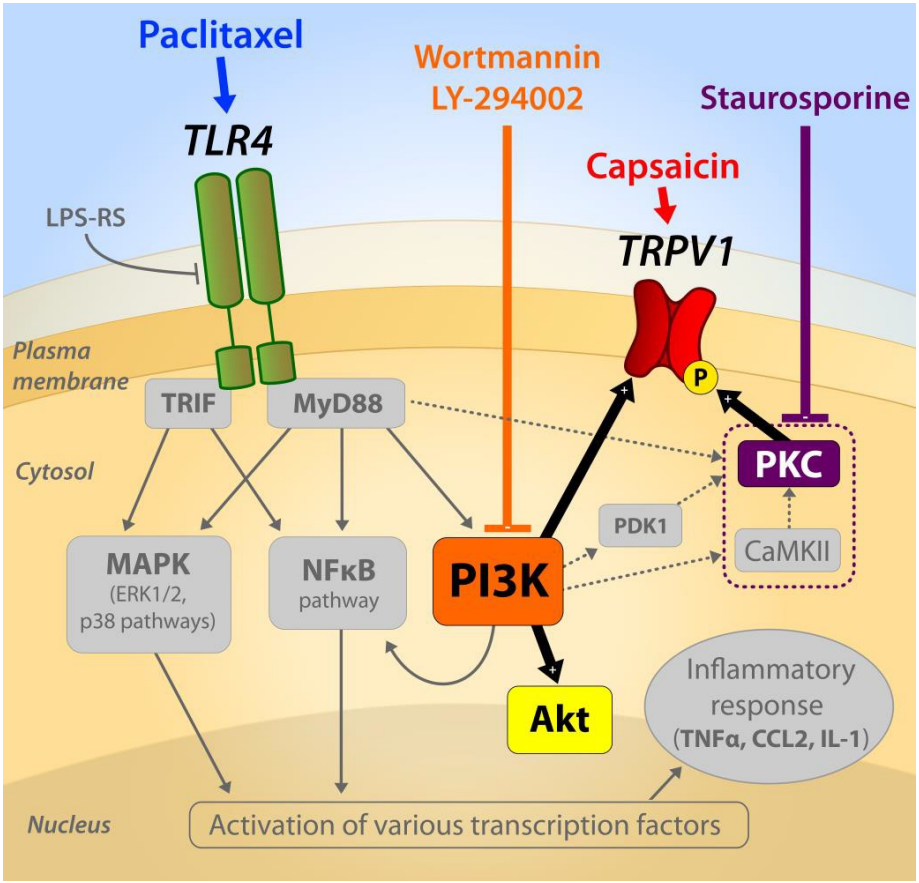


Fig. 2. Schematic diagram summarizing the proposed mechanism of the paclitaxel-induced modulation of the TRPV1 receptors function in nociceptive DRG neurons. For clarity, signaling tested in our study is in color, while other signaling pathways are in gray. Signal transduction following the TLR4 activation occurs via two adapter signaling molecules MyD88 and TRIF. The PI3K signaling pathway may be stimulated via MyD88-dependent mechanism after the TLR4 activation. The TLR4 activation may also activate PKC (the dottedlines) via MyD88, or PKC may be activated by PI3K via CaMKII or via PDK1. Our experiments showed that PI3K inhibitors wortmannin, LY-294002, and serine/threonine kinases inhibitor staurosporine prevented the paclitaxel-induced changes in tachyphylaxis of capsaicin-induced responses.

With the importance of TRPV1 receptors in the modulation of nociceptive signaling also goes the question of the possible ways of their activation, especially in the spinal cord. A number of endogenous substances were shown to activate TRPV1 receptors, while several of them including N-arachidonoylethanolamine (anandamide) activate both TRPV1 channels and CB1 receptors. Anandamide synthesis occurs through many metabolic pathways either in a Ca2+-insensitive or Ca2+-sensitive manner and N-arachidonoyl-phosphatidyl-ethanolamine (20:4-NAPE) constitutes the precursor for anandamide synthesis in all metabolic pathways. In our work we have shown the possible role of anandamide by providing substrate (20:4-NAPE) for anandamide-synthesizing pathways in the spinal cordfor the first time instead of flooding the entire preparation by exogenous anandamide. We have shown concentration dependent production of anandamide in spinal cord slices after application of (20:4-NAPE). Our data indicate that application of exogenous 20:4-NAPE induced mainly CB1 receptor-mediated inhibitory effects on excitatory transmission in naive animals while TRPV1 channel-mediated mechanisms were also involved after peripheral inflammation. Application of 20:4-NAPE, in our experiments, also provided a distinctiveopportunity to study the role of the spinal endocannabinoid system, by application of substrate for anandamide synthesis instead of anandamide directly. By this approach, physiological mechanisms of anandamide synthesis played an important role, including the level of their activity and local distribution, thus changing localized anandamide concentrations. By flooding the preparation, by applying

anandamide directly, it is more likely that other receptors and biological pathways would have been activated. This method of local'on demand' anandamide production from its precursor may prove to be of advantage also in the clinical settings for pain treatment. Especially now as clinical trials focused to increase anandamide levels, by reducing its hydrolysis with inhibitors of fatty acid amide hydrolase didnot show clinical efficacy. This work was done entirely by the team. Dr. I. Nagy from Imperial College London contributed to the writing of the manuscript.

Nerandžič V., Mrózková P., Adámek P., Špicarová D., Nagy, I., Paleček J. Peripheral inflammation affects modulation of nociceptive synaptic transmission in the spinal cord induced by N-arachidonoylphosphatidylethanolamine . British Journal of Pharmacology 2018,roč. 175, 12, p. 2322-2356. IF = 6.583

Neuroinflammatory changes in the central nervous system are known to be widely involved in the initiation and maintenance of different types painful states, especially of neuropathic origin. Our previous work studied the role of chemokines and cytokines in the modulation of TRPV1 function. Recently it was suggested that sartans, specific angiotensin type 1 receptor (AT1R) antagonists, have beside their antihypertensive effects also a potential role in attenuating neuronal inflammation. In our work we tested the effect of losartan (specific AT1R antagonist) administration on the development of neuropathic pain, neuroinflammation and macrophage involvement in a model of spinal nerve ligation model of neuropathic pain. Our new data show significant reduction of spinal cord and DRG neuroinflammation measured as reduced expression of different neuroinflammatory markers after losartan treatment, accompanied with attenuated infiltration of CD68 macrophages into the DRG and spinal roots and decreased hyperalgesia in this model of neuropathic pain. We have suggested that beside the direct action of losartan on the AT1R a possible mechanism of action could also involve its metabolite affecting activity of nuclear peroxisome proliferatorator activating receptor γ (PPARγ). Our results suggest that losartan based drugs may potentially represent a new therapy approach for neuropathic pain patients. Since then, we have also confirmed the beneficial effect of losartan treatment in a model of chemotherapy induced neuropathic pain (Journal of Cellular and Molecular Medicine, accepted, IF = 4.6). This work was done entirely by the team.

Kalynovska N., Diallo M., Paleček J. Losartan treatment attenuates the development of neuropathic thermal hyperalgesia induced by peripheral nerve injury in rats. Life Sciences 2019, 220, 147-155 . IF = 3.448

Controlling pain in burn injured patients poses a major clinical challenge. Recent findings suggested that reducing the activity of the voltage gated sodium channel Nav1.7 in primary sensory neurons could provide improved pain control in burn injured patients. In collaboration with I. Nagy from Imperial College London we tested this hypothesis in animal model. We found that burn type of injury upregulates Nav1.7 expression and phosphorylated cyclic adenosine monophosphate response element-binding protein (p-CREB) in primary sensory neurons 3 h following injury. The Nav1.7 blocker protoxin II (ProTxII) or morphine significantly reduced burn injury-induced spinal up-regulation in phosphorylated serine 10 in histone H3 and phosphorylated extracellular signal-regulated kinase 1/2, which are both markers for spinal nociceptive processing. The burn injury also induced a significant increase of the spontaneous excitatory postsynaptic currents frequency in the spinal dorsal horn neurons and this was reduced by the ProTxII application. Together, these findings indicate that using Nav1.7 blockers should be considered to control pain in burn injury patients. Our team (PA, JP) contributed all the electrophysiological data and related discussions.

Torres-Pérez J.V., Adámek P., Paleček J., Vizcaychipi M., Nagy, I. Varga A. The NA(v)1.7 blocker protoxin II reduces burn injury-induced spinal nociceptive processing . Journal of Molecular Medicine-Jmm 2018, roč. 96, 1, p. 75-84 . IF = 4.746

During this period we have continued with our intensive collaboration with Dr. Kudova from the

Institute of Organic Chemistry and Biochemistry of the CAS on translational research and development of new analgesic drugs. We have studied and tested number of compounds with analgesic properties. Our leading compound shows promising analgesic properties in different models of inflammatory, acute, chronic and neuropathic pain (Fig. 3). As the intellectual property of these results is not cover by patent we cannot disclose any more data at this moment.

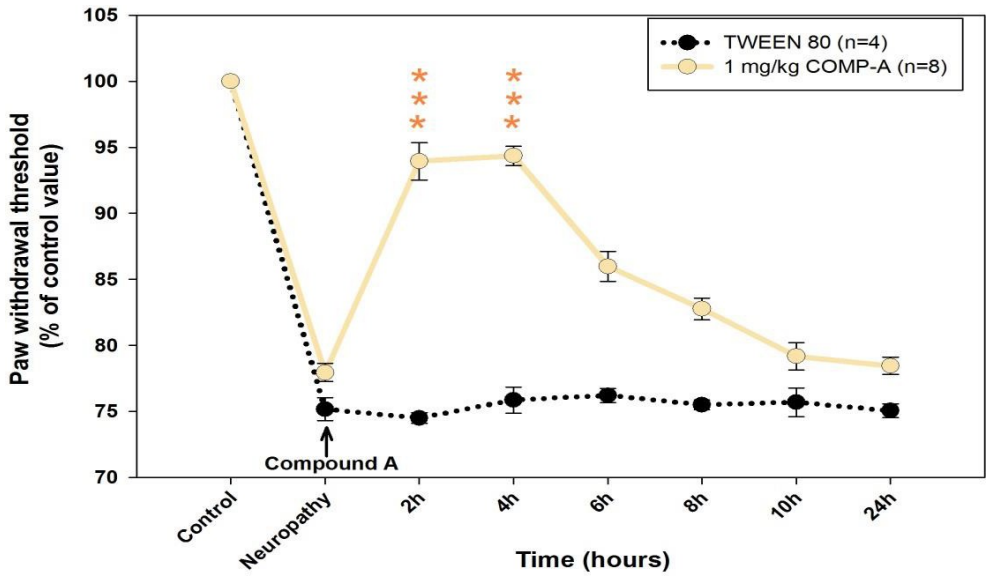


Fig. 3. A paclitaxel model of chemotherapy induced neuropathic pain leads to significant decrease of mechanical paw withdrawal threshold (mechanical allodynia). Application of experimental drug Compound-A leads to significant attenuation of the pathologicaly increased sensitivity.

During the whole evaluated period our team has published 17 papers in impacted journals. Beside those described above in detail, we have also published data on the role of TRPV1 receptors in the model of chemotherapy induced neuropathy, PAR2 receptors in modulation of nociceptive signaling at the spinal cord level and on the role of high concentration capsaicin treatment of the skin before surgical incision. Some of the papers were also directed towards the role of thyroid hormones (THs) and omega-3-polyunsaturatedfatty acid (n-3 PUFA) in the physiology of cardiac and skeletal muscles. Models of hyper- andhypothyroid status were used to analyze potential influence of n-3 PUFA on the induced pathological changes. The projects oriented on muscle physiology were headed by Dr. Tomas Soukup, who died after a tragic accident.

During this evaluation period we have also started several other projects that are ongoing and/or unpublished. These beside others include:

our project on the role of inhibitory neurons in modulation of nociceptive synaptic transmission at the spinal cord level using transgenic mice VGAT-ChR2-eYFP line that express yellow fluorescent protein and chanelorhodopsin (ChR2) in inhibitory neurons and enables their selective optical activation. Our results show significant depression of inhibitory currents under pathological conditions, contributing to pathological pain states. We study the mechanism of these changes.

project studying the interaction of opioid, TRPV1 receptors and the role of chemokines such as CCL2 in the modulation of nociceptive signaling. Our data show significant reduction of opioid induced inhibition at the spinal cord level after CCL2 administration. Our data also support the role of TRPV1 receptors in opioid induced hyperalgesia.

project studying the role of spinal protease-activated receptors 2 (PAR2) receptors in modulation of nociceptive synaptic transmission under inflammatory conditions. Our results show that in a model of acute peripheral inflammation intrathecal administration of PAR2 antagonist attenuated the development of hyperalgesia and application of PAR2 activating peptide pronounced hyperalgesia in a TRPV1 dependent manner. These results suggest the importance of PAR2 receptors in the spinal cord dorsal horn for nociceptive modulation.

Research activity and characterisation of the main scientific results

All vital processes at the cellular level can only function in a certain range of substrate, pH and solvent concentrations. Adaptation to stressful conditions requires cells to sense both changes in environmental conditions and changes of their own intracellular state.
One fundamental mechanism for this adaptation is regulated transport across cell membranes, i.e. an orchestrated uptake or release of substances across the plasma membrane, as well as intracellular membranes in eukaryotic cells. As mentioned above, our research activities in the evaluated period can be roughly divided in 4 domains, all related with transport processes across cell membrane.

Cation homeostasis and involved transporters

Ion homeostasis is a crucial requirement for all cells. They have developed efficient transport systems to acquire and store the desired elements and robust mechanisms to maintain homeostatic concentrations to avoid toxicity. Potassium is a key monovalent cation necessary for multiple aspects of cell growth and survival. It is accumulated in cells in high concentrations, whereas sodium accumulation is actively avoided because of its toxicity [26]. Tightly regulated potassium fluxes across the plasma and organellar membranes are also indispensable for the maintenance of proper membrane potential and intracellular pH, i.e. crucial physiological parameters contributing to cell fitness, which influences many processes (e.g. cell cycle, organelle biogenesis and cell volume, maintenance of membrane potential, cell response to osmotic and pH stresses etc.). In our studies we concentrated on two types of potassium transporters, Trk uniporters mediating an active uptake and accumulation of potassium and Na^+/H^+ antiporters, which serve to detoxify sodium cations, to optimize the intracellular concentration of potassium, or to regulate intracellular pH. These antiporters are conserved from bacteria to mammals and their malfunctioning is believed to be associated with various human diseases.

In the evaluated period, we studied the cation homeostasis and involved transporters both on the molecular (protein structure/function) and cellular (transporters regulation) levels.

One of our main results is the description of a hydrophobic filter in Na^+/H^+ antiporters, whose size determines the substrate specificity of the transporter (recognition and transport of K^+ or only smaller Na^+). Via modelling (in collaboration with P. Falson from France) we identified three amino-acid residues of three different transmembrane helices (Thr141, Ala179 and Val375) as components of the filter and our experimental results confirmed that the three residues play a central role in the determination of cation selectivity and transport activity (example shown in Fig. 1), and that the cation selectivity can be modulated by repositioning a single local methyl group within the filter region [3].

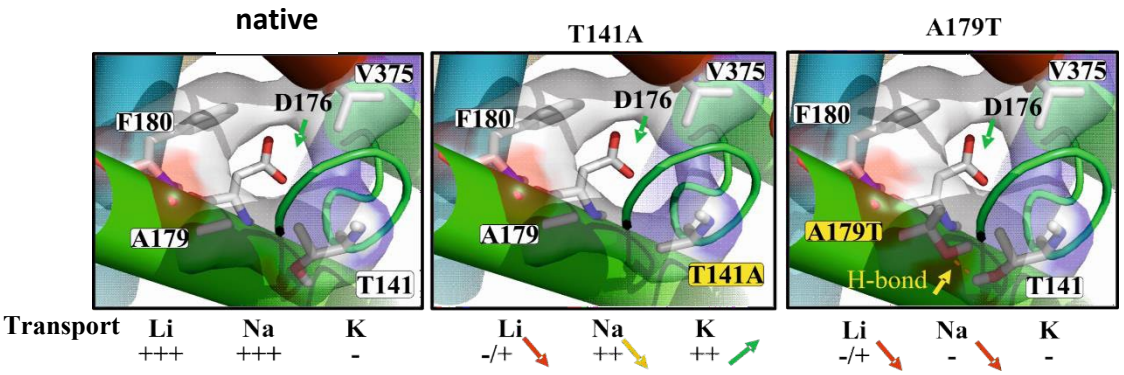


Fig. 1 Detailed view of impact of T141A or A179T mutations on structure of antiporter's cation filter. The 3D model of the protein is seen from the top, centred among helices 2, 4, 5 and 11. Residues Thr141, Ala179, Phe180 and Val375 form the cation filter. Mutated residues are highlighted in yellow boxes. The effect of mutations on cation transport and substrate specificity is indicated below.

On the cellular level, we paid (together with our long-standing Mexican collaborators) a lot of attention to the regulation of biogenesis and activity of the Na^+/H^+ antiporter and other cation transporters. We have described in detail the interaction of the yeast Na^+/H^+ antiporter with one of the cargo receptors of the secretory pathway, Erv14 [8], characterized the involvement of Erv14 in the yeast cation homeostasis, identified its new cargoes among potassium transporters [32], and described a structural motif of yeast and plant Erv14proteins necessary for proper targeting of their plasma-membrane cargo proteins [19].

For the first time, we showed that the activity of yeast Na^+/H^+ antiporter is negatively regulated via interaction with 14-3-3 proteins [36]. We identified Ser481 as the phosphorylated residue through which the antiporter interacts with the 14-3-3 protein, andthe crystal structure of the phosphopeptide containing Ser481 bound to 14-3-3 provided the structural basis of this interaction. 14-3-3 binding induced a disorder-to-order transition of the C-terminus of Nha1, and *in vivo* experiments showed that the lack of Ser481 (its substitution for Ala) increases antiporter's cation-efflux activity. Hence, 14-3-3 binding is apparentlyessential for the negative regulation of yeast Na^+/H^+ antiporter activity, which should be low under standard growth conditions, when toxic cations are not present and cells need to accumulate high amounts of K^+. These results were obtained in a new and very close collaboration with the IPHYS Laboratory of Structural Biology of Signalling Proteins [36].

Smaller part of our work was devoted to the elucidation of involvement of other proteins and (known or putative) transporters in cation and pH homeostases, these were Vcx1, Ist2 and Kch proteins. We were able to show that Vcx1 and ESCRT components regulate intracellular pH homeostasis in the response of yeast cells to calcium stress [2], Ist2 from the endoplasmic reticulum alters sodium accumulation in yeast cells [17], and that putative potassium channels Kch1 and Kch2 are probably not potassium uptake systems, nevertheless, they contribute to the maintenance of optimal cation homeostasis and membrane potential in the model yeast *S. cerevisiae* but not in pathogenic yeast *C. albicans* [20].

The second cation transporter which was in the focus of our studies is Trk1, a potassium specific uniporter. Genes encoding the Trk transporters have been identified in all yeast species with sequenced genomes, and they have orthologues in fungi and higher plants, not in animals. We used a series of mutant strains and expression tools constructedin our lab previously (cf. the report on previous evaluation period) to characterize in more detail the Trk system from the osmotolerant non-conventional yeasts *Z. rouxii* and to clone and characterize Trk systems from various pathogenic yeasts. We found that *Z. rouxii* Trk1 isextremely active, has a very high affinity for K^+, e.g. its activity (upon heterologous expression) prevents the non-

specific uptake of toxic cations and thereby assures *S. cerevisiae* cells much higher tolerance to toxic lithium cations than *S. cerevisiae* own Trk1 and Trk2 transporters [6]. Thus the *Zr*Trk1 transporter may serve for the improvement of cell fitness of industrial yeast strains in the future. As concerns potassium uptake proteins in pathogenic *Candida* species, we first characterized their needs for potassium in detail and performed an *in silico* analysis of their genes encoding putative potassium uptake systems [10]. Then, we cloned and partly characterized three different types of potassium uptake systems (not only Trk1 but also Hak1 K^+-H^+ symporter and Acu1 ATPase) identified by us in
C. albicans [14] and two Trk systems of *C. krusei* [28]. Most of our attention was paid to *C. glabrata* and its Trk1 [12,29,30]. *C. glabrata* is considered to be part of the human mycobiome in healthy individuals, even though in recent decades it has become a major fungal opportunistic pathogen, especially in immunocompromised patients. Nowadays, *C. glabrata* is considered to be the second most prevalent cause of Candida infections, due toits intrinsic high tolerance to most existing antifungal drugs. For this reason, the identification of new molecular targets for drug development is an important field of research, focusing on finding new ways to kill this pathogenic yeast. Our *in silico* analysis showed that *C. glabrata* genome contains only one gene encoding putative potassium transporter, a Trk1-like protein. Via heterologous expression in *S. cerevisiae* mutants lacking their own potassium uptake systems, we first confirmed, that the identified and cloned gene really encodes a K^+ uptake system [12]. Further, we constructed a *C. glabrata* knock-out mutant lacking the *TRK1* gene and compared its properties and phenotypes with those of the wild type. The obtained resultsshowed a pleiotropic effect on the cell physiology when the gene was deleted, affecting not only the ability of the mutant to grow at low potassium concentrations, but also the cell tolerance to toxic alkali-metal cations and cationic drugs, as well as the membrane potential and intracellular pH (Fig. 2, [12,29]).

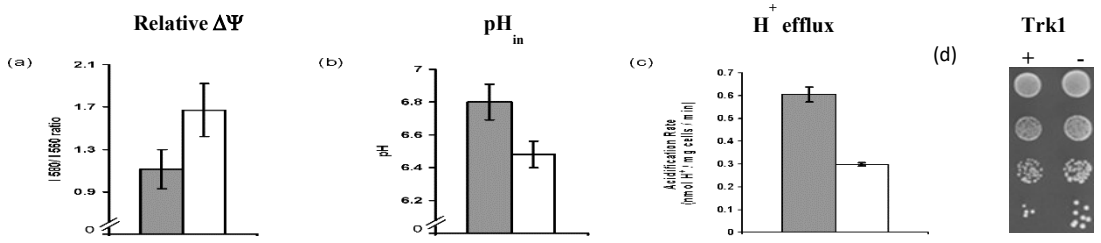


Fig. 2 Deletion of the TRK1 gene in C. glabrata (white columns) results, compared to the wild-type cells (grey columns), in plasma-membrane hyperpolarization (a), decreased intracellular pH (b) and partial inhibition of the Pma1 ATPase activity (c). The changes in these physiological parameters are visible even when the growth of mutant cells is not limited by low potassium (d; growth on 50 mM KCl).

When we observed the persistent changes in physiological parameters of *C. glabrata* cells lacking Trk1, we characterized the mutants further [30]. The absence of an active K^+ uptake system led to changes in cell surface properties, such as hydrophobicity and adherence, ability to form biofilms in vitro. The loss of an efficient potassium uptake system also results in diminished virulence as assessed by two insect host models and experiments with macrophages, which killed the mutant more effectively than wild-type cells [30]. The virulence studies were performed with colleagues from the ITN ImResFun consortium (cf, below Participation in large collaborations).

Taken together, our results found the sole potassium uptake system in *C. glabrata* to be a promising target in the search for its specific inhibitors and in developing new antifungal drugs [12, 30].

Antimicrobial peptides and multidrug resistence of pathogenic yeasts

Yeast species have several ways how to deal with toxic drugs. The main are drug-efflux systems, which eliminate toxic compounds from cells either via drug/H^+ antiporters or drug-efflux ATPases with a broad substrate specificity (called MDR pumps). Our results on the role of plasma-membrane lipids in multiple-drug resistance, cell salt tolerance and maintenance of membrane potential [7], together with our collection of different pathogenic *Candida* species and their mutants lacking various MDR pumps, and the know-how on testing *Candida* species performance and fitness (described above) led to a new collaboration with the team of Dr. V. Čeřovský from the CAS Institute of Organic chemistry and Biochemistry (IOCB). They isolated three types of potential antimicrobial peptides from venoms of various wild bees, characterized their molecules and synthesized a large collection of their analogues. Together, we successfully applied for a grant to the Technologyagency supporting applied research in the Czech Republic. Within a 3-year project, we characterized the antifungal activity of several members of each peptide family [15, 18, 34] and found that their activity against different *Candida* species is not the same. The level of killing efficiency is not only peptide-specific, but also species specific and proportional to the level of caused plasma-membrane damage. We found that this level of membrane damage reflects the plasma-membrane lipid composition [34]. The necessity to enlarge our methodology portfolio with lipid analysis led to a new collaboration with the group of Dr. I. Hapala from The Slovak Academy of Sciences (Institute of Animal Biochemistry and Genetics), which turned out to be mutually beneficial [34,39].

As the most important output of our work with antimicrobial peptides we can name successful Czech, EU and USA patent applications submitted together with the colleagues from IOCB [38].

Non-conventional yeast species, their osmotolerance and specific transporters

Tolerance to changes in environmental water activity is one of the key factors enabling microorganisms, including yeasts, to survive in nature. Glycerol, as a small and uncharged molecule, is the main and most frequently used compatible solute in yeastspecies. On the one hand, external glycerol may serve as a source of carbon for growth; and on the other, yeasts produce it for osmoadaptation purposes, for maintaining the redox balance, in response to temperature and oxidative stresses, and last, but not least as a precursor of glycerolphospholipid synthesis. The ability to both produce and consume glycerol, together with its broad-scale use in cell metabolism and physiology, requires a tight regulation of its synthesis, catabolism, uptake and release. Transporters mediating the uptake of external glycerol in symport with protons (Stl1) were previously described in *S. cerevisiae* and *C.*

albicans. In our studies, which started in the previous evaluated period, we focused on a highly osmotolerant non-conventional yeast *Z. rouxii* and we showed that its osmotolerance is based in the presence of two highly active glycerol uptake systems. We identified and cloned both copies of the *STL* gene from *Z. rouxii* genome, compared properties of corresponding transporters upon heterologous expression in *S. cerevisiae* lacking its own Stl1, and characterized phenotypes of their individual or joint deletion in *Z. rouxii* [5]. Construction of suitable *S. cerevisiae* mutants for the heterologous expression, together with the characterization of *Z. rouxii* mutants lacking *STL* genes led to the conclusion that glycerol transporters are involved in cell response to several types of stresses, not only osmotic stress, and surprisingly revealed a previously unknown role of Stl1 glycerol transporters in the regulation of intracellular pH. Both *S. cerevisiae* and *Z. rouxii* mutants lacking Stl transporters have a significantly higher intracellular pH even under non- stressed conditions [1,5].

Our work on glycerol transporters led to a participation in another MSCA ITN consortium within FP7 (Cornucopia, cf. below Participation in large collaborations), which started in the previous evaluated period, but whose results were published only recently. Within that ITN we studied, mainly together with Spanish partners from Valencia, osmotolerance and glycerol transporters of non-*cerevisiae Saccharomyces* species, which have a great potential in food and wine industries. We studied the glycerol homeostasis and its changes in various *Saccharomyces* species and in *Dekkera bruxelensis* [13, 23, 25], cloned and characterized the Stl1 transporter from *S. kudriavzevii* [27] and two Stl transporters from *D. bruxelensis* [25]. Finally, we tried to improve salt tolerance and fermentation performance of the osmosensitive *S. kudriavzevii* via expression of heterologous highly efficient transporters [24].

S. cerevisiae expressing laccases from filamentous fungi

We were involved also in a project of applied research aiming to produce recombinant enzymes (Laccases) in *S. cerevisiae*. Laccases are enzymes with a broad range of biotechnological applications and have, for example, the ability to oxidize many xenobiotics including synthetic dyes. The work started in the previous evaluated period, but its results were published in 2015-2019. We constructed *S. cerevisiae* strains expressing different laccases from filamentous fungi and optimized the culture growth/laccase production ratio [9,21]. Production strains were further used by our partners (group headed by Dr. I. Pichova) at IOCB for the successful purification and characterization of individual laccases (a joint PhD student defended the related thesis in 2018). In the context of this work, we also optimized the use of PVA hydrogel for immobilization of various yeast species and found, that this immobilization dramatically enhances storage stability of most of tested yeast species, and moreover, enables a long-term reusability of *S. cerevisiae* producing recombinant laccases [22].

Tools for nonconventional and pathogenic yeast species

In order to perform above mentioned experimental work, we needed to optimize various tools used for *S. cerevisiae* for non-conventional and pathogenic yeast species or develop new ones, as well as enlarge our already existing *S. cerevisiae* mutants for expression of heterologous proteins.

For example, we constructed a new series of mutant strains suitable for the expression of plant proteins and used it for the characterization of rice Hkt1;3 transporter's substrate specificty and study of its interaction with cornichon protein from the endoplasmic reticulum [4].

In collaboration with Italian colleagues, we optimized our tools for genetic engineering

of *Z. rouxii* cells (plasmids, deletion casettes, transformation protocol) and constructed new ones [33], which helped to elucidate the pecularities of mating-type loci in *Z. rouxii* hybrids [31].

In collaboration with the physicists of the Charles University and colleagues of the CAS Institute of Macromolecular Chemistry we developed a method based on nuclearmagnetic resonance, which can be used to monitor water transport through the plasma membrane of various yeast species [37].

A lot of our attention was given to the improvement of fluorescence techniques used in our team and in development of their new applications. We have been using a pH- sensitive variant of GFP pHluorin in *S. cerevisiae* for many years. In the evaluated period, weprepared new constructs with the pHluorin coding sequence optimized for the use in non- conventional and pathogenic yeast species. In most of the research activities described above we used these constructs to monitor the changes in intracellular pH resulting from gene deletions/overexpression, stress conditions or drug presence. Nowadays, we have the possibility to monitor intracellular pH and its changes in both auxotrophic and prototrophic strains of all non-*cerevisiae Saccharomyces* species, in *Z. rouxii, C. albicans* and *C. glabrata* [e.g. 5,6,12,23,29]. Together with Belgian colleagues, we prepared a new pHluorin construct, which is targeted to the yeast Golgi apparatus and enables to monitor pH changes inside this organelle [40].

To assess the activity of antifungal peptides, we developed a high-throughput fluorescence screening assay monitoring the yeast plasma-membrane integrity and survival of various stresses [15]. We used this method to characterize the activity of various antifungal peptides, drugs and disinfectants [15,18,34]. The fluorescent diS-C3(3) compound (3,3'-dipropylthiacarbocyanine iodide), which is usually used to monitor changes in the relative membrane potential or the activity of MDR pumps in the yeast cells, is used to monitor the level of plasma-membrane integrity damage (Fig. 3). The new assay enables a quick screening of several yeast species and several drugs in parallel in a 96-well plate in fluorescence reader. Moreover, this technique requires much lower amounts of cells, growth media and peptides or drugs.

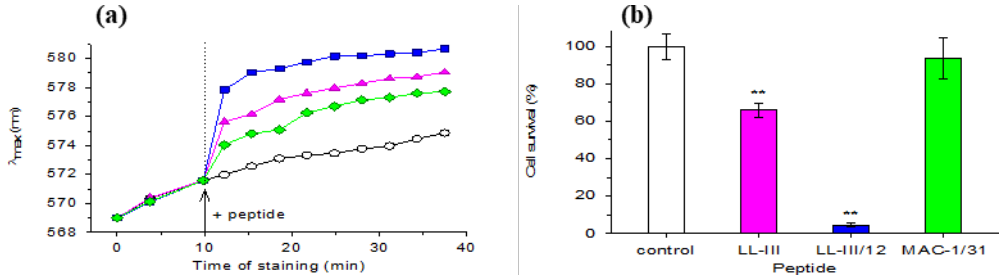


Fig. 3 Quick fluorescence assay monitoring the level of plasma-membrane damage by 3 antifungal peptides (a), and the validation of its results via classical microbiology survival test (b). For (a), the peptides were added to 100 μl of yeast cells 10 min after the diS-C3(3) probe and the measurement of fluorescence in the reader continued for 30 min. In (b) cells were incubated with peptides for 15 min, then plated on YPD plates and survival (colony forming units) was read after 2 days. The highest membrane damage in the fluorescence assay was observed for LL-III/12 (blue curve in (a)) and confirmed in plating cells where almost no cells survived the LL-III/A2 treatment (Blue column in (b)).

Publications and other outputs of the team

Altogether our results obtained in 2015-2019 were published in **39 papers** in journals with IF, **1 book** and formed a basis for successful **Czech, European and USA patent applications**. In 25 papers, the first author was from the team; in 24 papers, the corresponding

author was from the team; and 23 papers resulted from international cooperation.

Last but not least, we would like to mention that H. Sychrova was one of the 3 editors of a book (Yeast Membrane Transport) published by Springer in the series of Advances in Experimental Medicine and Biology in 2016. The book summarizes the current knowledge of the field in 15 chapters (380 pages).

Team members in bold.

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37. Šoltésová M., **Elicharová H.,** Srb P. **Růžička M.**, Janisova L., **Sychrova H.**, Lang J. Nuclear magnetic resonance investigation of water transport through the plasma membrane of various yeast species, *FEMS Microbiol. Lett.* **366,** fnz220 (2019)

38. Czech (307055 in 2019) , European (16721001.2-1111/3280722 in 2019) and US (10,160,785 B2 in 2018) patents on new antifungal peptides, patent owned by the Institute of Organic chemistry and Biochenistry CAS and Institute of Physiology, CAS, 5 inventors, **M. Kodedova** and **H. Sychrova** among them

*39. Csaky Z., Garaiová M., **Kodedová M.,** Valachavič M., **Sychrová H.,** Hapala I. Squalene lipotoxicity in a lipid droplet-less yeast mutant is linked to plasma membrane dysfunction. Yeast **37:** 45-62 (2020), submitted in 2019*

*40. Deschamps A., Colinet A.-S., **Zimmermannova O., Sychrova H**., Morsomme P. A new pH sensor localized in the Golgi apparatus of Saccharomyces cerevisiae reveals unexpected roles of Vph1p and Stv1p isoforms. Sci. Rep. 10:1881 (2020), submitted in 2019*

Research activity and characterisation of the main scientific results

The main areas of interest and key scientific achievements in the period 2015-2019 are listed below.

1. Basic mechanisms of beneficial effects of Omega-3 and related clinical research

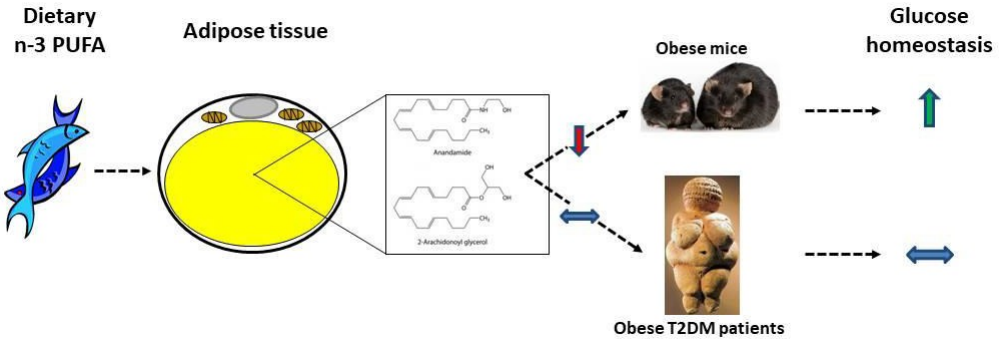
In spite of multiple beneficial effects of Omega-3 on health, the underlying mechanisms are still poorly characterised. In our previous studies in mice with diet-induced obesity, we mostly focused on beneficial metabolic effects of dietary Omega-3 supplementation, including changes in immunometabolic properties of adipose tissue [reviewed in (Masoodi et al., *Biochim Biophys Acta* **1851**: 503, 2015;Kuda et al., *Mol Aspects Med* **64**: 147, 2018). The following is an overview of results and publications demonstrating team activity in a specific area of research related to Omega-3 in 2015-2019.

Omega-3 prevent proliferation of fat cells in mice fed a high-fat diet
We have previously found that white adipose tissue (**WAT**) hyperplasia in obese mice was limited when the high-fat diet was supplemented by Omega-3 (Ruzickova et al., *Lipids* **39**: 1177, 2004) (>200 citations). We now show by flow cytometry that Omega-3 ameliorate immunometabolic properties of WAT in high-fat diet-fed mice, while suppressing proliferation of preadipocytes and endothelial cells (Adamcova et al., *Mar Drugs* **16**: 2018). Our new results thus provide further insight into the beneficial effect of Omega-3 on WAT and explain their anti-obesity effects in mice. They also open the way for clinical research into the effects of Omega-3 on adipose tissue of obese patients. See ref. (Adamcova et al., *Mar Drugs* **16**: 2018). IF = 3.772 (2018).

Team's contribution: Supported by the Czech Science Foundation (16-05151S; 2016-2018; PI: Jan Kopecký). All the co-authors were from the Dept. of Adipose Tissue Biology.

Omega-3 differentially modulate adipose tissue endocannabinoid levels and glucose metabolism in obese mice and type 2 diabetic patients
Our previous studies suggest that Omega-3 supplementation can ameliorate impaired glucose metabolism in obese mice (Kuda et al., *Diabetologia* **52**: 941, 2009;Jelenik et al., *Diabetes* **59**: 2737, 2010), but is less effective in obese patients with type 2 diabetes (Veleba et al., *Nutrition & metabolism* **12**: 52, 2015). This difference could reflect in part dissimilar regulation of endocannabinoid system activity in adipose tissue.
Our new study shows that in dietary obese mice long-term intake of Omega-3 leads to a decrease in the levels of major endocannabinoids 2-AG and AEA in WAT, while improving glucose homeostasis. In contrast, Omega-3 administration of the same type and duration failed to decrease endocannabinoid levels both in adipose tissue and serum of obese type 2 diabetic patients. These results indicate that the inability of Omega-3 to reduce endocannabinoid levels could also partly explain the absence of beneficial effects of these fatty acids on glucose homeostasis in diabetic patients (Rossmeisl et al., *Biochim Biophys Acta* **1863**: 712, 2018). IF = 4.402 (2018).

Team's contribution: Supported by the Czech Science Foundation (14-09347S; 2014-2016; PI: Martin Rossmeisl). The first and the last (corresponding) author are members of theteam. The contribution of the team members (64 %) was: (i) conceptual, (ii) all animal experiments, (iii) lipidomic analyses, and (iv) writing the publication. This was a collaborative project between the Institute of Physiology CAS and the Institute for Clinical and Experimental Medicine.



Combined intervention with insulin-sensitizing drugs and Omega-3 improves postprandial lipid metabolism in type 2 diabetic patients

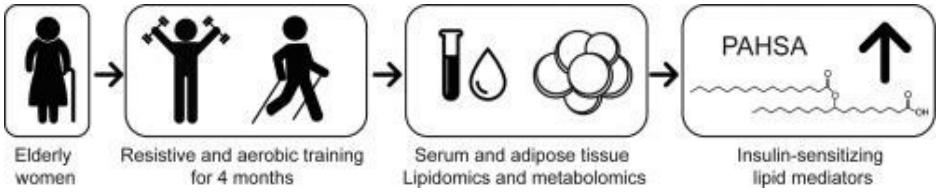
Previously, we found that a combined intervention with rosiglitazone (i.e. an insulin- sensitizing drug from the thiazolidinedione family) and Omega-3 markedly improved the function of adipose tissue and insulin-sensitivity in obese mice (Kuda et al., *Diabetologia* **52**: 941, 2009). Subsequently (2010 – 2014), in collaboration with the Institute for Clinical and Experimental Medicine, we used this approach to treat type 2 diabetic patients on stable metformin therapy. We found that combination therapy (pioglitazone + Omega-3) had a significant beneficial effect primarily at the level of postprandial lipid metabolism. The results of this clinical study were published in 2015 (Veleba et al., *Nutrition & metabolism* **12**: 52, 2015). IF = 3.280 (2015).

Team's contribution: Supported by the the Ministry of Health of the Czech Republic (NT13763-4; 2012-2015; PI: Jan Kopecký). The penultimate (corresponding) author is a member of the team. The contribution of the team members (29 %) was: (i) conceptual, (ii) biochemical and lipidomics analyses, and (iii) writing the publication.

Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women

Our long-term goal is to study the metabolic effects of Omega-3 in relation to the lipid form in which these fatty acids are administered to the body (see also paragraph below). Wax esters, contained in Calanus oil, represent one of those novel lipid forms of Omega-3. We collaborated with the 3rd Faculty of Medicine and the Faculty of Physical Education and Sport of Charles University in Prague on the clinical study EXODYA, which was aimed at studying effects of exercise training alone or in combination with Calanus oil supplementation. Using lipidomic analyses we show that Calanus oil favorably affects insulin sensitivity in sedentary older women undergoing a 4-month exercise program. Furthermore, exercise training itself improved the function of adipose tissue while raising the levels of insulin-sensitizing PAHSA lipokines in both adipose tissue and serum (Brezinova et al., *Biochim Biophys Acta Mol Cell Biol Lipids* **1865**: 158576, 2020). Our results document the importance of adipose tissue function for whole body metabolism and implicate PAHSA lipokines in the beneficial effects of exercise on insulin sensitivity. See ref. [(Brezinova et al., *Biochim Biophys Acta Mol Cell Biol Lipids* **1865**: 158576, 2020); published on-line in 2019]. IF = 4.402 (2018).

Team's contribution: Supported by the Ministry of Health of the Czech Republic (16-29182A; 2016-2019; co-PI: Martin Rossmeisl). The first and the last two (corresponding) authors are members of the team. The contribution of the team members (40 %) was: (i) conceptual, (ii) lipidomic analyses, (iii) data analyses, and (iv) writing the publication.



The effects of Omega-3 on insulin sensitivity and intestinal tissue metabolism depend on the lipid form of their administration

Our previous studies suggested that the effects of Omega-3 supplementation on glucose metabolism and liver fat accumulation in dietary obese mice could be augmented when these fatty acids are administered in the form of marine Omega-3-containing phospholipids (Rossmeisl et al., *PLoS One* **7**: e38834, 2012;Rossmeisl et al., *Biochim Biophys Acta* **1841**: 267, 2014). Furthermore, we also observed that Omega-3 administration is associated with the induction of fatty acid oxidation in the intestine (van Schothorst et al., *BMC Genomics* **10**:110, 2009). The new unpublished studies carried out during 2015 – 2019 demonstrate that marine phospholipids (Krill oil) alleviate insulin resistance associated with high-fat feeding more effectively than triacylglycerol (**TAG**)-based Omega-3 supplementation, while improvingWAT function and reducing endocannabinoids levels in the circulation (unpublished). This was consistent with their superior effects on intestinal metabolism and induction of fatty acid oxidation. In these studies was also observed inhibitory effect of antidiabetic drug metformin on glucose uptake in the intestine (Horakova et al., *Sci Rep* **9**: 6156, 2019). IF = 4.011 (2018).

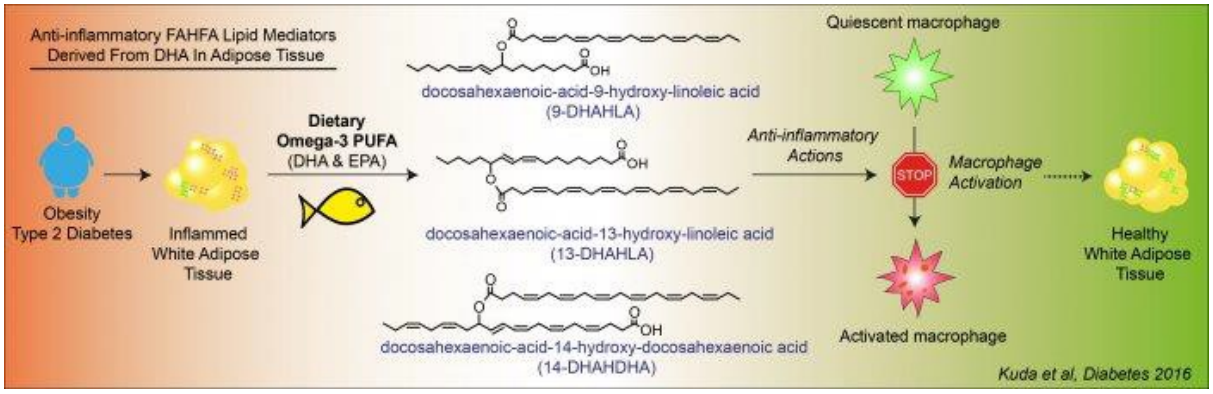
Team's contribution: Supported by the Czech Science Foundation (17-11027S; 2017-2019; PI: Martin Rossmeisl; and 16-08124S, 2016-2018, PI: Olga Horakova).

2. Lipid mediators

In 2014, a new class of lipid mediators with anti-diabetic and anti-inflammatory properties was discovered by prof. Kahn (Harvard University). Since then, we have systematically explored this new field connecting metabolism and inflammation. The three most important discoveries were published in *Diabetes* journal (Kuda et al., *Diabetes* **65**: 2580, 2016;Kuda etal., *Diabetes* **67**: 1190, 2018;Paluchova et al., *Diabetes* **69**: 300, 2020) and two human studies of FAHFAs were finished (Brezinova et al., *Biochim Biophys Acta Mol Cell Biol Lipids* **1865**: 158576, 2020;Brezinova et al., *Biochim Biophys Acta* **1863**: 126, 2018).

Anti-inflammatory effects of omega-3 PUFA are mediated by new lipid mediators from adipose tissue

A complex research of omega-3-related mechanisms of action in mouse models of obesity at the Institute of Physiology CAS, clinical research on obese patients with type 2 diabetes in the Institute for Clinical and Experimental Medicine, and a collaboration with the Institute of Organic Chemistry and Biochemistry CAS led to the identification of structures of novel signaling molecules of lipid origin - esters of fatty acids and hydroxyl-fatty acids (FAHFA) - derived from DHA: 13-DHAHLA, 9-DHAHLA a 14-DHAHDHA. These molecules, which are synthesized by adipose cells and exert anti-inflammatory effects, were detected in the serum and adipose tissue of both obese mice and diabetic patients following dietary intervention with omega-3. These newly discovered molecules, which can be endogenously synthesized when eating an appropriate diet, are involved in the beneficial health effects of omega-3 and have the potential for their wide use in the prevention and treatment of severe diseases. See ref. (Kuda et al., *Diabetes* **65**: 2580, 2016). IF = 8.684 (2016).



Levels of palmitic acid ester of hydroxystearic acid (PAHSA) are reduced in the breast milk of obese mothers

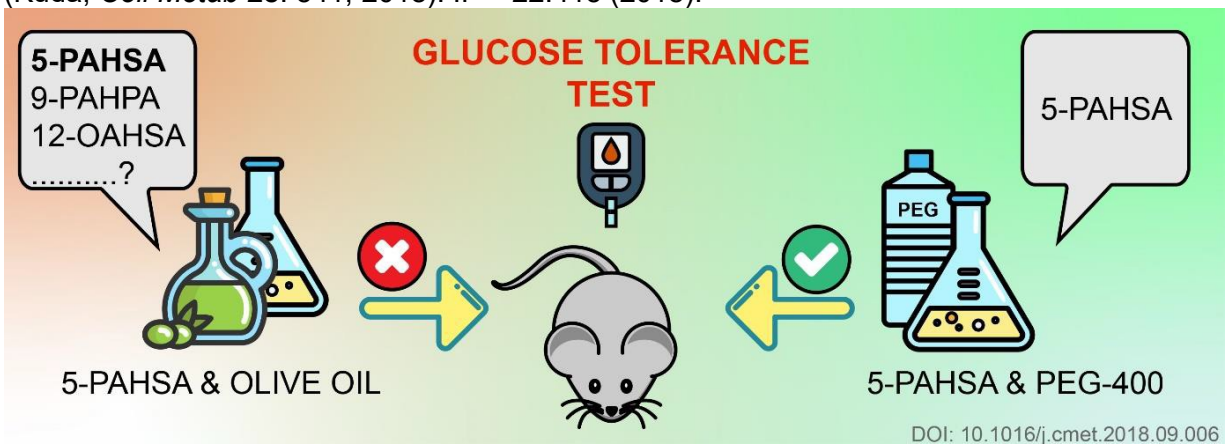
We have identified FAHFAs, especially 5-PAHSA, as an important component of human breast milk. Levels of 5-PAHSA are negatively affected by obesity of the nursing mothers. Moreover, we also explored the stereochemistry of PAHSA regioisomers and identified (R)- configuration as prevailing. (Brezinova et al., *Biochim Biophys Acta* **1863**: 126, 2018). IF = 4.402 (2018).

Nrf2-mediated Antioxidant Defense and Peroxiredoxin 6 are Linked to Biosynthesis of Palmitic Acid Ester of 9-Hydroxystearic Acid.

Here we have explored the biosynthetic pathways of FAHFAs. Comprehensive lipidomic analysis of rat white adipose tissue samples identified ~160 FAHFA regioisomers and QTL analysis highlighted several positional candidate genes in PAHSA metabolism. The results indicate that the synthesis of PAHSAs via carbohydrate-responsive element-binding protein (ChREBP)-driven *de novo* lipogenesis (**DNL**) is linked to the adaptive antioxidant system and the remodeling of phospholipid hydroperoxides. (Kuda et al., *Diabetes* **67**: 1190, 2018). IF = 7.199 (2018).

On the Complexity of PAHSA Research

Comments on the methodological and conceptual problems when working with FAHFAs. (Kuda, *Cell Metab* **28**: 541, 2018). IF = 22.415 (2018).



Lipokine 5-PAHSA is Regulated by Adipose Triglyceride Lipase and Primes Adipocytes for DNL in Mice

Insight into molecular mechanisms of 5-PAHSA action revealed that 5-PAHSA primes adipocytes for glucose metabolism in a different way from insulin, promoting DNL and impeding TAG synthesis, and uncovered a metabolite reservoir of TAG-bound PAHSAs (TAG estolides). [(Paluchova et al., *Diabetes* **69**: 300, 2020); published on-line in 2019]. IF = 7.199 (2018).

Team's contribution: Members of the Metabolomics Unit were mostly involved in the FAHFA research. FAHFA-related research was supported by the Czech Science Foundation (17-10088Y) and the Ministry of Education, Youth and Sports of the Czech Republic (grants LH14040, LTAUSA17173, LTAUSA18104; PI: Ondrej Kuda) during the period 2015-2019.

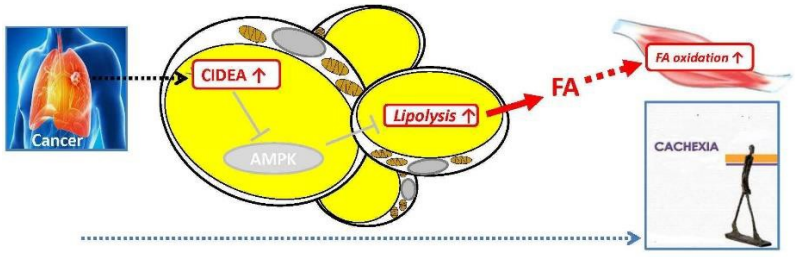
3. Systemic effects of white adipose tissue metabolism

Our special interest in the systemic effects of WAT metabolism was triggered by the discovery that induction of energy expenditure by uncoupling oxidative phosphorylation in WAT mitochondria could counteract obesity in *aP2-Ucp1* transgenic mice (Kopecky et al., *Journal of Clinical Investigation* **96**: 2914, 1995) (>400 citations).

Cancer cachexia can reflect aberrant metabolism of adipose tissue

The role of WAT metabolism in the propensity to obesity, but also in aberrant wasting of adipose tissue in cachexia, became also one of the key research topics of the EU FP7 project DIABAT, in which the team at the Dept. of Adipose Tissue Biology was involved as one of the partners (see below). Cachexia, which is associated with cancer, heart failure, sepsis and burn injuries, worsens the patient's prognosis. It reflects the global metabolic response. Our new results showed that the loss of WAT in cachexia could be caused by „aberrant regulation" of metabolism of WAT itself, namely by inhibition of AMPK activity by high CIDEA level in adipocytes. This results in increased lipolytic activity, while fatty acids are „burned" in skeletal muscle and other tissues. Energy dissipation due to futile cycling in WAT, based on lipolytic release of fatty acids from intracellular TAG and their re-esterification (TAG/FA cycling), also contributes to WAT loss and cachexia. These results published in high impact journal represented one of the major outcomes of the whole DIABAT project and indicated that treatment of patients with cachexia should also directly target WAT metabolism (Rohm et al., *Nat Med* **22**: 1120, 2016). IF = 29.886 (2016).

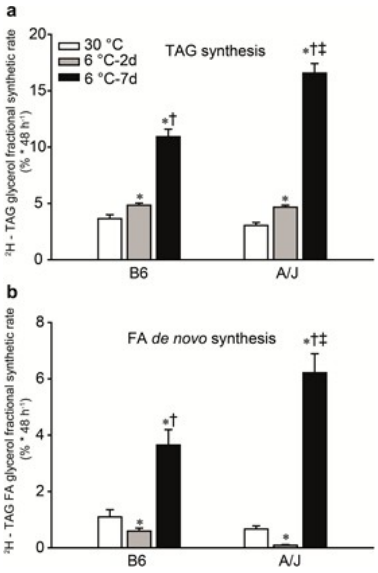
Team's contribution: Supported by the EU FP7 project DIABAT (2011 – 2015; HEALTH-F2-2011-278373), in which the Prague team (headed by J. Kopecky) contributed (P. Janovska and J. Kopecky) by (i) characterization of the AMPK activity in adipose tissue of the mice, and (ii) participating in the study concept. Collaborative work of 24 scientists from 13 institutes from 7 EU countries.



Correlation between obesity resistance and lipid synthesis in adipose tissue

Propensity to obesity depends on the genetic background of the individual. Futile **TAG/FA** cycling as well as DNL represent the core energy-consuming biochemical activities of WAT. We aimed to characterize their roles in cold-induced thermogenesis and energy homeostasis. We have found in mice that changes in WAT metabolism during cold exposure reflected propensity to obesity. The main novel results are that TAG/FA cycling and, somehow paradoxically, also DNL in WAT, as well as hepatic lipoprotein production, are integrated to provide energy fuels for thermogenesis and thus could contribute to lean phenotype. These results were listed among the best achievements of the Czech Academy of Sciences in 2017 (Flachs et al., *Int J Obes (Lond)* **41**: 372, 2017). IF = 5.159 (2017).

Team's contribution: Supported by the EU FP7 project DIABAT (2010 – 2015; HEALTH-F2-2011-278373) – see above; and by the Czech Science Foundation (13-00871S; 2013-2017; PI: Jan Kopecký). The first and the last (corresponding) author are members of the team. The contribution of co-authors from the Dept. of Adipose Tissue Biology was 59 %. Other DIABAT project partners from Norway and the Netherlands were involved in the study.



*Effect of cold exposure on metabolicactivity of WAT of obesity-prone B6 mice and obesity-resistant A/J mice: **a**) triacylglycerol turnover; **b**) de novo fattyacid synthesis.*

4. Early postnatal changes of energy metabolism – imprinting of lean or obese phenotype

Metabolic changes and other variables that occur during perinatal development may have lasting effects on the metabolism and health of an adult. Adaptation to extrauterine environment depends on the transition from glycolysis to catabolism of fatty acids provided as milk lipids.

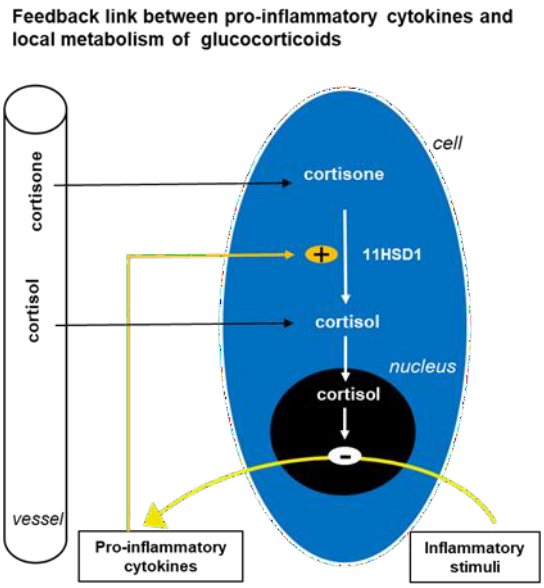
Postnatal induction of muscle fatty acid oxidation in mice differing in propensity to obesity: a role of pyruvate dehydrogenase

We sought to learn whether the postnatal induction of muscle fatty acid oxidation in mice could reflect propensity to obesity and to characterize the mechanisms controlling this induction. We have found in mice that changes in metabolism during suckling could contribute to a different susceptibility to obesity later in life. The main new results concern thepotential involvement of transient suppression of pyruvate dehydrogenase activity in muscle, which (i) results in the induction of fatty acid oxidation, and (ii) is linked to postnatal increase in leptinaemia. We provide a new perspective on the role of early ontogenesis in the lasting control of energy metabolism [(Buresova et al., *Int J Obes (Lond)* **44**: 235, 2020); published on-line in 2018]. IF = 4.514 (2018)

Team's contribution: Supported by the the Czech Science Foundation (MITOCENTRE, GB14-36804G; 2014-2018; PI: Jan Kopecký). The first and the last (corresponding) author are members of the team. The contribution of co-authors from the Dept. of Adipose Tissue Biology was 66 %. Collaboration with the Wageningen University (the Netherlands) and the 1st Faculty of Medicine, Charles University in Prague.

Research activity and characterization of the main scientific results

Topic 1: *Effect of psychological and inflammatory stress on activation of HPA axis, local metabolism of glucocorticoids and the cardiovascular response to stress*



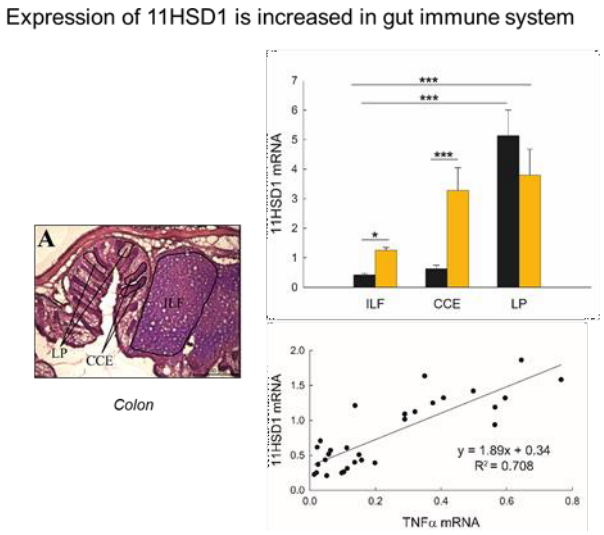
Stress is an important risk factor for human diseases. It activates HPA axis and increases plasma glucocorticoids, which are powerful regulators of many physiological systems including immunity, cardiovascular system, reproduction etc. In addition, HPA axis is a self-regulatory network utilizing its end-product, corticosterone in rats and mice and cortisol in humans, to regulate its own activity through negative feedback mechanisms at the level of brain and pituitary. The level of the target cells to glucocorticoids depends not only on the level of free hormone in blood and the receptor density in target cells, but also on the prereceptor metabolism of glucocorticoids catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase (11HSD). The isoform 1 (11HSD1) converts biologically inactive 11-oxo-steroids (cortisone, 11-dehydrocorticosterone) to cortisol and corticosterone and thus amplifies cellular glucocorticoid signal. In contrast, the isoform type 2 (11HSD2) catalyzes the oxidation of cortisol and corticosterone to inactive 11-oxo-derivatives, thereby reducing the local glucocorticoid signal. The expression of 11HSD1 in the principal components of the HPA axis and in brain areas that are responsible for the positive and negative regulation of this axis suggests that 11HSD1 might modulate the activity of the HPA axis.

These finding together with the well-known effect of genetic background on reactivity of HPA axis led us to study whether the phenotypic differences in the reactivity of HPA axis translate into differences of 11HSD and glucocorticoid receptors (GR) in structures associated with the HPA axis. We demonstrated that inbred stress hyper-responsive Fisher 344 (F344) rats and their stress hypo-responsive Lewis (LEW) rats exhibit not only the well-known phenotypic differences in the HPA axis activity but also strain- and stress-dependent differences in the expression of 11HSD1 and neuropeptides associated with this axis. Psychological stress did not modulate the expression of 11HSD1 in canonical components of the HPA axis but selectively upregulated 11HSD1 in brain structures associated with regulation of this axis, specifically in F344 rats. The findings suggest different local concentration of corticosterone and access to GR in canonical and noncanonical structures of the HPA axis (**Ergang et al. *Psychoneuroendocrinology* 53:49, 2015**). *Team's contribution: The contribution of team members to these results was strongly predominant, the first and the corresponding authors are members of our team. P. Zach (Inst. of Anatomy,Charles Univ., Prague) provided expertise on brain neuroanatomy.*

In our subsequent study we analyzed the effect of psychologic stress onglucocorticoid metabolism in lymphoid organs of F344 and LEW rats, which exhibit marked differences in their HPA axis response not only to psychological stressors but also to inflammation.

We showed that repeated stress increased the regeneration of corticosterone from inactive 11-dehydrocorticosterone via 11HSD1 both in primary (thymus) and secondary (spleen, lymphatic nodes). As this regeneration was higher in LEW than F344 rats, it is reasonable to assume that these strain-dependent differences might participate in the higher susceptibility of the LEW strain to inflammatory diseases (**Ergang et al. *Endocr Connect* 7:1389, 2018**). *Team's contribution: The contribution of team members to these results was predominant, the first and the corresponding authors are members of our team. A. Mikulecká (Lab. of Developmental Epileptology) participated in the development of resident-intruder stress model and I. Mikšík (Lab. of Translational Metabolism) in HPLC analysis of steroids.*

In our earlier studies, we have demonstrated increased bioavailability of endogenous glucocorticoids in bioptic samples of patients with inflammatory bowel disease and in colonic mucosa and mesenteric lymphatic nodes (MLN) of rats and mice with experimental colitis.



These changes in bioavailability reflected regulatory effects of pro-inflammatory cytokines on colonic 11HSD1 and 11HSD2. Using laser microdissection and qRT-PCR, we analyzed the expression of 11HSD1 and cytokines in microanatomical compartments of the immune system associated with colitis, particularly in colon (colonic crypt epithelium, lamina propria, intestinal lymphoid follicles) and MLN (cortex, paracortex, medulla). The experiments identified topo-graphically distinct changes in the regulation of 11HSD1 expression, which correlated with local expression of cytokines. These findings demonstrate different bioavailability of endogenous glucocorticoids in individual compartments of gut immune system, which might facilitate a regulatory role of glucocorticoids in immune processes generated in response to activation of the gut-associated lymphoid tissue (**Ergang et al. *Steroids* 126:66, 2017**). *Team's contribution: The contribution of team members to these results was strongly predominant, the first and the corresponding authors are membersof our team. M. Kment, a gastroenterologist from the University Hospital Královské Vinohrady, Prague, provided expertise on pathology of colitis.*

Stress-induced response involves not only activation of the HPA axis but also sympathetic nervous system and sympathetic adrenomedullary system, which lead to immediate cardiovascular responses to stressors typically consisting of the increased blood pressure, heart rate and cardiac output. Elevated circulating glucocorticoids then potentiate numerous sympathetically mediated effects such as peripheral constriction and impaired adaptation to repeated stressor can participate in the pathogenesis of cardiovascular diseases. We tested therefore the hypothesis whether chronic stress might result in allostatic overload and maladaptive changes of cardiovascular responses. For these experiments wereused F344 rats, which are characterized not only by HPA axis hyper-reactivity to single stressor but also by impaired ability of adaptation to chronic stress. As their counterpartswere used hypo-responsive LEW rats. We showed that hyper-reactivity of F344 rats to stresswas accompanied with a higher blood pressure in both single and repeated stress while repeated stress revealed additional differences in heart rate and baroreceptor reflex sensitivity between F344 and LEW rats. The study showed (i) that the poor adaptation of F344 rats to chronic stress is

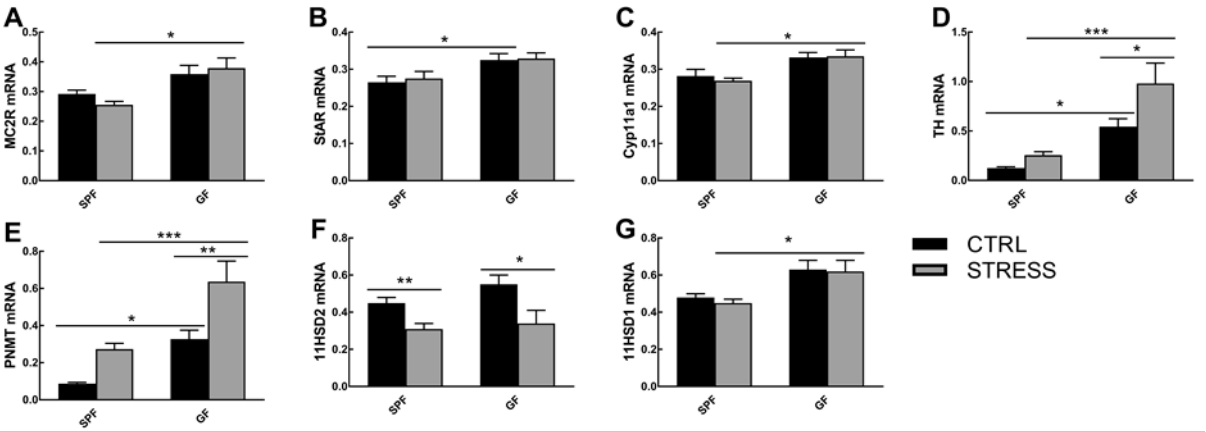
not limited to neuroendocrine response, but also has cardiovascular and behavioral consequences and (ii) detrimental effects of inadequate stress on cardiovascular system (**Vodička et al. *Stress*, 2019, submitted**). *Team's contribution: The collaboration between our team (M. Vodička, first author), the team of the Lab. of Experimental Hypertension (A. Vavřínová, corresponding author, J. Zicha, M. Behuliak) and A. Mikulecká (Lab. of Developmental Epilepsy). We provided the expertise, designing and realization of stress experiments, analyzed the behavior and wrote the manuscript. The team of Exp. Hypertension performed all cardiovascular analyzes and measurement of plasma hormones and participated on writing the manuscript. A. Mikulecká provided the expertise on designing stress experiments and analyzing behavior.* The young members of our team (M. Vodička, P. Ergang) and the team of Lab. of Experimental Hypertension (A.Vavřínová, M. Behuliak, M. Bencze) further collaborated in the study characterizing the effect of chemical sympathectomy on cardiovascular parameters and the compensatory role of adrenal hormones in hypertensive SHR and normotensive WKY rats. Sympathectomy transiently decreased blood pressure, chronically decreased heart rate and improved baroreflex sensitivity. In addition, sympathectomy elevated expression of adrenal catecholamine biosynthetic enzymes, plasma adrenaline (more in WKY than SHR) and plasma aldosterone and corticosterone (only in WKY). The data show that the effect of sympathectomy on blood pressure was counteracted by increased vascular sensitivity to catecholamines in WKY and SHR rats and/or by the enhanced secretion of adrenal hormones, which was more pronounced in WKY rats (**Vavřínová et al. *Hypertens Res* 42:1872, 2019**). *Team's contribution: The collaboration between the team of the Lab. of Experimental Hypertension (A. Vavřínová, corresponding author) and our team (M. Vodička, P. Ergang). The work was done mostly by the collaborators, we participated in the study of adrenal gland using laser microdissection and RT-PCR.*

The studies were supported by grants from Czech Science Foundation P303/10/0969, 15-07268S and 18-02993S awarded to J. Pácha. The studies of interaction between stress, adrenal gland and cardiovascular system (Vavřínová et al. 2019, Vodička et al. 2019) were supported by grant 16-10349Y from Czech Science Foundation (PI: M. Behuliak, Lab. of Experimental Hypertension)

Topic 2: *Gut microbiota and neuroendocrine regulatory pathways during stress*

Accumulating clinical evidence suggests that dysbiosis of gut microbiota might be associated or even a causal factor for various illnesses as varied as inflammatory bowel disease and psychiatric illnesses. The findings emerging from antibiotic, probiotic, infection and germ-free (GF) animal studies strongly suggest that the commensal microbiota residing in the gut affects brain functioning, emotional behavior and acute stress response. However, little is known about the role of the microbiota in shaping the chronic stress response in peripheral components of the HPA axis and peripheral organs. We showed that microbiota attenuated in the pituitary gland the glucocorticoid receptor (GR) sensitivity followed by decreased efficiency of the negative feedback of the HPA. In the adrenal gland, microbiota attenuated the expression of genes encoding steroidogenesis and biogenesis of catecholamines whereas in colon, it attenuated 11HSD1 but potentiated the expression of cytokines, which was downregulated by stress. Our experiments confirmed the effect of gut microbiota in shaping the response of peripheral tissues to chronic stress and indicated the possible pathways by which the environment can interact with glucocorticoid signaling/local prereceptor metabolism of glucocorticoids (**Vodička et al. *Brain Behav Immun* 73:615, 2018**). This paper was selected as an outstanding publication of the Institute of Physiology in the year 2018 and

M. Vodička was awarded Pavel Flachs Prize for young authors. *Team's contribution: The contribution of our team (first and corresponding author) and the team of Lab. of Gnotobiology of the Institute of Microbiology, CAS, were equivalent. Analysis of pituitary, adrenal gland, colon and behavior was done by our team, whereas isolation of MLN cells, their immunophenotyping and quantification of cytokine secretion was done by the collaborating team. Work with animals incl. stress was done by both teams. A. Mikulecká (Lab. of Developmental Epileptology) participated in the analysis of animal behavior.*



Responses of genes encoding adrenal steroidogenesis, catecholamine biogenesis and glucocorticoid metabolism following chronic stress in specific pathogen-free (SPF) and germ-free (GF) mice. MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; Cyp11a1, cholesterol side-chain cleavage enzyme; TH, tyrosine hydroxylase; PNMT, phenylethanolamine N-methyltransferase; 11HSD1 and 11HSD2, 11beta-hydroxysteroid dehydrogenase type 1 and type 2. The data are expressed as the means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

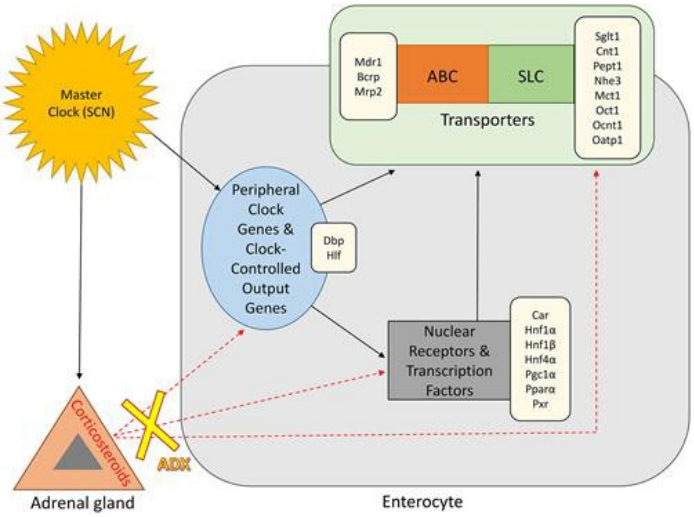
In contrast to the observed effect of microbiota on attenuation of GR sensitivity in pituitary during chronic stress, acute stress did not attenuate either the expression of pituitary GR or its sensitivity. However, microbiota significantly downregulated pituitary proopiomelanocortin (POMC) and corticotropin-releasing hormone receptor (CRHR1), which suggests the possibility that the higher expression of POMC and CRHR1 in GF mice might contribute to the exaggerated HPA response to acute stress in GF animals. The effect of microbiota on adrenal glucocorticoid biogenesis was weak but the extra-adrenal steroid biogenesis in the intestine significantly depended on microbiota, stress and stress x microbiota interaction. These findings show that similar to chronic stress response, the acute stress response is shaped by microbiota not only in the components of the HPA axis but alsoin peripheral organs and that the activation of intestinal steroidogenesis is controlled differently from that in the adrenals (**Vagnerová et al. *Front Immunol* 10:2655, 2019**). *Team's contribution: Collaborative work of our team (first and corresponding author) and the team of Lab. of Gnotobiology of the Institute of Microbiology CAS. Preparation of GF animals was done by the team of Lab. of Gnotobiology, animal work incl. stress was done by both teams, biochemical analysis of pituitary, adrenal gland, ileum and colon together with preparation of the manuscript was done by our team.*

The studies summarized in this chapter were supported by Czech Science Foundation grants 15-07268S and 18-02993S awarded to J. Pácha.

Topic 3: *Role of corticosteroid hormones in the regulation of peripheral circadian clock in the intestine*

Glucocorticoids are considered to synchronize the rhythmicity of clock genes in peripheral tissues and to operate as a link mediating the central clock in suprachiasmatic nucleus of hypothalamus with peripheral clocks due to circadian regulation of adrenal glucocorticoids. To determine the role of endogenous glucocorticoids and their diurnal variations in the regulation of circadian rhythmicity, amplitude and phase of the clock genes and clock-output genes, we compared the effect of adrenalectomy on circadian clock in the intestine, kidney cortex, liver, adipose tissue and splenocytes. We found that adrenalectomy modulated the rhythmicity in tissue and gene-specific manner, the rhythmicity persisted but the amplitude of the rhythms was reduced. The results suggest that endogenous glucocorticoids are necessary for maintaining the high-amplitude rhythmicity of the peripheral clock but itsrhythmicity is not necessary for the phase of the clock (**Soták et al. *Chronobiol Int* 33:520, 2016**). *Team's contribution: The contribution of our team members to these results was predominant (first and corresponding author). A. Sumová (Lab. of Biological Rhythms) provided the expertise on designing the experiment and on the text of the manuscript.* In a subsequent study was analyzed the mechanisms of hormonal regulation of colonic circadian clock. Adrenalectomy decreased substantially the amplitude of clock gene Per2 and repeated administration of glucocorticoid dexamethasone restored the amplitude of Per2 andrescued its circadian rhythm. In contrast pinealectomy had no effect on the colonic clock. Theresults provide evidence for differences in the role of diurnal hormonal signal on maintaining high-amplitude rhythms of peripheral clock (**Polidarová et al. *Chronobiol Int* 34:1, 2017**). *Team's contribution: The concept and majority of the work was done by the team of Lab. of Biological Rhythms, our team prepared the adrenalectomy animals, synchronized them with chronic administration of dexamethasone and participated in sampling of the colonic tissue.*

Significant effect of adrenalectomy was observed not only on the amplitude of circadian clock but also on some transporters. In recent paper we analyzed the diurnal rhythmicity of intestinal transporters of the solute carrier (SLC) and ATP-binding cassette (ABC) families, which participate in intestinal barrier for absorption of nutrients, non-nutrients and oral drugs. Whereas the SLC transporters for nutrients such as glucose and peptides and some efflux pumps of ABC family were rhythmic, the SLC transporters of organic cation, organic anion and nucleoside transporter subfamilies were arhythmic. Adrenalectomy decreased the expression both of arrythmic and rhythmic transporters. In case of rhythmic transporters adrenalectomy decreased the amplitude without any change of the phase of the rhythm. These findings demonstrate that signals from adrenal gland are important for regulation of rhythmic and arhythmic intestinal transporters and that changes of adrenal secretion of glucocorticoid



associated with stress might reorganize intestinal transport barrier and modulate xenobiotic/drug transport and diurnal changes of pharmacokinetics (**Vagnerová et al. *Comp Biochem Physiol C* 266:108607, 2019**). *Team's contribution: The work was done by our team.*

The studies summarized in the papers Soták et al. (2016) and Vagnerová et al. (2019) were supported by the Czech Science Foundation grant 13-08304S (PI: J. Pácha); the studies summarize in the paper Polidarová et al. (2017) were predominantly supportedby the grant 14-07711S awarded to A. Sumová (Lab. of Biological Rhythms) and partially by the grant 13-08304S awarded to J. Pácha.

Our research activities in the evaluated period significantly benefited from intensive fruitful partnership with several research and service laboratories within Institute of Physiology. In particular, we have a long-lasting cooperation with the teams of I. Mikšík (Lab. of Translational Metabolism, **LTM**), J.Zicha/M. Behuliak (Lab. of Experimental Hypertension, **LEH**) and A. Sumová (Lab. of Biological Rhythms, **LBR**). Other intensive collaboration duringthe reported period represents the collaboration with the Lab. of Developmental Epileptology (**LDE**), particularly with A. Mikulecká.

Mikšík's team of analytical chemists in **LTM** was indispensable in the projects in which it was necessary to analyze and quantify corticosteroids using high pressure liquid chromatography. In the evaluated period, we published two joint papers (Ergang et al. *Endocr Connect* 7:1389, 2018; Jágr et al. *Eur J Oral Sci* 127:112, 2019).

Long-lasting collaboration with the team of **LBR** facilitated the research of circadian clock in the gastrointestinal tract under physiological and pathophysiological conditions,which was supported by the 5-year grant from Czech Science Foundation (13-08304S, 2013 – 2017, PI: J. Pácha). In the evaluated period, we published two joint papers with LBR (Soták et al. *Chronobiol Int* 33:520, 2016; Polidarová et al. *Chronobiol Int* 34:1, 2017).

The successful cooperation with **LDE** was based on experiments, which analyzed behavior of animals under specific conditions and on expertise in molecular biological techniques. This cooperation led to three joint papers (Szczurowska et al. *Exp Neurol*283:97, 2016; Ergang et al. *Endocr Connect* 7:1389, 2018; Vodička et al. *Brain Behav Immun* 73:615, 2018).

Our collaboration with **LEH** was focused on the experiments, which tried to analyze the effect of stress and corticosteroids on cardiovascular system. This collaborative effort led to successful application for a junior grant under the leadership of M. Behuliak (16-10349Y) from LEH, in which participated young scientists of LEH and of our group. The cooperation led to two joint papers (Behuliak et al. *J Hypertens* 33:2443, 2015; Vavřínová et al. *Hypertens Res* 42:1872, 2019), the third is submitted after revision (Vodička et al. *Stress*, 2019, submitted).

Research activity and characterisation of the main scientific results

Note: Metabolomics-oriented projects have been initiated after the change of the Head of the laboratory and are therefore limited to years 2018–2019. Proteomics-oriented projects cover the full evaluation period (2015–2019).

(A) Metabolomics-oriented research

Bioactive lipids related to T2D. Branched esters of palmitic acid and hydroxy-stearic acid (PAHSA) are anti-inflammatory and anti-diabetic lipokines that connect glucose and lipid metabolism. Our data revealed new cellular and physiological mechanisms underlying the beneficial effects of 5-PAHSA, its relation to insulin action in adipocytes, and independently confirmed a PAHSA metabolite reservoir linked to adipose triglyceride lipase (ATGL)-mediated lipolysis. We found that PAHSAs are liberated from triacylglycerol (TAG) estolides, TAG-like molecules containing esterified PAHSA during lipolysis. We reported TAG estolides extracted from epididymal white adipose tissue (eWAT) mouse extracts and analyzed by LC–MS/MS (**Figure 1**).

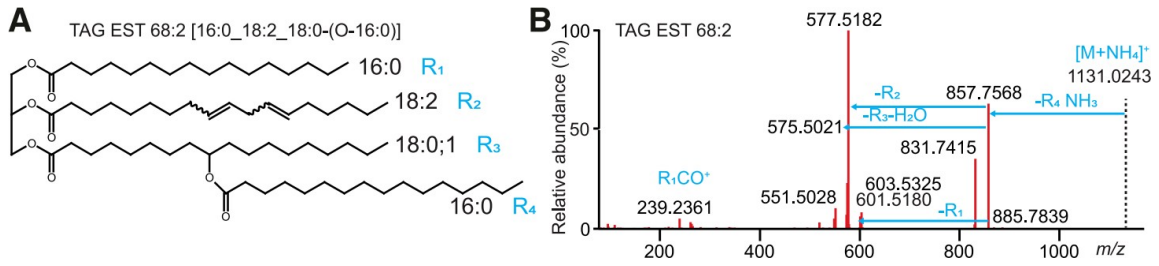


Figure 1. Tentative structure of a TAG estolide containing 9-PAHSA. (A) This structure was deduced from MS/MS spectra observed in eWAT mouse extracts. (B) MS/MS spectrum of the measured analyte.

- Paluchova V. *et al.*, 5-PAHSA is regulated by adipose triglyceride lipase and primes adipocytes for de novo lipogenesis in mice. *Diabetes* 69 (2020) 300–312. Published online December 5, 2019. doi: 10.2337/db19-0494, IF = 7.2 (Q1)
- In cooperation with the Laboratory of Metabolism of Bioactive Lipids and Laboratory of Adipose Tissue Biology, IPHYS
- The contribution of the team member (5%): (i) coordination and performing of metabolomics and lipidomics analyses; (ii) advanced data processing of acquired data set; (iii) data curation.

Lipidome changes due to exercise training. Aging is associated with redistribution of adipose tissue characterized by increased visceral and ectopic fat deposition. In the elderly, lifestyle interventions based on increased physical activity are primarily aimed to improve muscle function and/or cardiovascular fitness. Our data suggest that exercise training stimulates also beneficial metabolic changes in adipose tissue, including the synthesis of branched esters of palmitic acid and hydroxy-stearic acid (PAHSA) and PAHSA-containing triacylglycerol estolides. Although the benefit of omega-3 polyunsaturated fatty acid (PUFA) supplementation was not proven, our discovery can help understand the nature of the metabolic benefits of exercise.

- Brezinova M. *et al.*, Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* 1865 (2020) 158576. Published online November 16, 2019. doi: 10.1016/j.bbalip.2019.158576, IF = 4.4 (Q1)
- In cooperation with the Laboratory of Metabolism of Bioactive Lipids and Laboratory of Adipose Tissue Biology

- The contribution of the team member (10%): (i) coordination and performing of metabolomics and lipidomics analyses; (ii) advanced data processing of acquired data set; (iii) data curation.

Computational metabolomics for metabolomics and lipidomics data processing. Mass spectrometry raw data repositories have contributed to increased transparency in metabolomics studies and the discovery of novel insights in biology by reanalysis with updated computational metabolomics tools. We reanalyzed the previously published lipidomics data sets resulting in the annotation of 1437 lipids achieving a 40% increase in annotation compared to the previous results. Our data shows that integrated analyses of untargeted and targeted approaches are necessary to extract the maximum amount ofmetabolome information (**Figure 2**).

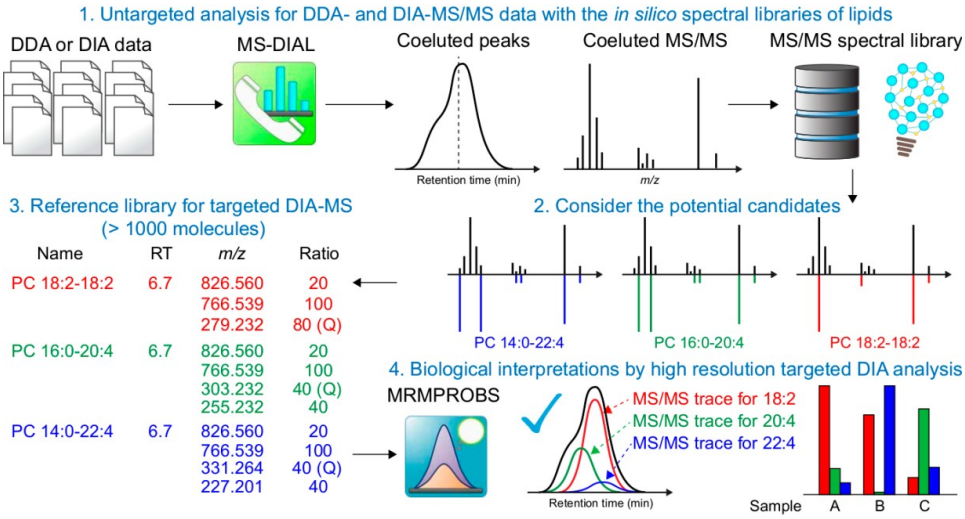


Figure 2. Integrated strategy of untargeted and targeted analyses to increase the coverage of annotated lipids. First, the data dependent (DDA) and independent (DIA) acquisition data are analyzed using the untargeted analysis pipeline where peak-picking, MS/MS assignment, and peak alignment are performed. Second, all potential lipid candidates that exceeded the cut-off of mass spectral similarity are obtained. Third, a reference library containing the target metabolite name, retention time, precursor ion *m*/*z*, product ion *m*/*z* list, and ion abundance ratios is automatically generated.

- Tsugawa H. *et al*., Mass spectrometry data repository enhances novel metabolite discoveries with advances in computational metabolomics. *Metabolites* 9(6) (2019) 119. doi: 10.3390/metabo9060119, IF = 3.3 (Q2)
- In cooperation with RIKEN Center for Sustainable Resource Science (Japan)
- The contribution of the team member (17%): designing the research

Novel approach for normalizing large-scale untargeted lipidomics data. Large-scale untargeted lipidomics experiments involve the measurement of hundreds to thousands of samples. Such extensive data acquisition processes introduce a variety of systematic errors, including batch differences, longitudinal drifts, or even instrument-to-instrument variation. Technical data variance can obscure the true biological signal and hinder biological discoveries. We developed a novel quality control (QC) sample-based data normalization algorithm, systematic error removal using random forest, SERRF. SERRF corrects batch effects and time-dependent drifts in large-scale plasma lipidomics human cohort studies. SERRF reduced the average technical errors for these data sets to 5% RSD. We conclude that SERRF outperforms other existing methods and can significantly reduce the unwanted systematic variation, revealing biological variance of interest.

- Fan S. *et al*., Systematic error removal using random forest for normalizing large- scale untargeted lipidomics data. *Analytical Chemistry* 91(5) (2019) 3590–3596. doi: 10.1021/acs.analchem.8b05592, IF 6.4 (Q1)
- In cooperation with West Coast Metabolomics Center (California)
- The contribution of the team member (10%): (i) coordination and performing of lipidomics analyses on a large cohort study (>1200 patients); (ii) advanced data processing of acquired data set; (iii) data curation.

Lipid composition of T cell membrane rafts. An emerging alternative to the use of detergents in biochemical studies on membrane proteins is the use of styrene-maleic acid (SMA) amphipathic copolymers. Lipidomic profiling provided an in-depth characterization of several lipid classes of different gel filtration fractions. Our results support the use of SMA as a potentially better (less artifact prone) alternative to detergents for studies on membrane proteins and their complexes, including membrane rafts.

- Angelisová P *et al.*, The use of styrene-maleic acid copolymer (SMA) for studies on T cell membrane rafts. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1861(1) (2019) 130–141. doi: 10.1016/j.bbamem.2018.08.006, IF 3.8 (Q1)
- In cooperation with the Institute of Molecular Genetics of the Czech Academy of Sciences
- The contribution of the team member (13%): (i) performing of lipidomics analyses; (ii) advanced data processing of acquired data set; (iii) data curation.

(B) Proteomics-oriented research

Clubfoot. Clubfoot belongs to a group of fibro-proliferative disorders but its origin remains unknown. Our research aimed to achieve complex proteomic comparison of affected tissue with controls (**Figure 3**). Most of the differently expressed proteins seem to be promising targets for future investigations and treatment of clubfoot.

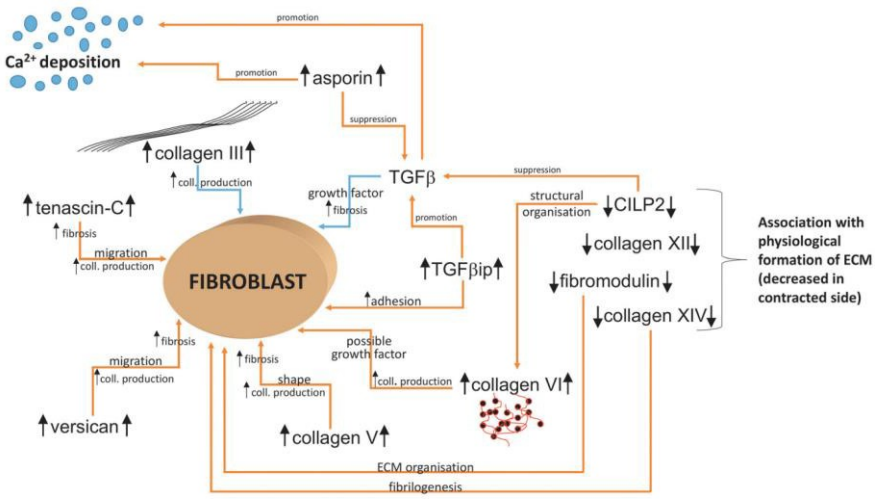


Figure 3. Doubled arrows express upregulation (↑protein↑) or downregulation (↓protein↓) of proteins to the M-side (contracted side, "disc-like tissue"). All upregulated extracellular matrix (ECM) proteins have some relationship to fibroblasts. Some upregulated proteins are implicated in fibrosis (↑fibrosis) and/or take part in migration and/or enhanced collagen production (↑coll. production) in dermal. Orange arrows designate predicted interactions based on the upregulated proteins in the contracted side. The upregulation of these proteins was detected in this type of fibrotic tissue for the first time. Already described possible pathways are expressed in blue lines. All observed downregulated proteins associate with the physiological formation of ECM.

- Eckhardt A. *et al*., Novel contribution to clubfoot pathogenesis: The possible role of extracellular matrix proteins. *Journal of Orthopaedic Research* 37 (2019) 769-778. doi: 10.1002/jor.24211, IF = 3.0 (Q1)
- In cooperation with the Laboratory of Biomaterials and Tissue Engineering and Laboratory of Biomathematics and Department of Orthopedics, University Hospital Bulovka, Charles University
- The contribution of the team members (60%): performing most measurements and evaluations. The first and corresponding author is a member of the team.

Cardiac proteomics. The major result of our research revealed for the first time vertical protein concentration gradient in the heart by significant baso-apical differences (both in the left ventricle and right ventricle). Described changes were more pronounced in the harder working left ventricle.

- Eckhardt A. *et al*., Proteomic analysis of cardiac ventricles: baso-apical differences. *Molecular and Cellular Biochemistry* 445 (2018) 211-219. doi: 10.1007/s11010-017-3266-8, IF = 2.8 (Q1)
- In cooperation with the Laboratory of Developmental Cardiology
- The contribution of the team members (75%): performing most measurements and evaluations. The first and corresponding author is a member of the team.

Pulmonary hypertension. Pulmonary hypertension is a serious disease characterized by the structural remodeling of pulmonary arteries. Our research aimed to the relationship between changes of arterial cells and the extracellular matrix. The main result was the evidence of the loss of collagen VI in the tunica media during the development of hypoxic pulmonary hypertension, which is a novel step in pulmonary arterial remodeling (**Figure 4**).

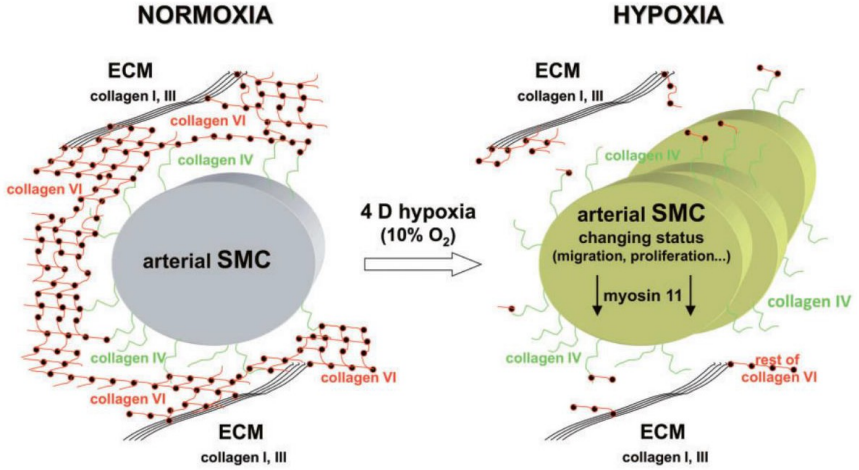


Figure 4. Reduction of collagen VI content (red lines with black dots: collagen VI forms "beaded" fibrils) in the tunica media of pulmonary arteries (about 300 mm diameter) during hypoxia. On the contrary, the amount of collagen type IV (in green on the surface of smooth muscle cell, SMCs) was unaltered. Fibrillar collagens (type I and III) are in black and collagen VI anchorages them to collagen IV. The status of some SMCs during hypoxic pulmonary hypertension development is changed (decreasing concentration of protein myosin 11: marker of SMC status). The loss of collagen VI around cells in media (disorganization of chemical and physical contacts) could be a new step in this process.

- Zaloudikova M. *et al.*, Decreased collagen VI in the tunica media of pulmonaryvessels during exposure to hypoxia: a novel step in pulmonary arterial remodeling. *Pulmonary Circulation* 9(3) (2019). doi: 10.1177/2045894019860747, IF = 2.1 (Q2)
- In cooperation with the Laboratory of Biomaterials and Tissue Engineering and Department of Physiology, 2nd Faculty of Medicine of Charles University
- The contribution of the team members (13%): performing proteomic analyses. The corresponding author is a member of the team.

Tooth and saliva proteins. The research of human oral health, human teeth as well as saliva is nowadays an accelerating task. In our research, we looked at proteins of tooth and saliva. We presented for the first time a proteomic two-dimensional difference gel electrophoresis (2D-DIGE) analysis to elucidate the changes in expression between the third molar tooth pulp of caries-resistant and caries-susceptible people (**Figure 5**). This study found eight overexpressed proteins in caries-resistant samples. We assume that theseproteins are likely involved in the natural resistance of teeth to the development of dental caries. Our findings suggest that resistance to the development of dental caries is a multifactorial phenomenon.

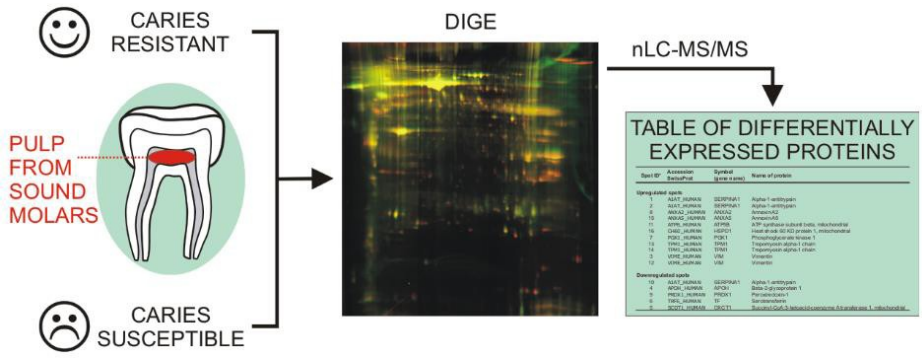


Figure 5. Tooth proteomic comparison of caries-free and caries-positive people for detection of anti-caries compounds.

- Jágr M. *et al.*, Proteomic analysis of human tooth pulp proteomes – Comparison of caries-resistant and caries-susceptible persons. *Journal of Proteomics* 145 (2016) 127–136. doi: 10.1016/j.jprot.2016.04.022, IF = 3.5 (Q1)
- In cooperation with the Department of Stomatology, First Faculty of Medicine, Charles University
- The contribution of the team members (67%): Conducting proteomics analysis. The first and corresponding author is a member of the team.

We also looked at differences in saliva proteins. In this study, we compared the differences in the abundances of proteins in the saliva between caries-resistant and caries-susceptible females and males. Our results demonstrate that the observed differences in the protein levels might have an influence on anti-caries resistance. A total of 19 potential markers of tooth caries were found.

- Kulhava L. *et al*. Differences of saliva composition in relation to tooth decay and gender. *Folia Biologica* 64 (2018) 195-203. IF = 1.1 (Q3)
- In cooperation with the Department of Stomatology, First Faculty of Medicine, Charles University, Prague
- The contribution of the team members (67%): Conducting proteomics analysis. The first and corresponding author is a member of the team.

Further, we also studied dentin–enamel junction at teeth — the border between two different mineralized structures — enamel and dentin. In this work we used e.g. technique laser capture microdissection. We identified 46 proteins in dentin–enamel junction and enamel organic matrix. Our findings revealed new insight into proteomics of highly mineralizedtissues that are obviously difficult to analyze.

- Jágr M. *et al.*, Proteomic analysis of dentin-enamel junction and adjacent protein-containing enamel matrix layer of healthy human molar teeth. *European Journal of Oral Sciences* 127 (2019) 112–121. doi: 10.1111/eos.12594, IF 1.8 (Q1)
- In cooperation with Quality of Plant Products, Crop Research Institute, Prague and Institute of Dental Medicine, First Faculty of Medicine, Charles University
- The contribution of the team members (67%): Conducting proteomics analysis. The first and corresponding author is a member of the team.

Eggshells. We studied eggshells of birds as well as crocodiles. The eggshell is a barrier that plays an important role in the defense of the egg against microbial and other infections. Properties of eggshells (colors etc.) are important in biological consequences. In our laboratory, we measured pigments at eggshells as well as we first described the proteome of crocodile eggshells.

- Maurer G. *et al*., First light for avian embryos: eggshell thickness and pigmentation mediate variation in development and UV exposure in wild bird eggs. *Functional Ecology* 29 (2015) 209–218. doi: 10.1111/1365-2435.12314, IF 5.0 (Q1)
- In cooperation with the School of Earth & Environmental Sciences, University of Adelaide
- The contribution of the team member (17%): Conducting proteomics analysis.
- Duval C. *et al*., Maternal influence on eggshell maculation: implications for cryptic camouflaged eggs. *Journal of Ornithology* 157(1) (2016) 303–310. doi: 10.1007/s10336-015-1278-2, IF = 2.0 (Q1)
- In cooperation with the School of Psychology and Neuroscience, University of St Andrews, UK
- The contribution of the team member (17%): Conducting proteomics analysis.
- Brulez K. *et al*., Eggshell pigment composition covaries with phylogeny but not with life history or with nesting ecology traits of British passerines. *Ecology and Evolution* 6 (2016) 1637–1645. doi: 10.1002/ece3.1960, IF = 2.4 (Q1)
- In cooperation with Centre for Ornithology, School of Biosciences, College of Life & Environmental Sciences, University of Birmingham, UK
- The contribution of the team member (10%): Conducting proteomics analysis.
- Poláček M. *et al*., Eggshell coloration and its importance in postmating sexual selection. *Ecology and Evolution* 7 (2017) 941–949. doi: 10.1002/ece3.2664, IF = 2.4 (Q1)
- In cooperation with the Department of Biology, University of Padova, Padova, Italy
- The contribution of the team member (17%): Conducting proteomics analysis.
- Mikšík I. *et al*., Analysis of siamese crocodile (*Crocodylus siamensis*) eggshell proteome. *Protein Journal* 37(1) (2018) 21–37. doi: 10.1007/s10930-017-9750-x, IF = 1.0 (Q3)
- In cooperation with the Institute of Anatomy, First Faculty of Medicine, Charles University, Prague

- The contribution of the team member (75%): Conducting proteomics analysis.

Horns. The purpose of this study was to find synthetic alternatives to Saiga horn extract, which is used in traditional Chinese medicine, by identifying potentially biologically active compounds in the extracts. It should be emphasized that saiga is a critically endangered animal. As possible antipyretic and procoagulant compounds were identified short-chain poly-3-hydroxybutyrates.

- Pataridis S. *et al*., Identification of short-chain poly-3-hydroxybutyrates in Saiga horn extracts using LC-MS/MS. *Journal of Separation Science* 42 (2019) 797–808. doi: 10.1002/jssc.201800910, IF = 2.5 (Q2)
- In cooperation with prof. Romanov (Kalmykian State University, Elista, Russia)
- The contribution of the team members (67%): Conducting LC–MS analysis. The first and corresponding author is a member of the team.

Research activity and characterisation of the main scientific results

References to publications authored by the team members within the evaluated period (2015 – 2019) and their contribution are highlighted in **bold letters** and in *italics*, respectively.

Topic 1. Circadian clocks during the early and late parts of the lifespan

Ontogenesis of circadian clocks. Our significant contribution to this topic has been recognized by invitation to submit a review on ontogenesis of the circadian clocks within a Special edition in European Journal of Neuroscience (***Sumová A., Čečmanová V.: Mystery of rhythmic signal emergence within the suprachiasmatic nuclei. Eur. J. Neurosci.51:300-309, 2020, published on line in 2018*** – *both authors are from the team*)(Fig.1).

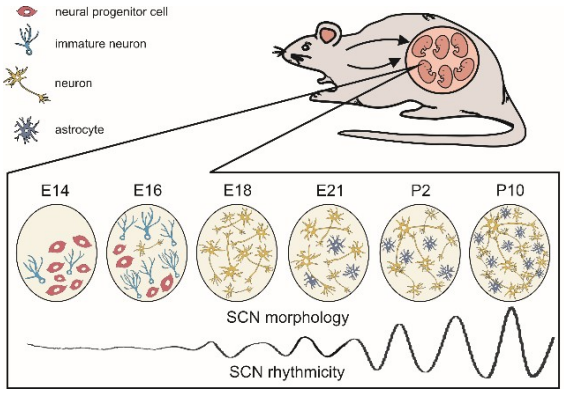


Fig. 1: from Sumová and Čečmanová, 2020

Our previous studies demonstrated that mammalian suprachiasmatic nuclei (SCN) and their intrinsic rhythmicity develop during ontogenesis gradually from fetal to postnatal period(Sladek at al., 2004). In the rat, the SCN forms morphologically between embryonic day (E) 14 and E17, with gestation terminating at E21-22. Overt SCN rhythmicity is already presentin the late embryonic stage. Base on the result, we formulated a hypothesis that the over rhythmicity observed in the fetal SCN is driven by rhythmic maternal cues rather thanfunctional fetal SCN clock (Sumova et al., 2012). To test the hypothesis, we performed a detailed study to ascertain when exactly during the fetal stage SCN clock develops in vivo and whether overt rhythmicity observed during prenatal period results from a functional fetal clock. To achieve this goal, we assessed the prenatal development of rhythmic expression profiles of clock genes (*Per2*, *Nr1d1* and *Bmal1*) and genes related to cellular activity (*c-fos*, *Avp* and *Vip*) with a highly sensitive and specific method for detection of the clock gene expression (RT qPCR in laser dissected fetal SCN). We found that at E19, rhythms in expression of canonical clock genes *Per2* and *Bmal1* were absent in the fetal SCN; however,the expression of *Nr1d1* and the other genes related to cellular activity was driven rhythmically. The results confirmed our previous results and supported our hypothesis that at the early stage in vivo the developing fetal SCN clock is likely entrained via oscillation of genes involved in cellular activity driven by maternal rather than fetal circadian clock (***Houdek P. and Sumová A.: In vivo initiation of clock gene expression rhythmicity in fetal rat suprachiasmatic nuclei. PLoS One 2014; 9(9):e107360*** – *both authors belong to the team*).

The maternal cues entraining the fetal SCN clock have been rather elusive. In our studies, we focused on the hormonal pathways, namely on the role of melatonin and glucocorticoids. Whereas melatonin has been studied in this context previously, a potential role of glucocorticoids has been proposed originally by our team.
To ascertain the role of maternal hormone melatonin in entrainment of the fetal circadian clocks, we aimed at providing a lacking evidence that melatonin affects gene expression in the fetal/neonatal SCN. We performed a study with intact and pinealectomized pregnant rats which were exposed to constant light throughout pregnancy to abolish/suppress the endogenous melatonin and behavioral rhythms. During the last 5 days of gestation, we injected the rats with melatonin or vehicle or left them untreated. After delivery, we measured daily expression profiles of *c-fos* and *Avp* in the suprachiasmatic nuclei (SCN), and *Per1*, *Per2, Rev-erbα* and *Bmal1* in the liver in 1-day-old pups. We found that due to the

administration of melatonin to pregnant rats, the gene expression profiles were entrained in the newborn pups' SCN, but not the liver. Monitoring of maternal activity revealed that the effect was not mediated via melatonin-induced changes in maternal behavior. Melatonin and vehicle entrained the SCN gene expression profiles to different phases, demonstrating that the effect of melatonin was apparently not due to the treatment procedure per se. This result provides the first evidence that melatonin affects the SCN fetal clock in vivo at the gene expression level. Additionally, our data demonstrate that in pregnant rats with suppressed endogenous melatonin levels, pharmacological doses of melatonin affect the fetal clock in the SCN, but not in the liver. The result may be of potential interest to neonatologist as they may provide recommendations regarding to the light exposure and/or the drug treatment (melatonin) to pregnant women (***Houdek P., Polidarová L., Nováková M., Matějů K., KubíkŠ., Sumová A.: Melatonin administered during the fetal stage affects circadian clock in the suprachiasmatic nucleus but not in the liver. Dev. Neurobiol. 75(2): 131-144, 2015 -*** *the authors P.H., L.P., M.N., K.M., A.S. belong to the team, that includes the first and the last (corresponding) author. They designed and performed the study and wrote the manuscript. The co-author K.Š. is from Laboratory of Neurophysiology of Memory, IPHYS, CAS, and he was helping us with the brain surgery*).

Intriguingly, our previous studies suggested that entrainment of the fetal SCN may occur also in absence of maternal melatonin. Manipulation with daily feeding/activity regimens of pregnant rats who were maintained in constant light (that is, when their endogenous melatonin levels were suppressed) significantly entrained the SCN clock in newborn pups (Novakova et al., 2010). Therefore, we hypothesized that other rhythmically released hormones may be involved. Although the adult SCN is resilient to glucocorticoids (GC), they represented the hottest candidates for us. The idea fitted into our hypothesis that the fetal rodent SCN resemble in their organization GC-sensitive peripheral tissues (Sumova et al., 2006). We first examined mechanism how GC reach the fetal SCN and we found that maternal but not fetal part of the placenta harbors the autonomous circadian clock, which is reset by dexamethasone (DEX) and rhythmically expresses *Hsd11b2*. These results provided a new mechanism for rhythmic GC passage through the placental barrier and its adjustment according to actual GC levels. Using the knock-in mouse model (*mPer2^{Luc}* mice) we found that hypothalamic explants containing the SCN prepared at embryonic day (E)15(in mice that is about 4 days before delivery) spontaneously develop rhythmicity within several days in culture with dynamics varying among fetuses from the same litter. Culturing of these explants in media enriched with DEX accelerated the development. At E17, treatment of the explants with DEX induced phase advances and phase delays of therhythms depending on timing of the treatments and the shifts were completely blocked by theGC receptor antagonist mifepristone. Vehicle treatment also greatly shifted the clock but the DEX-induced phase response curve differed to that induced by vehicle. We also confirmed sensitivity of the fetal SCN to GCs in vivo because DEX administration to pregnant rats acutely downregulated *c-fos* expression specifically in the laser-dissected fetal SCN. Our results provide the first evidence that whereas the adult SCN is resilient to GC, the fetal SCN clock may respond to changes in GC levels. These results pawed a pathway for a new research topic on impact of stress and glucocorticoid treatment in pregnancy on developmentof the circadian clock in the SCN (***Čečmanová V., Houdek P., Šuchmanová K., Sládek M., Sumová A.: Development and entrainment of the fetal clock in the suprachiasmatic nuclei: The role of glucocorticoids. J. Biol. Rhythms, 34(3):307-322, 2019*** *– all authors belong to the team*).

In a parallel set of studies on ontogenesis of the circadian clocks, we employed Spontaneously Hypertensive Rats (SHR), a rat strain which develops cardiovascular and metabolic pathology in adulthood and in which we identified aberrances in its circadian system compared with healthy control rats (Sladek et al., 2012; Polidarova et al., 2013). An analysis of ontogenetic development of clock gene expression rhythms in the SCN and peripheral tissues (liver and colon) revealed significant differences in SHR compared with

Wistar rats. In SHR the development of a high amplitude expression rhythm selectively for *Bmal1* was delayed in the SCN and liver. Additionally, in pregnant SHR restriction of maternal feeding to inactivity period shifted the fetal SCN clocks despite the fact that the mothers were maintained under the light/dark cycle when they produce melatonin. In contrast, no shifts were detected in Wistar rats. Our findings of the diverse development and higher sensitivity of the developing circadian system of SHR to maternal cues might result from our previous data on differences in the SHR circadian genotype. They provided a background for our further studies on their potentially causal link to cardiovascular and metabolic diseases, which the animal model spontaneously develops later in adulthood (***Olejníková L., Polidarová L., Paušlyová L., Sládek M., Sumová A.: Diverse development and higher sensitivity of the circadian clocks to changes in maternal feeding regime in a rat model of cardio-metabolic disease. Chronobiol. Int., 32(4): 531- 47, 2015*** - *all authors belong to the team*). As these two rat strains differ in their sensitivity to stress, we tested our speculation about a higher sensitivity of SHR's SCN to maternal signalsin a follow-up study in which we compared sensitivity to maternal arousal between the neonatal SHR and Wistar rats' SCN. We confirmed that neonatal SCN of SHR contains higher levels of GC receptors than that of Wistar rats and demonstrated that maternal stress-elevated GC levels in plasma of pups during the first 4 days after birth affect strain- dependently *Bmal1* gene expression profiles in the neonatal SCN via GC-dependent pathway. Our results demonstrate that the SCN is sensitive to stressful stimuli also early after birth and the effect is mediated via glucocorticoid-dependent pathways (***Olejníková L., Polidarová L., Sumová A.: Stress affects expression of the clock gene Bmal1 in the suprachiasmatic nucleus of neonatal rats via glucocorticoid-dependent mechanism. Acta Physiol (Oxf). May;223(1):e13020, 2018*** – *all authors belong to the team*).

These findings on atypical development and sensitivity of the immature circadian clocks in SHR prompted us to elucidate whether this might arise from aberrant maternal behavior of SHR compared to and Wistar rats that we recorded soon after delivery. We assessed the impact of altered timing and quality of maternal care on the offspring's circadian system using a cross-strain fostering approach. The „abnormal", i.e., circadian misaligned, care was represented by SHR and "normal" care by Wistar rats. Worse maternal care provided to Wistar pups by SHR mother impaired entrainment of their central clock parameters duringthe early developmental stages. Better maternal care provided to SHR pups facilitateddevelopment of amplitudes of *Bmal1* clock gene expression in their central clock, as well as the clock-driven activity/rest rhythm, and its entrainment to the external light/dark cycle. Italso remedied the dampened amplitudes of the colonic clock during postnatal development. Intriguingly, better maternal care improved not only the circadian clocks, but also cardiovascular parameters in SHR offspring because it protected them from increased heart rate, which spontaneously develops in those reared by their genetic mothers. The results provided compelling evidence that the circadian phenotype of a foster mother may affect the pathological symptoms of the offspring even if they are genetically programmed. (***OlejníkováL., Polidarová L., Behuliak M., Sládek M, Sumová A.: Circadian alignment in a foster mother improves the offspring's pathological phenotype. J. Physiol. (London), 596, 23:5757-5775, 2018*** – *the authors L.O., L.P.,M.S. and A.S belong to the team, which includes the first and last (corresponding) author. They designed and performed the study and wrote the manuscript. M.B. is from the Laboratory of Experimental hypertension, IPHYS, CAS and he performed the measurement of heart rate in one group of rats*).

Aging of circadian clocks. It is well known that circadian regulation of behavior worsens with age, however, the mechanism behind this phenomenon has not been ascertained. Specifically, it is not clear to what extend the ability of the circadian clock in the suprachiasmatic nuclei (SCN) to generate the rhythm is affected by aging. Therefore, we aimed our study at ascertaining the effect of aging on the functioning of the SCN of *mPer2*^{Luciferase} mice in vitro in standard LD12:12 cycle, constant darkness (DD) and constant light (LL) conditions. We found that under LD12:12 and DD, the SCN ability to produce

bioluminescence rhythms in vitro was not compromised in aged mice although aging significantly affected their SCN-driven locomotor activity rhythms. In mice exposed to LL, which worsened the age-induced effect on behavioral rhythms, only a marginal age- dependent effect on in vitro rhythmicity in explants was apparent. The results suggest that in the aged animals, LL may weaken the coupling among oscillators in specific sub-regions of the SCN, leaving other sub-regions better synchronized. Our results demonstrate that although age worsened the SCN output rhythm, the SCN molecular core clock mechanism itself was relatively resilient to aging in these same animals. The results suggest the involvement of pathways downstream of the core clock mechanism which are responsible forthis phenomenon (***Polidarová L., Sládek M., Novosadová Z., Sumová A.: Aging does notcompromise in vitro oscillation of the suprachiasmatic nuclei but makes it more vulnerable to constant light. Chronobiol. Int. 34(1):105-117, 2017*** *– all authors belong theteam*).

This result was rather surprising and prompted us to study the effect of aging on the peripheral clocks. We revealed that our aged *mPer2^{Luc}* mice develop abnormal glucose homeostasis represented by hyperinsulinemic hypoglycemia. Therefore, we focused on clocks in peripheral tissues involved in glucose regulation, namely the pancreas. We found that age-induced impairment of glucose homeostasis is related to the *Pclo*-mediated insulin hyper-secretion and *Slc2a2*-mediated glucose transport impairment in the pancreas, and the alterations in *Pp1r3c*-related glycogen storage and *Sgk1*-related glucose transport in the liver. Aging affected only marginally clock in the pancreas in vivo and had no effect on the clock in pancreatic organotypic explants in vitro. However, the pancreatic clock was highly sensitive to exposure of animals to constant light conditions. This finding was similar to resultof our study in the effect of aging on the SCN clock (see above). These findings may provide an explanation for the previously demonstrated relationship between disturbances in the circadian system and disordered glucose homeostasis, including diabetes mellitus type 2, in subjects exposed to long-term shift work (***Novosadová Z., Polidarová L., Sládek M., Sumová A.: Alteration in glucose homeostasis and persistence of the pancreatic clockin aged mPer2^{Luc} mice. Sci. Rep. 2018 Aug 3;8(1):11668*** *– all authors belong to the team*)**.**

Topic 2. Systemic neurohumoral regulations within the circadian system

The concept of humoral regulation of the circadian clocks within the mammalian circadian system has been established a long time ago. It is well documented for GC but for other hormones, namely melatonin, the role in circadian regulation is highly cited in literature but direct evidence for this effect is rather sparse. Therefore, we decided to shed light on this area and perform a study in which we investigated impact of long-term absence of melatonin on regulation of circadian system in melatonin proficient rodent model (Wistar rat). We recorded behavior and circadian clock gene expression in rats, which were devoid of the melatonin signal due to pinealectomy (PINX) for more than one year and compared them with those of intact age-matched controls. We found that PINX led to a decrease in spontaneous locomotor activity and a shortening of the free-running period of the activity rhythm driven by the central clock in the SCN in DD, but did not affect behavior and feeding under LD12:12. However, PINX had a significant effect on amplitudes of the clock gene expression rhythms in the duodenum and partially in the liver. These results demonstrate the significant impact of long-term melatonin absence on period of the central clock and the amplitudes of the peripheral clocks, and suggest that melatonin might be a redundant but effective endocrine signal for these clocks (***Houdek P., Nováková M., Polidarová L., Sládek M., Sumová A.: Melatonin is a redundant entraining signal in the rat circadian system. Hormones and Behavior, 83: 1 -5, 2016*** *– all authors belong to the team*).

Previously, our group provided an important discovery that circadian clock is located in the colonic epithelial cells where, in spite of their short half-life, drives processes related to cellular cycle (Sladek et al., 2007). This opened a new avenue for our interest in characterizing the properties of the colonic clock and mechanisms of its entrainment via hormonal pathways. We found that in rat colon, adrenalectomy decreased and repeated applications of DEX selectively rescued circadian rhythm in the expression of the clock gene *Per1*. Moreover, DEX entrained the colonic clock in explants from mPer2^{Luc} mice *in vitro*. In contrast, melatonin is likely not involved in entrainment of the colonic clock because pinealectomy had no effect, and repeated melatonin injections were not able to rescue the dampened colonic clock in animals maintained in constant light. Additionally, melatonin did not entrain the clock in colonic explants from mPer2^{Luc} mice *in vitro*. The findings provided a novel insight into possible beneficial effects of glucocorticoids in the treatment of digestive tract-related diseases, greatly exceeding their anti-inflammatory action (***Polidarová L., Houdek P., Sládek M., Novosadová Z., Pácha J., Sumová A.: Mechanisms of hormonal regulation of the peripheral circadian clock in the colon. Chronobiol.Int. 34(1):1-16, 2017*** – L.P., P.H., M.S. Z.N. and A.S. *belong to the team, which includes the first and last (corresponding) author. They designed and performed the study and wrote the manuscript. J.P. is from the Laboratory of Epithelial physiology, IPHYS, CAS and he provided us expertise on the colonic physiological functions*)

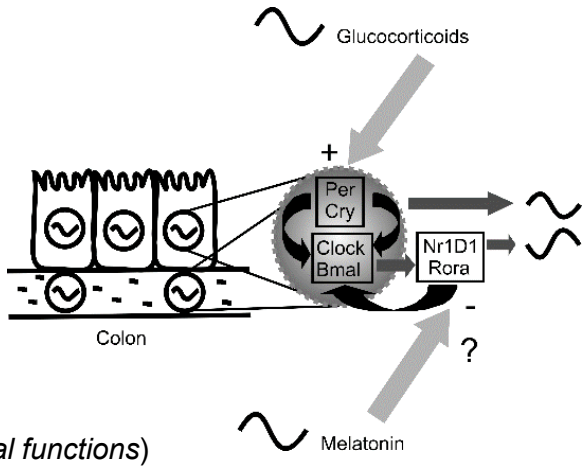


Fig.2. Hormonal control of colonic clock from Polidarová et al., 2017

Related to these results, we participated in a collaborative study, in which we tested whether GC are required to maintain transcriptional oscillations of core clock genes (*Bmal1, Per1-3, Cry1, RevErbalpha, Rora*, clock-output genes (*Dbp, E4bp4*) and a GC- and clock-controlled gene (*Gilz*) in selected peripheral tissues (liver, jejunum, kidney cortex, splenocytes and visceral adipose tissue, VAT). The findings suggested that adrenalectomy alters the expression profiles of core clock genes and clock-output genes in peripheral organs and may cause tissue-specific modulations of the circadian expression profiles, which are reflected in changes in their amplitudes, but not the phases. These results question the potential exclusive role of GC in maintaining synchrony among peripheral clocks under physiological conditions, even if they are able to modulate their expression (***Soták M., Bryndová J., Ergang P., Vagnerová K., Kvapilová P., Vodička M., Pácha J., Sumová A.: Peripheral circadian clocks are diversely affected by adrenalectomy. Chronobiol Int, 33: 520-529, 2016*** *– M.S., J.B., P.E., K.V., P.K., M.V. and J.P. (corresponding author) are from the Laboratory of Epithelial physiology, IPHYS, CAS; only A.S. belongs to the team, she participated in the study design, data interpretation, writing and revision of the manuscript*)

The sensitivity of the colonic clock to GC is tightly related to the sensitivity to immune-stimulation. Therefore, we were interested whether environmental and life-style related factors, which relate to alteration of GC regulation, may per se induce pro-inflammatory state in the colon. Previously, such effect was demonstrated only in conditions of immunostimulant-challenged immune system. We exposed rats to various conditions threatening the behavioral activity/rest cycle regulation, namely aging with or without melatonin, 6-h advance/delay phase shifts in the light/dark cycle repeated with a 2-day frequency and constant light, and tested their effect on expression of immune markers in the rat colonic mucosa (*Tnf*, *Il1a*,*Il17ra*, *Stat3*, and *Rsg16*). Of all tested conditions, the exposure to LL, which perturbed the interval of inactivity (sleep) and led to the complete abolishment of

activity/inactivity cycles, activated robustly proinflammatory state in the colon selectively via *Stat3*-dependent pathway. Our results provided the first evidence that disruptions in the behavioral activity/inactivity cycles may spontaneously (without immuno-stimulant) induce selective proinflammatory responses in the colonic mucosa. Such effects may take part in the mechanisms of modern lifestyle-induced inflammatory diseases of the gut (***Polidarová L., Houdek P., Sumová A.: Chronic disruptions of circadian sleep regulation induce specific proinflammatory responses in the rat colon. Chronobiol. Int., 34: 1273-1287, 2017*** *– all authors belong to the team*).

Recently, we became interested in the role of endocannabinoid (EC) signaling in the circadian clock function and entrainment. Previous reports based on behavioral and electrophysiological data suggested that EC might reduce the ability of the SCN clock to respond to light, however, evidence at the level of clock gene expression was lacking. We first constructed a complete phase response curve for NMDA receptor activation in vitrousing luminescence recording of cultured SCN slices from *mPer2*^{Luc} mice. We found that a stable "singularity" point, in the course of which the clock seemingly stops while the overall phase is caught between delays and advances, can occur in response to NMDA at a narrow interval during the PER2 level decrease. NMDA-induced phase delays were affected neither by the agonist (WIN 55,212-2 mesylate) nor by the antagonist (rimonabant hydrochloride) of EC receptors. However, the agonist significantly reduced the NMDA-induced phase advance of the clock, while the antagonist enhanced the phase advance, causing a shift in the sensitivity window of the SCN to NMDA. The modulation of EC signaling in the SCN had no effect by itself on the phase of the PER2 rhythm. The results provide evidence for a modulatory role of EC in photic entrainment of the circadian clock in the SCN (***Sládek M., and Sumová A.: Modulation of NMDA-mediated clock resetting in the suprachiasmatic nuclei of mPer2^{Luc} mouse by endocannabinoids. Front. Physiol. 2019 Mar 29;10:361*** *– both authors belong to the team*). EC signaling is involved in food intake and energy balance which are under circadian control. However, despite the obvious link between the circadian and EC systems, evidence for their interaction in periphery started to emerge only recently. To ascertain the mechanisms, we used targeted lipidomics to analyze circadian variations in EC tone in rat plasma, liver and adrenal tissue. We found that ECs, monoacylglycerols, N-acylethanolamines and their precursors oscillate with a tissue-specific circadian phase in plasma and liver. We then identified a set of rhythmically expressed genes likely responsible for the variations in EC tissue tone. Interestingly, EC levels did not oscillate in the adrenal glands. To explore the impact of metabolic signals on expression of these genes, we used daytime-restricted feeding schedule. Daytime feeding strongly suppressed liver-expressed fatty acid binding protein 5 (*Fabp5*) and adrenal-expressed non-canonical endocannabinoid receptors *Gpr55* and *Trpv1*, whereas it upregulated liver-expressed *Trpv1* and glycerophosphodiester phosphodiesterase 1 (*Gde1*). Our results reveal tissue-specific mechanisms involved in interaction between endocannabinoid signaling, circadian system and metabolism (***Sládek M., Houdek P., Sumová A.: Circadian profiling reveals distinct regulation ofendocannabinoid system in the rat plasma, liver and adrenal glands by light-dark and feeding cycles. BBA - Molecular and Cell Biology of Lipids, Vol. 1864, Issue 2 158533, 2019*** *– all authors belong to the team*).

Topic 3. Circadian regulation of brain function, learning and memory

In the larger collaborative project, we contributed to characterization of an animal model of the familial form of Alzheimer's disease (AD),

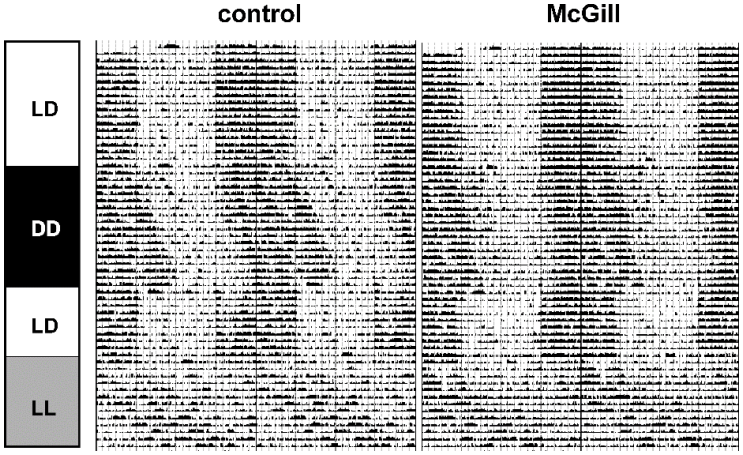


Fig. 3. Representative activity records of rats (from Petrásek et al., 2018)

McGill-R-Thy1-APP transgenic rat, which mirrors many neuropathological hallmarks of the disease and gradual deterioration of cognitive functions. This study characterized the model thoroughly in several domains. In our part, in which we analyzed circadian phenotype of the model, we found that McGill rats exhibit changes in circadian profile, with shorter free – running period and increased total activity during subjective night, without signs of sleep disturbances during the inactive phase. Expression of circadian clock gene *Bmal1* was increased in the parietal cortex and cerebellum, while *Nr1d1* expression was not changed. The clock-controlled gene *Prok2* expression was elevated in the parietal cortex and hippocampus, which might contribute to the observed changes in circadian phenotype.Altogether, the data show that the model parallels the spectrum of symptoms observed in human patients and enables development of new therapeutic approaches targeting not only memory decline but also other symptoms decreasing the quality of life of the patients**(*Petrásek T., Vojtěchová I., Lobellová V., Popelíková A., Janíková M., Brožka H., Houdek P., Sládek M., Sumová A., Krištofiková Z., Valeš K., Stuchlík A.: McGill transgenic rat model of Alzheimer's disease displays cognitive and motor impairment, changes in anxiety and social behavior and altered circadian activity. Front Aging Neurosci, 10:250; 2018*- *our team (P.H., M.S. and A.S.) was fully responsible for circadian phenotyping using monitoring circadian locomotor activity, sleep parameters, detection of clock gene expression levels in various brain areas: Results depicted in Figs. 12, 13, 14, Tables 15, 16, and writing the representative part of the manuscript*).

In collaboration with a team from University of Toronto we also attempted to contribute to understanding the phenomenon of time memory, which means that animals create implicit memories of the time of day that significant events occur and then anticipate the recurrence of those conditions at the same time on subsequent days. We tested the hypothesis that implicit time memory for daily encounters relies on the setting of the canonical circadian clockwork in brain areas involved in the formation or expression of context memories. Of the tested brain regions (SCN, dorsal striatum, nucleus accumbens, cingulate cortex, hippocampal complex, and amygdala), the evidence for conditioned resetting of a molecular circadian oscillation was found only in the dorsal striatum, and specifically only for *Per2* expression. The results are consistent with a model in which the dorsal striatum is selectivelyresponsive to temporal conditioning, and where the conditioned response of *Per2* can occur without resetting the entire circadian clockwork (***Shrestha TC, Šuchmanová K, Houdek P, Sumová A, Ralph MR: Implicit time-place conditioning alters Per2 mRNA expression selectively in dorsal striatum without shifting its circadian clock. Sci. Rep. 2018,8:15547*** *– T.C.S. and M.R.R. from UTo performed the conditioning of animals and collecting the brain samples, our team (K.Š., P.H., A.S.) was responsible for laser dissection of thebrain areas of interest and detection of transcript levels by RT qPCR, A.S. also participatedin interpretation of the data and writing the manuscript*).

<u>Topic 4. Human circadian system</u>

We continued in our projects based on collaboration with clinics in order to examine relationship between neuropsychiatric disorders, namely bipolar disorder (BD) and Alzheimer disease (AD), and functional state of the circadian system in patients.

BD is a common psychiatric disease characterized by disturbances in the sleep/wake cycle. Importantly, in BD patients mood changes with alternating episodes of mania and depression. Although disturbances in circadian system of BD patients have been reported, none of the studies focused on these effects separately in the episode of mania and depression. We found that in patients in mania, the daily profiles of melatonin differed compared with healthy controls, as well as patients in depression, mainly due to elevated melatonin levels during the daytime. The *Per1* and *Nr1d1* profiles in buccal cells were

advanced in patients in mania compared with those in depression and compared withcontrols, a trend toward an advance was apparent in the profiles of patients during anepisode of mania. Intriguingly, in depression no differences were found between melatonin and clock gene expression profiles of controls and patients. Our results revealed for the first time that the functional state of the circadian system can differ in bipolar patients depending on manic and depressive episodes (***Nováková M., Praško J., Látalová K., Sládek M., Sumová A.: The circadian system of patients with bipolar disorder differs in episodes of mania and depression. Bipolar Disorders, 17: 303-3014, 2015*** *– J.P. and K.L. are clinicians who were responsible for care of the patients, their diagnosis, and organization of the human sample collection. Our team (M.N., M.S., and A.S.), which includes the first and last (corresponding) author, designed the study, performed all assays (melatonin and clock gene expression profiles), analyzed and interpreted the data, and wrote the manuscript*).

AD is another neurodegenerative disease often accompanied with disruption of sleep-wake cycle. In this study, we aimed to assess the circadian system in patients with a mild form of AD in their home environment. We selected AD patients who lived together with their healthy spouses who served us as controls exposed to exactly the same environmental conditions. We found that AD patients exhibited significantly longer inactivity interval during the 24 h and significantly higher number of daytime naps than controls. Compared with controls, decline in amplitude of the melatonin rhythm in AD patients was not significant, however, in AD patients more melatonin profiles were dampened or had atypical waveforms. The clock genes *PER1* and *BMAL1* were expressed rhythmically in buccal cells with high amplitudes in both groups and no significant differences in phases between both groups were detected. Our results demonstrate only moderate differences in functional state of the circadian system in patients with mild form of AD compared with healthy controls which are present in conditions of their home dwelling (***Weissová K., Bartoš A., Sládek M., Nováková M, Sumová A.: Moderate Changes in the Circadian System of Alzheimer's Disease Patients Detected in their Home Environment. PLoS One. 2016; 11(1):e0146200*** – A.B. is a clinician who *who was responsible for care of the patients and, their diagnosis. Our team (K.W., M.S., M.N. and A.S), which includes the first and last (corresponding) author, designed the study, organized the human sample collection in home dwelling, performed all assays (actigraphy, sleep parameters, melatonin and clock gene expression profiles), analyzed and interpreted the data, and wrote the manuscript*).

As a by-project, we contributed to a study in which hormonal profiles were analyzed in patients with surgically removed pineal glands due to pinealcystoma. Our group was involved in analyses of melatonin profiles in plasma. The results provided evidence that presence of pineal cyst does not impair production of melatonin and pinealectomy abolishes the melatonin levels to the detection limit of the assay (***Májovský M, Řezáčová L, Sumová A, Pospíšilová L, Netuka D, Bradáč O, Beneš V.: Melatonin and cortisol secretion profile in patients with pineal cyst before and after pineal cyst resection. J Clin Neurosci. 39:155-163, 2017*** *– of the authors, only A.S. belongs to the team – analyses of melatonin levels in plasma samples*).

FIVE SELECTED BREAKTHROUGH FINDINGS ACHIEVED DURING THE EVALUATED PERIOD

- **Although the adult SCN clock is resilient to glucocorticoids, the hormones are able to entrain the fetal and neonatal SCN clock.**
- **Clock located in maternal placenta serves as a glucocorticoid sensitive gate keeper to control and deliver proper amount of glucocorticoids to the fetal SCN.**
- **Proper maternal care may protect offspring from development of pathological symptoms even if they are genetically programmed.**
- **Aging does not impair ability of the circadian clocks in the SCN and pancreas to generate rhythmic signal but impairs their ability to control output rhythms**

- **Functional state of the circadian system in patients with bipolar disorder may vary depending on arousal state as accompanied with the episodes of mania and depression**

RESEARCH FOR PRACTICE

We have an ongoing collaboration with Dr. Jiří Voller, who is affiliated with Palacký University (PI Miroslav Strnad) and with Institute of Experimental Botany (PI Marián Hajdúch) in Olomouc, CZ. The collaboration started in 2014 and is currently funded by **Strategy AV21 QUALITAS - Wellbeing in health and disease (for start-up projects)**. We have discovereda new class of circadian modulators in the form of phytohormones cytokinins. Circadian rhythms (CR) can be modulated not only by light or social cues but also by xenobiotics. Suchcompounds could be used as chronotherapeutics – drugs for clock-related diseases including hereditary aberrations in period length, jet-lag or social jet-lag caused by chronic dissonance between circadian period and sleep-wake schedule (e.g. due to shift-work). The number of known CR modulators remains low (N<50) even after several high-throughput screening campaigns. Moreover, many of them have issues with bioavailability, selectivityand toxicity. Search for compounds with new mechanisms of action therefore continues to bean important research field. However, discovery of new ligand classes for established targetsis also of interest, as they can have better properties than known ligands including distinct target/off-target selectivity profiles. We have shown that cytokinins and their derivatives effectively modulate CR in mammalian cells and tissue explants. We have recently applied jointly with Palacký University for **CZ patent PV2019-757** to cover the discovery of circadian modulator activity in one of the cytokinins and we are planning to apply for an international patent in 2020. We are also seeking to expand the collaboration. We have unsuccessfully applied for additional funding from the Czech Science Agency (Impacts of circadian clock modulation by novel casein kinase 1 inhibitors in spontaneously hypertensive rat, 2014; A proof of concept, molecular mode of action and preclinical of cytokinin modulators of circadian rhythm, 2018) and from Ministry of Education as a joint project with India (THERAPEUTIC EFFECTS OF PHYTOHORMONES IN CIRCADIAN RHYTHM DYSFUNCTION, 2019).

References used in the text can be found in the publication record of the team listed elsewhere.

Research activity and characterisation of the main scientific results

Our research team had four major topics within the evaluated period 2015-2019.

1) Altered central regulation of BP and sympathetic tone in hypertension

Augmented sympathetic vasoconstriction plays a major role in the development and maintenance of various forms of experimental hypertension. This is true for genetic hypertension of SHR (Zicha et al. Physiol Res 63: 13, 2014; Behuliak et al. Hypertension 72: 676, 2018; Vavřínová et al. Hypertens Res 42: 949, 2019), angiotensin II-dependent hypertension of TGR (Vaněčková et al. J Hypertens 33: 161, 2015; Řezáčová et al. Biomed Pharmacother 116: 108996, 2019) as well as for salt hypertension of Dahl rats (Zicha et al. Acta Physiol 202: 29, 2011; Zicha et al. Acta Physiol 205: 124, 2012, Zicha et al. Physiol Res68: 873, 2019). Using these three models we have obtained several important results.

First of all, we compared the influence of chronic systemic or intracerebroventricular administration of losartan (angiotensin II type 1 receptor antagonist) on blood pressure and its sympathetic component in adult Ren-2 TGR. Řezáčová et al. (Biomed Pharmacother 116: 108996, 2019) demonstrated that both systemic and central losartan treatment lowered blood pressure through a reduction of sympathetic vasoconstriction. Similar results were also obtained in TGR subjected to chronic central or systemic administration of direct renin inhibitor aliskiren. These data indicate the major importance of central angiotensin II in the control of sympathetic tone in TGR with angiotensin II-dependent hypertension. Surprisingly, there is no enhancement of sympathetic tone in another strain - Cyp1a1-Ren-2 transgenic rats in which malignant angiotensin II-dependent hypertension is induced by xenobiotic indole-3-carbinol administration in adulthood (Zicha et al. Physiol Res 68: 329, 2019).

Second, there is a long-term discussion on the role of brain and kidney in the pathogenesis of hypertension. Several years ago, Fujita et al. (Nat Med 17: 573, 2011; J Am Soc Nephrol 25: 1148, 2014) suggested that enhanced beta-adrenergic stimulation can augment the activity of thiazide-sensitive sodium-chloride cotransporter (NCC) in renal proximal tubules and thus cause sodium retention leading to salt hypertension development. Therefore, Zicha et al. (Physiol Res 68: 873, 2019) tested whether chronic beta-adrenergic blockade with propranolol would attenuate salt hypertension development in Dahl rats through the prevention of NCC-mediated sodium retention. In our experiments chronic beta- adrenergic blockade did not influence salt hypertension development, although heart rate was lowered in propranolol-treated rats. In addition, the natriuresis induced by the acute injection of hydrochlorothiazide was neither augmented in salt hypertensive Dahl rats nor attenuated in propranolol-treated Dahl salt-sensitive rats. Thus, our data indicate that sympathetic nervous system participates in the pathogenesis of salt hypertension through its alpha-adrenergic vasoconstrictor effects rather than via beta-adrenergic stimulation of renal sodium retention.

Finally, as far as the role of sympathetic nervous system (SNS) in genetic hypertension is concerned, we have paid our attention to SNS changes in prehypertensive SHR (Vavřínová et al. Hypertens Res 42: 949, 2019), to the sympathoadrenal mechanisms compensating blood pressure reduction in sympathectomized SHR (Vavřínová et al.

Hypertens Res 42: 1872, 2019) and to the role of different types of voltage-dependent calcium channels (VDCC) in blood pressure maintenance of SHR (Behuliak et al. Hypertension 72: 676, 2018).

The alterations of sympathoadrenal and sympathoneural systems participate in the pathogenesis of hypertension in SHR. The evaluation of ontogenetic changes indicated increased adrenal catecholamine content in prehypertensive SHR, which was reduced in SHR with established hypertension. The downregulation of catecholamine biosynthetic enzymes, which was observed in both the adrenal medulla and sympathetic ganglia of prehypertensive and hypertensive SHR, might be a compensatory mechanism that counteracts vascular sympathetic hyperinnervation and/or

enhanced sympathetic outflow seen in SHR at both ages (Vavřínová et al. Hypertens Res 42: 949, 2019).

Furthermore, Vavřínová et al. (Hypertens Res 42: 1872, 2019) studied the effects of guanethidine-induced sympathectomy on cardiovascular parameters in adult SHR with established hypertension. Sympathectomy induced only a transient reduction of blood pressure but a permanent lowering of heart rate as well as the improvement of baroreflex sensitivity. Nevertheless, the sympathectomy attenuated blood pressure and heart rate responses to restraint stress which are mediated by the activation of sympathetic nervous system. The effect of sympathectomy on basal blood pressure was counteracted by the increased vascular sensitivity to catecholamines and/or by enhanced secretion of adrenal hormones.

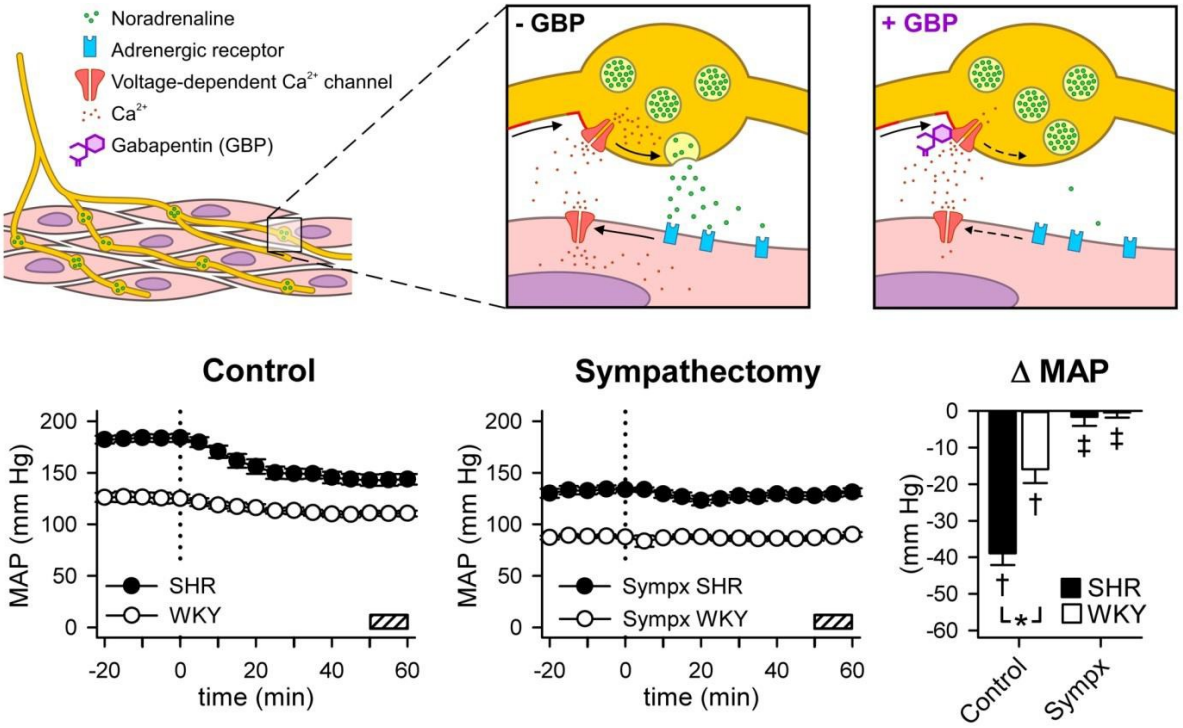


Fig 1 Behuliak et al. Hypertension 72: 676, 2018

Behuliak et al. (Hypertension 72: 676, 2018) **(Fig 1)** demonstrated that the acute administration of gabapentin (ligand to alpha2delta subunit of VDCC) lowered blood pressureof SHR through its action on N-type VDCC which was associated with the changes of sympathetic nerve transmission. This mechanism of blood pressure control through N-type VDCC located on peripheral sympathetic nervous system seems to be complementary to the well-known role of L-type VDCC in the control of vascular smooth muscle tone (Fig 1).

3) Abnormal vascular smooth muscle contraction in hypertension

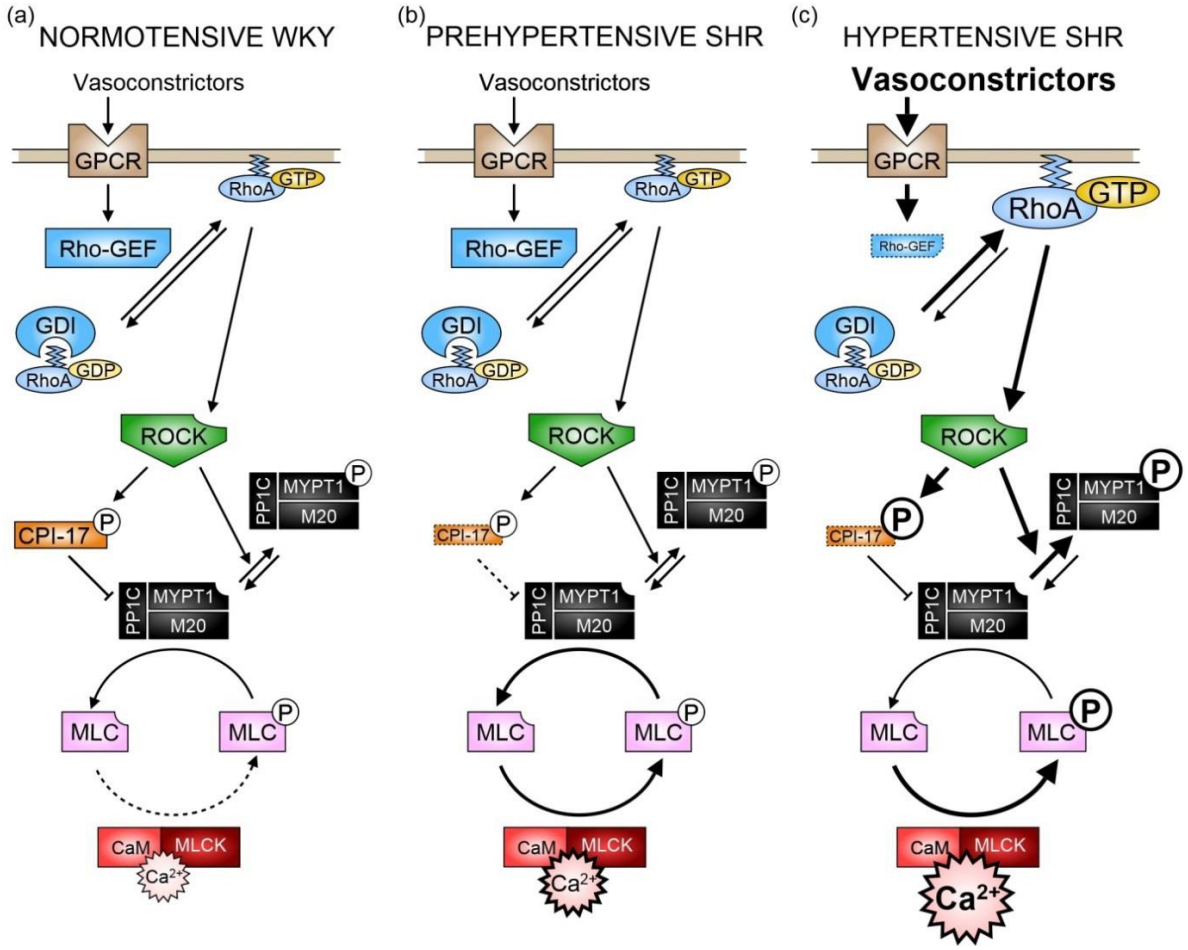


Fig 2 Behuliak et al, J Hypertens 33: 2443, 2015

Although the role of enhanced sympathetic outflow is dominant in most forms of experimental hypertension (SHR, TGR, Dahl rats), the altered vascular smooth muscle reactivity in hypertensive animals (Bencze et al. Life Sci 166: 46, 2016) to various constrictor stimuli is also dependent on the changes in intracellular calcium concentration and/or sensitivity of actomyosin complex to cytosolic calcium. We have demonstrated that in both normotension and hypertension there is an important interaction between the participation of calcium influx (through L type voltage-dependent calcium channels, L-VDCC) and calcium sensitization (mediated by RhoA/Rho kinase pathway) in blood pressure maintenance (Zicha et al. Physiol Res 63 (S1): S19, 2014). Moreover, Behuliak et al. (Biomed Res Int 2017: 8029728, 2017) found that the contribution of calcium influx and calcium sensitization might be different in various types of experimental hypertension. Enhanced calcium influx and decreased calcium sensitization are the characteristic features in spontaneous hypertension (SHR) and in angiotensin II-dependent hypertension (TGR), whereas increased calcium sensitization is typical for salt-sensitive Dahl rats (Behuliak et al. Biomed Res Int 2017: 8029728, 2017) and NO-deficient hypertension (Brunová et al. Physiol Res 64: 447, 2015). The above mentioned changes originally demonstrated in adult SHR with established hypertension (Behuliak et al. J Hypertens 31: 2025, 2013) are also present in young prehypertensive animals (Behuliak et al. J Hypertens 33: 2443, 2015) **(Fig 2).** The same was true also for TGR (Vaněčková et al. Hypertens Res 42: 145, 2019). The reduced calcium sensitization in genetic hypertension might be a compensation of enhanced calcium influx through L-VDCC on vascular smooth muscle (Behuliak et al. J Hypertens 33: 2443, 2015).

We also paid a considerable attention to the acute and chronic impact of main vasoactive systems (renin-angiotensin system, sympathetic nervous system, nitric oxide) on calcium sensitization. Brunová et al. (Physiol Res 64: 447, 2015) demonstrated that calcium sensitization is largely attenuated by nitric oxide (both under the acute and chronic conditions). On the other hand, there is only a minimal influence of both the above mentionedpressor systems on calcium sensitization. Our recent study (Vaněčková et al. Hypertens Res 42: 145, 2019) disclosed why the acute reduction of calcium sensitization (blockade of Rho kinase by fasudil) elicited a greater blood pressure reduction in hypertensive TGR, although their calcium sensitization was attenuated compared to the normotensive Hannover Sprague-Dawley controls. This rather surprising blood pressure response to fasudil can be explained by the impaired baroreflex efficiency in hypertensive rats because fasudil-induced BP reduction causes smaller compensatory activation of sympathetic nervous system as compared to the normotensive controls. Similar role of impaired baroreflex efficiency was also demonstrated in salt hypertensive Dahl rats (Zicha et al., to be published).

4) Contribution of renin-angiotensin and endothelin systems to BP regulation andrenal damage

Angiotensin II (the key element of the renin-angiotensin system) and endothelin-1 (the key element of the endothelin system) are two most potent vasoconstrictors known to date. They are involved in blood pressure regulation and also in organ damage. The inhibitors of RAS are used in human medicine for more than 40 years. By contrast, the antihypertensive and nephroprotective effects of endothelin receptor blockers, especially of the ET_A receptor blockers, were demonstrated 20 years ago. There are debates on the preferential non-selective ET_A/ET_B versus selective ET_A receptor blockers associated with the antiproliferative and anti-inflammatory effects mediated by ET_A receptors. Our experimental results support the use of selective ET_A receptor blockers in chronic kidney disease (Vaněčková et al, 35:382, 2012; Čertíková Chábová et al, Life Sci, 118:297, 2014; Vaněčková et al, Front Physiol, 10:1145, 2019). There are many other diseases, such as sickle cell disease, renal artery stenosis, or ischemic reperfusion injury in which ET_A receptor blockers are now experimentally tested (for review see Vaněčková et al, Physiol Res, 67 (Suppl 1):S55, 2018). Based on our previous studies evaluating the contribution of major vasoactive systems (RAS, SNS and NO) to blood pressure regulation in various hypertensive modelsand using different antihypertensives, we were interested in the effects of ET_A receptorblockade on these systems in Ren-2 transgenic rats (Vaněčková et al, J Hypertens, 33:161,2015). Our study demonstrated that the reduced calcium influx through the voltage-dependent calcium channels (L-VDCC) due to the missing ET_A receptor-dependentvasoconstriction and attenuated Ang II-dependent vasoconstriction is responsible for the BP-lowering effect of atrasentan, selective ET_A blocker.

We also examined the effects of combination therapy using atrasentan with different RAS blockers (angiotensin converting enzyme inhibitor, angiotensin receptor blocker and direct renin inhibitor). We found normalization of BP with all three types of RAS blockers with no additional BP effect of atrasentan. The BP-lowering effect of different RAS blockers was always mediated by substantially attenuated RAS-dependent vasoconstriction and moderately reduced sympathetic vasoconstriction. Interestingly, in atrasentan-treated groups we observed not only significantly reduced calcium influx through L-VDCC but also substantially reduced NO-dependent vasodilation. Thus, although most of the BP-lowering effect is mediated by RAS blockade, there are other important effects on major vasoactive systems attributed to ET_A blockade (Vaněčková et al, Life Sci, 159:127, 2016). Similar findings (reduced calcium influx through L-VDCC and attenuated NO-dependent vasodilation) were obtained with a non-selective ET_A /ET_B receptor blocker bosentan (Vaněčková et al, Physiol Res, 68:717, 2019) suggesting that these effects might be the common property of ET receptor blockers.

Within our focus on the translational medical research we continued our research on therapeutic potential of ET_A blockade in chronic kidney disease induced by 5/6 nephrectomy in hypertensive Ren-2 transgenic rats. We have analyzed the effects of the diuretic added to

the combined RAS+ ET_A blockade since ET_A blockers are known to cause edema andseveral big clinical trials were terminated due to this reason. We demonstrated that diuretic could not only prevent this adverse effect but it can be even advantageous if administered fora very long period. Indeed, diuretic treatment improved renal function (lowered proteinuria and increased creatinine clearance), renal morphology (decreased kidney mass and glomerular area) and histological markers of renal injury (reduction of glomerulosclerosis index and tubulointerstitial injury) compared to all other groups (Vaněčková et al, Front Physiol, 10, 1145, 2019) (**Fig 3).**

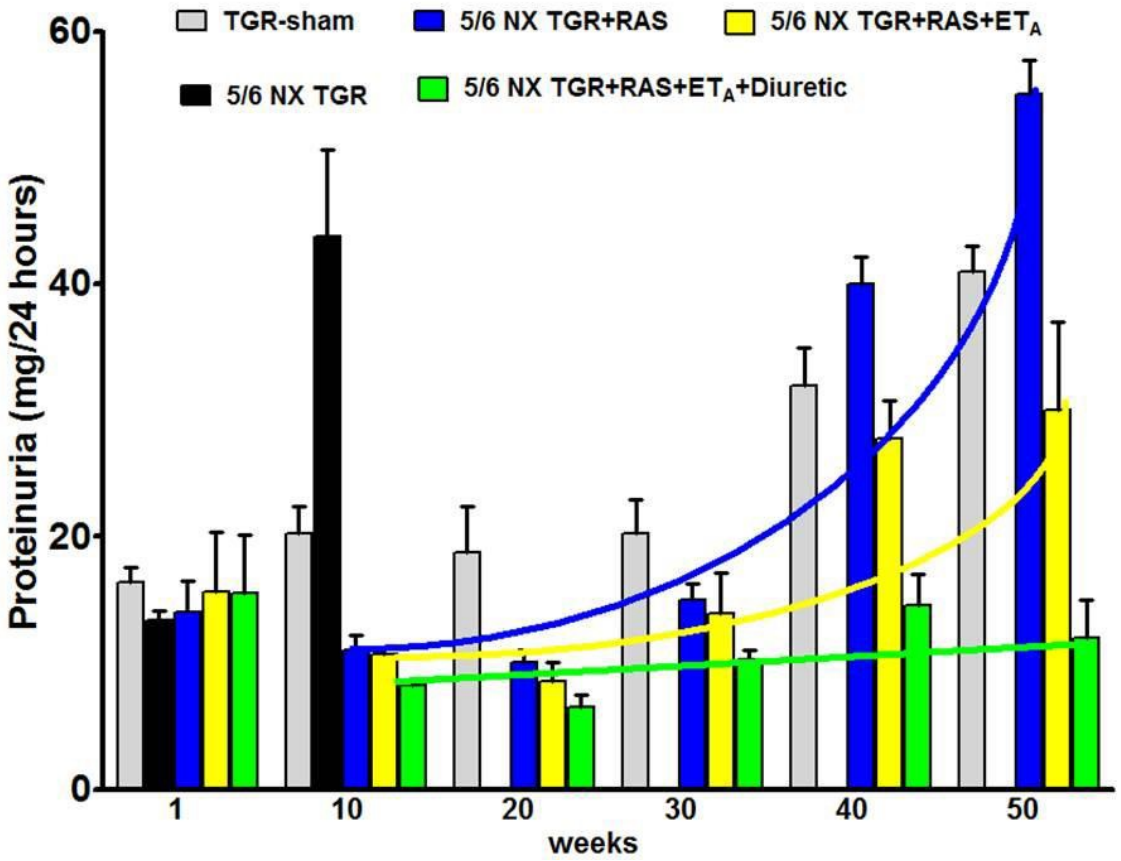


Fig 3 Vaněčková et al, Front Physiol, 10, 1145, 2019

Our experiments performed in cooperation with the researchers from the Institute for Clinical and Experimental Medicine in the animals with established chronic kidney disease (CKD) demonstrated that the results from the experimental studies must always be translated into the clinical practice with caution. These experiments were done in animals in which the treatment was postponed to the phase of the established CKD, i.e. 6 weeks after 5/6 nephrectomy. We reported that atrasentan added to RAS blockade brought no further improvement of the survival rate and unexpectedly even worsened albuminuria at the end of the study. Moreover, it did not reduce the markers of renal injury and did not prevent the decrease in renal function (evaluated as creatinine clearance) (Sedláková et al, Clin Exp Hypertens, 39:183, 2017). Another our study using a triple therapy consisting of RAS+ ET_A blockade+blockade of soluble epoxide hydrolase (sEH - the enzyme responsible for the degradation of beneficial epoxyeicosatrienoic acids into the inactive hydroxytetraenoic acids) showed that sEH inhibition added to RAS blockade improved survival rate, reduced albuminuria, decreased renal damage and attenuated the decline in renal function suggesting that sEH inhibition might be a novel tool to alleviate the progression of CKD. By contrast, ET_A blockade spoiled the beneficial effects of sEH inhibition and, unexpectedly, alsoabolished the

beneficial effects of adding sEH inhibitor to the RAS blockade (Čertíková Chábová et al, Kidney Blood Press Res, 44:1493, 2019).

5) Cardiovascular effects of neuropeptides controlling food intake

Obesity and type 2 diabetes mellitus, as important symptoms of metabolic syndrome, are frequent metabolic disorders but an effective therapy is still missing. Anorexigenic neuropeptides, such as prolactin-releasing peptide (PrRP), produced and acting in the brain, have the potential to decrease food intake and ameliorate obesity but are ineffective after peripheral application. Our collaborators at the Institute for Organic Chemistry and Biochemistry have designed lipidized analogs of PrRP, which have a high affinity and ability to activate the PrRP receptor GPR10 and they are involved in energy balance regulation. These lipidized analogs showed binding affinity and signaling in PrRP receptor-expressing cells similar to the natural PrRP. Moreover, these analogs showed high binding affinity alsoto anorexigenic neuropeptide FF-2 receptor. Peripheral administration of myristoylated and palmitoylated PrRP analogs to fasted mice induced strong and long-lasting anorexigenic effects and neuronal activation in the brain areas involved in food intake regulation. Moreover, two-week subcutaneous administration of palmitoylated PrRP31 and myristoylated PrRP20 lowered food intake, body weight and improved metabolic parameters, and attenuated lipogenesis in mice with diet-induced obesity (Maletínská et al, Int J Obes, 39: 986, 2015) **(Fig 4).**

In order to show the best application routes for the effect of lipidized PrRP, a palmitoylated analog of human PrRP (palm-PrRP31) was injected in free-fed Wistar rats by three routes: subcutaneous (s.c.), intraperitoneal (i.p) (both 5 mg/kg) and intravenous (i.v.) (from 0.01 to 0.5 mg/kg). We found a circulating compound in the blood after all three applications with the highest concentration after i.v. administration. This corresponds to the effect on food intake, which was also strongest after i.v. injection. Moreover, this is in agreement with the fact that the expression of c-Fos in specific brain regions involved in food intake regulation was also highest after intravenous application. Human palm-PrRP31 analog showed a strong tendency to micellize, and formation of aggregates suggested lower availability after i.p. or s.c. application. We have demonstrated that palm-PrRP influenced food intake even in free-fed rats. Not surprisingly, the maximal effect was achieved after the intravenous application even though two orders of magnitude lower dose was used as compared to both other applications (Mikulášková et al, Peptides, 75: 109, 2016).

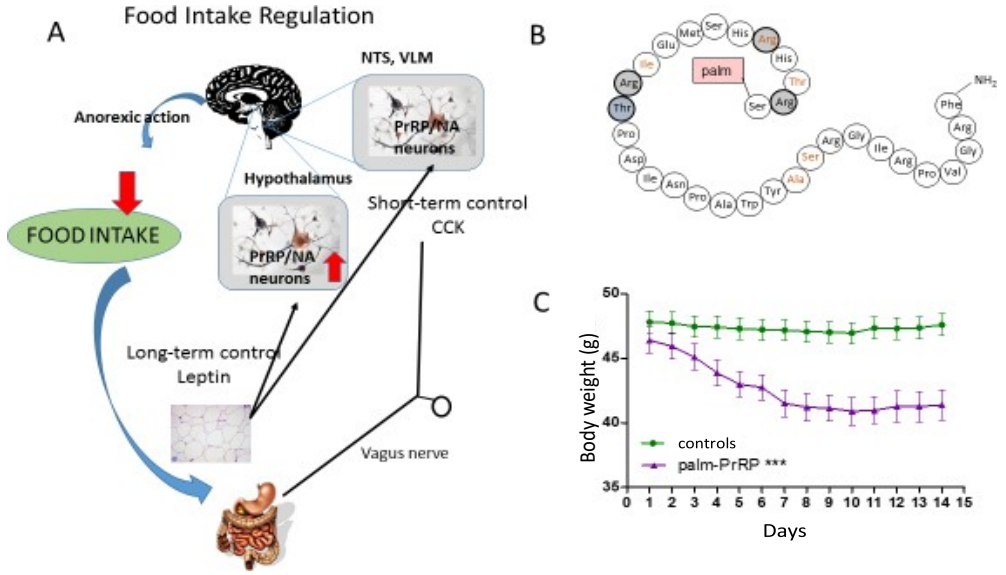


Fig 4 Maletínská, L. et al. Int. J. Obesity. 39: 986, 2015

We have also studied if the subchronic administration of another potent palmitoylated PrRP analog (palm11-PrRP31) to Koletsky spontaneously hypertensive obese rats (SHROB) could lower body weight and glucose intolerance as well as other metabolic parameters. Palm11-PrRP31 was administered intraperitoneally to spontaneously hypertensive rats (SHR) and SHROB at a dose of 5 mg/kg once-daily for 3 weeks. At the end of the experiment, vehicle-treated SHROB rats showed typical metabolic syndrome parameters, including obesity, glucose intolerance, dyslipidemia, and hypertension. Peripheral treatment with palm11-PrRP31 progressively decreased the body weight in SHR rats but not in SHROBrats, though glucose tolerance was markedly improved in both strains. Moreover, in SHROB palm11-PrRP31 ameliorated the HOMA index, insulin/glucagon ratio, and increased insulin receptor substrate 1 and 2 expression in fat as well as insulin signaling in the hypothalamus, while it had no effect on blood pressure (Mikulášková et al, Nutr Diabetes, 8:5, 2018).

The only drug which is recently used for type 2 diabetes treatment is liraglutide (glucagon-like peptide-1 receptor agonist). Recently, liraglutide has been demonstrated to decrease cardiovascular morbidity and mortality in patients with type 2 diabetes and high cardiovascular risk. However, its detailed action at the metabolic level has not been studied. We explored the effect of 2-week liraglutide treatment in C57BL/6 male mice with obesity and diabetes induced by 13 weeks of high-fat diet using NMR spectroscopy to capture the changes in urine metabolic profile induced by the therapy. Liraglutide treatment decreased body weight and fat pads weight along with blood glucose and triglyceride levels. NMR spectroscopy identified 11 metabolites significantly affected by liraglutide treatment as compared to the control group fed a high-fat diet. These metabolites included those involved in nicotinamide adenine dinucleotide metabolism, β-oxidation of fatty acids and microbiome changes. Moreover, taurine and trigonelline were also specific for liraglutide administration. The significance of these changes and its possible use in the personalization of antidiabetic therapy in humans require further research (Bugáňová et al., J Endocrinol, 233:93, 2017).

Research activity and characterisation of the main scientific results

(i) Ion channels and electrical excitability of pituitary cells

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 focusedon characterization of ligand-gated receptor channels, including the GABA-A, nicotinic, and ATP-gated P2X receptor channels, in several subpopulations of pituitary cells (we *reviewed in*: Stojilkovic et al., *Frontiers in Endocrinology* **8**:126, 2016; Zemkova et al., *Mol Cell Endocrinol* **463**:49-64, 2017). More recently, in an international collaboration with the National Institute of Child Health and Human Development, and the National Institute of Diabetes and Digestive and Kidney Diseases, NIH (USA), we have characterized ion channels involved in spontaneous and hypothalamic neuropeptide-stimulated electrical activity of corticotrophs, and performed mathematical modeling of spontaneous as well as neuropeptide-evoked activity patterns. The results on ion channels in corticotrophs are described in detail bellow.

<u>Ion channels involved in electrical activity and calcium signaling in pituitary corticotrophs</u>
Pituitary corticotrophs fire action potentials spontaneously and in response to stimulation with corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), and such electrical activity is critical for calcium signaling and calcium-dependent adrenocorticotropic hormone secretion. The ionic mechanisms responsible for CRH-triggered electrical activity in corticotrophs are poorly characterized because these cells are very small and represent only 2-5% subpopulation of anterior pituitary cells. We used transgenic mice expressing the tdimer2(12) form of *Discosoma* red fluorescent protein under control of theproopiomelanocortin gene's regulatory elements to investigate the ion channels, calcium signaling and mechanisms controlling excitability in identified corticotorphs (Zemková et al., *Endocrinology* **157**: 1576-1589. 2016). We showed that corticotrophs are either quiescent or electrically active, with a 22-mV difference in the resting membrane potential (RMP) between the 2 groups. In quiescent cells, CRH depolarized the membrane, leading to initial single spiking and sustained bursting; in active cells, CRH further facilitated or inhibited electrical activity and calcium spiking, depending on the initial activity pattern and CRH concentration. The stimulatory but not inhibitory action of CRH on electrical activity was mimicked by cAMP.Removal of bath sodium silenced spiking and hyperpolarized the majority of cells; in contrast,the removal of bath calcium did not affect RMP but reduced CRH-induced depolarization. Corticotrophs with inhibited voltage-gated sodium channels fired calcium-dependent action potentials, whereas cells with inhibited L-type calcium channels fired sodium-dependent spikes; blockade of both channels abolished spiking without affecting the RMP. These results indicate that the background voltage-insensitive sodium conductance influences RMP, the CRH-induced depolarization current is driven by a cationic conductance, and the interplay between voltage-gated sodium and calcium channels plays a critical role in determining the status and pattern of electrical activity and calcium signaling in corticotrophs.

This research was supported by the Grant Agency of the Czech Republic (grant # P304/12/G069 to Hana Zemkova), the Ministry of Education, Youth and Sports of the Czech Republic, within the LQ1604 National Sustainability Program II (Project BIOCEV-FAR) and by the project “BIOCEV” (CZ.1.05/1.1.00/02.0109) with 33% involvement of team member, the first author is also a corresponding author and team member. This work was done in collaboration with the Section on Cellular Signalling of the National Institute of Child Health and Human Development, NIH) (USA). Collaborative laboratory provided transgenic mice,

performed calcium imaging experiments and contributed to writing of the manuscript.

Modeling the diversity of spontaneous and agonist-induced electrical activity in corticotrophs

There is a variability in reports of the fraction of corticotrophs that are electrically active, as well as their patterns of activity, and the sources of this variation are not well understood. Various modes of bursting activity in CRH- and AVP-stimulated corticotrophs are also poorly characterized. We used electrophysiology and mathematical modeling to investigate possible sources of variability in patterns of spontaneous and agonist-induced corticotroph electrical activity (Fletcher et al., *J Neurophysiol* **117**:2298-2311, 2017). We generated a mathematical model, where variation in as few as two parameters can give rise to many of the types of patterns observed in electrophysiological recordings of corticotrophs. We compared theknown mechanisms for CRH, and AVP action, and found that different ionic mechanisms can contribute in different but complementary ways to generate the complex time courses of CRH and AVP responses. The results of our modeling suggest that corticotrophs have several mechanisms at their disposal to achieve their primary function of pacemaking depolarization and increased electrical activity in response to CRH and AVP.

This study was supported by the Grant Agency of the Czech Republic (grant # 16-12695S PI: Hana Zemkova) with 25% involvement of team member, Hana Zemkova performed electrophysiological experiments and contributed to writing of this manuscript.This work was done in collaboration with the Section on Cellular Signalling of the National Institute of Child Health and Human Development, and Laboratory of Biological Modeling, National Institute of Diabetes and Digestive and Kidney Diseases, NIH (USA). Collaborative laboratories provided transgenic mice, performed mathematical modeling, and wrote the manuscript.

(ii) Purinergic signaling in hypotalamic suprachiasmatic and supraoptic nuclei

Background – Extracellular ATP acts on its plasma membrane receptors termed purinergic P2 receptors, composed of two families: the ion-conducting P2X receptor channels (P2XRs) and G protein-coupled P2Y receptors (P2YRs). Seven distinct genes encode family of P2XRs, labeled P2X1-7, which are permeable to calcium, in addition to monovalent cations. ATP acts as autocrine and/or paracrine extracellular messenger that controls the accumulation of calcium in the cytoplasm, and modulates secretion of numerousneurotransmitters, hormones and other signaling molecules, including ATP itself, both in neuronal and non-neuronal cells. Earlier, we have showed that extracellular ATP produces depolarization and facilitates glutamate and GABA release from nerve terminals in hypothalamic supraoptic (SON) and suprachiasmatic (SCN) nuclei, and that P2X2Rs and P2X4Rs are involved in both presynaptic and somatic action of ATP in these neurons. Our recent work was focused on endogenous sources of ATP, functional expression of other P2 receptor subtypes, and physiological role of P2XRs in hypothalamic SCN and SON neurons and glia cells. The results on circadian release of ATP, role of P2X7R in the SCN, and changes in P2X2R and P2Y1R expression in SON neurons stimulated *in vivo* by refeeding after fasting are described in detail bellow.

Role of P2X7R a P2YRs in circadian accumulation of extracellular ATP in the SCN

Using 7-day old organotypic cultures of rat brain slices containing the SCN, we investigated the mechanism by which SCN astrocytes rhythmically release ATP under physiological conditions (Svobodová et al., *Frontiers in Pharmacology* **9**:192, 2018).The circadian rhythms in many physiological and behavioral functions are driven by a pacemaker located in the SCN neurons. There is also a rhythm in extracellular accumulation of astrocytic ATP in the SCN, which continues in constant darkness and depends on cell-cell communication between neurons and glia, but molecular mechanisms of ATP release are poorly understood.We tested the hypothesis that ATP is released via the plasma membrane P2X7Rs and release is controlled by P2YRs which have been previously shown to be expressed in the SCN at transcriptional level. We have investigated this hypothesis using ATP bioluminescent assays, immunohistochemistry, patch-clamping, and calcium imaging. We found that extracellular ATP accumulation in medium with organotypic cultures follows a circadianrhythm, with a peak between 24:00 and 04:00 h, and the trough at ~12:00 h. ATP rhythm was inhibited by application of AZ10606120, A438079, and BBG, specific blockers of P2X7R,and potentiated

by GW791343, a positive allosteric modulator of this receptor. Double- immunohistochemical staining revealed high expression of the P2X7R protein in SCN astrocytes. PPADS, a non-specific P2 antagonist, MRS2179, specific P2Y1R antagonist, andthe pannexin-1 hemichannel blocker carbenoxolone also abolished ATP rhythm. The P2Y1R agonist MRS2365, and the P2Y2R agonist MRS2768 potentiated ATP release in organotypic cultures and increase intracellular calcium level in cultured astrocytes. Together, these results provided evidence that SCN astrocytes utilize P2X7R and multiple P2YRs, in addition to pannexin-1 hemichannels, to release ATP.

This research was supported by the Grant Agency of the Czech Republic (grants #16- 12695S PI: Hana Zemkova, and 304/12/G069, Project excellence, PI: Ladislav Vyklicky), the Ministry of Education, Youth and Sports of the Czech Republic within the LQ1604 National Sustainability Program II (Project BIOCEVFAR), and by the project BIOCEV (CZ.1.05/1.1.00/02.0109) with 80% involvement of team members; the first and last (corresponding) author are members of the team. This work was done in collaboration with Faculty of Science, Charles University, Prague, that contributed with immunohistochemical studies.

Increased expression and function of P2X2Rs in SON stimulated by refeeding after fasting

We used a rat model of refeeding after fasting to examine the effect of physiological stimulation of SON neurons on P2XRs mRNA expression and ATP-induced electrophysiological responses (Ivetic et al., *Frontiers in Cell Neurosci* **13**:284, 2019). Magnocellular neurons in the SON, which synthesize and release arginine vasopressin(AVP) and oxytocin (OT), express several subtypes of ATP-stimulated purinergic P2XRs that modulate neuronal activity as well as neurotransmitter and hormone release. However, the physiological impact of purinergic modulation is not well understood. We tested a hypothesis that P2XRs play a role in the increased release of hormones from SON neurons stimulated through fasting/refeeding. We studied the effect of 2 h of refeeding after 48 h of fasting on several P2XR and P2YR mRNA expression and ATP-induced presynaptic and postsynaptic responses in SON of acutely isolated brain slices from 30-day-old rats. Quantitative real-time PCR revealed that the expression of P2X2R and AVP mRNA was upregulated, whereas P2X4R, P2X7R, P2Y2R, and OT mRNA levels were not significantly changed and P2Y1R mRNA expression was decreased. Whole-cell patch clamp recordings showed that the amplitude of the ATP-stimulated somatic current and the ATP-induced increases in the frequency of spontaneous GABAergic inhibitory postsynaptic currents were significantly higher in SON neurons from fasted/refed rats than in SON neurons from normally fed rats. No evidence was found for changes in the presynaptic effect of ATP in SON neurons not expressing somatic P2XRs, most probably OT neurons. These results provided evidence that the expression of P2X2Rs increases proportionally to the AVP hormone synthesis and secretion in the SON neurons stimulated *in vivo* by refeeding after fasting.

This research was supported by the Grant Agency of the Czech Republic (grants #16- 12695S and #18-054138; PI: Hana Zemkova) and the Ministry of Education, Youth and Sports of the Czech Republic within the LQ1604National Sustainability Program II (Project BIOCEVFAR) and by the project BIOCEV (CZ.1.05/1.1.00/02.0109). This work was done with 100% involvement of team members.

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

Background – Seven distinct genes encode family of P2XRs, labeled P2X1-7. Each P2XR subunit has two transmembrane (TM) domains connected by a large extracellular loop with ATP binding site, and 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. Despite this fact, the P2XR subtypes differ with respect to their ligand-selectivity profiles, antagonist sensitivity, and cation selectivity. The functional channels are composed of three subunits, which form ion permeable pore through homo- and heteropolymerization. Earlier, our work contributed to identification of ectodomain residues involved in ATP binding and transmembrane residues involved in P2X4R gating or interaction with receptor-specific

allosteric modulator ivermectin (IVM). More recently, in an international collaboration with the National Institute of Child Health and Human Development and the National Institute of Diabetes and Digestive and Kidney Diseases, NIH (USA), and the McGill University, Montreal, Quebec (Canada), we have investigated molecular mechanisms of the P2X4R and P2X7R large pore formation and generated 2 kinetic models of IVM interaction with P2X4R. Our ongoing work is also focused on identification of new molecule that are able to interact with allosteric binding sites at P2XRs. These molecules are synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, and characterized at Faculty of Sciences and Faculty of Mathematics and Physics, CharlesUniversity, Prague. The results on structure-function relationship of P2XRs obtained with major contribution of our laboratory are described in detail bellow.

Ivermectin-induced regulation of the P2X4R channel pore formation and its modeling

Allosteric modulators of ligand-gated receptor channels induce conformational changes of the entire protein that alter potencies and efficacies for orthosteric ligands, expressed as the half maximal effective concentration (EC50) and maximum current amplitude, respectively. We studied the influence of allostery on P2X4R channel pore formation, an issue not previously addressed, using electrophysiology and mathematical modeling. Experiments were done using the rat P2X4Rr expressed in HEK293T cells and gated by ATP in the presence and absence of IVM, an established specific allosteric regulator of this channel. In the absence of IVM, this channel activates and deactivates rapidly, does not show transition from open to dilated states, desensitizes completely with a moderate rate, and recovers only fractionally during washout. IVM treatment increases the efficacy of ATP to activate the channel and slows receptor desensitization during sustained ATP application and receptor deactivation after ATP washout. The rescue of the receptor from desensitization temporally coincides with pore formation, and the channel can be reactivated after washout of ATP. Our experiments with vestibular and transmembrane domain mutants established that IVM 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 EC50 and kinetics of receptor deactivation. We developed kinetic (Markov state) model showing that the IVM- 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 P2X4R thus provides sustained signaling during prolonged and repetitive ATP stimulation (Zemkova et al., *Pflugers Arch* **467**:713-726, 2015). Next study (Mackay et al.,*PloS Computational Biol* **13**: , e1005643, 2017) was focused on development of a substantially larger kinetic model of IVM action, that not only fitted the data more closely but offered better insights into how the kinetics of ATP and IVM sequential binding to P2X4R affect receptor activation, desensitization, and deactivation. The novelty of this model is prediction of the existence of receptor states that are not directly observable in the experimental current recordings.

This research was supported by Grant Agency of the Czech Republic (grant # 16-12695S; PI: Hana Zemkova) and with 20-50 % involvement of team members who performed electrophysiological experiments and contributed to the writing of manuscripts. This work was done in collaboration with the McGill University, Montreal, Quebec (Canada), Laboratoryof Biological Modeling, National Institute of Diabetes and Digestive and Kidney Diseases, and Section on Cellular Signalling, the National Institute of Child Health and Human Development, NIH (USA) that performed mathematical modelling and wrote of the manuscript.

Identification of transmembrane residues that regulate P2X7R conductivity and sensitivity

We investigated molecular mechanism of large pore formation of the rat P2X7R expressed in HEK293T cells using mutagenesis (Jindrichova et al., *J Neurochem*, **133**:815-827, 2015). In the sustained presence of agonist, the opening of P2X7R channel is followed by pore formation which is accompanied by receptor sensitization. To explore the molecular mechanisms by which the conductivity and sensitivity are increased, we analyzed the electrophysiological properties of selected alanine mutants in the first and second transmembrane domains (TM1 and TM2) of the rat P2X7R, measured permeability to larger organic cations of wild type and

mutated receptors, and dye (YO-PRO-1) uptake by HEK293cells expressing these receptors. The results revealed that alanine substitution of residues Y40, F43 and G338 reduced membrane trafficking, and the D352A mutant was practically non-functional. The Y40A and F43A mutants, that were expressed in the membrane, lacked pore formation ability. Moreover, the Y40A and Y40F currents displayed desensitization, which is absent in wild type P2X7R, whereas the Y40W partially recovered receptor function. The G338A/S mutations favored the open state of the channel and displayed instantaneous permeability to larger organic cations, substitution with proline did not rescue receptor function, indicating that glycine 338 might be a hinge. The L341A and G345A displayed normal trafficking, current amplitude, and sensitization, but both mutations resulted in a decreased pore formation and dye uptake. These results showed that the increase in P2X7R conductivity and sensitivity, observed during prolonged receptor exposure
to agonist, is critically dependent on TM1 and that the region located at the intersection of TM2 helices controls the rate of large pore opening.

This research was supported by the Grant Agency of the Czech Republic (grants # P304/12/P371, PI: Marie Jinrichova, and P304/12/G069, PI: Laislav Vyklicky) and by the project BIOCEV (CZ.1.05/1.1.00/02.0109), with 84 % involvement of team members, the first and last (corresponding) author are members of the team. Collaborative work with Faculty of Science, Charles University, Prague, that performed homology modelling.

Modulation of rat P2X2R and P2X4R channel gating by synthetic testosterone derivatives

P2XRs are allosterically modulated by numerous compounds, including steroids and neurosteroids. These compounds may both inhibit and potentiate the activity of P2X receptors, but sex steroids such as 17beta-estradiol or progesterone are reported to be inactive. We tested a hypothesis that testosterone, another sex hormone, modulates activity of P2XRs. We designed and synthesized new derivatives of testosterone to increase its potential activity, and analyzed the mechanism of action at P2X2, P2X4 and P2X7 receptors (Sivcev et al., *J Neurochem* **150**: 28-43, 2019). We showed that 17beta-ester derivatives of testosterone rapidly and positively modulated the ATP-evoked currents in P2X2R- and P2X4R-expressing cells, but not agonist-evoked currents in P2X7R-expressing cells. Most of the tested testosterone derivatives were more potent modulators than endogenous testosterone. The comparison of chemical structures and whole-cell recordings revealed that their interactions with P2XRs depend on the lipophilicity and length of the alkyl chain at position C-17. Pre-treatment with testosterone butyrate or testosterone valerate increasesthe sensitivity of P2X2R and P2X4R to ATP by several fold, reduces the rate of P2X4R desensitization, accelerates resensitization, and enhances ethidium uptake by cellsexpressing P2X4R. Native P2X2Rs and P2X4Rs endogenously expressed in pituitary cells orhypothalamic neurons are also potentiated by testosterone derivatives, while endogenously expressed GABA receptors type A are inhibited. The effect of IVM, a P2X4R-specific allosteric modulator, on deactivation is antagonized by testosterone derivatives in a concentration-dependent manner. Together, our results provided evidence for potentiation of particular subtypes of P2XR by testosterone derivatives and suggest a potential role of IVM binding site for steroid-induced modulation.

This project was supported by the Grant Agency of the Czech Republic (grants #16-12695S and #18-054138; PI: Hana Zemkova), the Ministry of Education, Youth and Sports of the Czech Republic within the LQ1604National Sustainability Program II (Project BIOCEVFAR) and by the project BIOCEV (CZ.1.05/1.1.00/02.0109), and by Charles University Grant Agency (grant # 918120, PI:Sonja Sivcev), and with 50 % involvement of team members, thefirst and last (corresponding) author are members of the team. This work was done in collaboration with the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, that designed and synthesized new testosterone derivatives, and the Faculty of Mathematics and Physics, Charles University, Prague, that performed mathematical calculations of lipophilicity.

Research activity and characterisation of the main scientific results

Abbreviations used in the text: LCN - Laboratory of Cellular Neurophysiology, NMDAR - NMDA receptor, TRP channel - transient receptor potential channel, IOCB - Institute of Organic Chemistry and Biochemistry of the CAS

A. Structural and functional analysis of NMDA receptor and TRP ion channels

Using a combined approach – involving electrophysiological examination with rapid solution exchange as well as kinetic analysis and modeling, we have elucidated several aspects of NMDAR and TRP channel function.

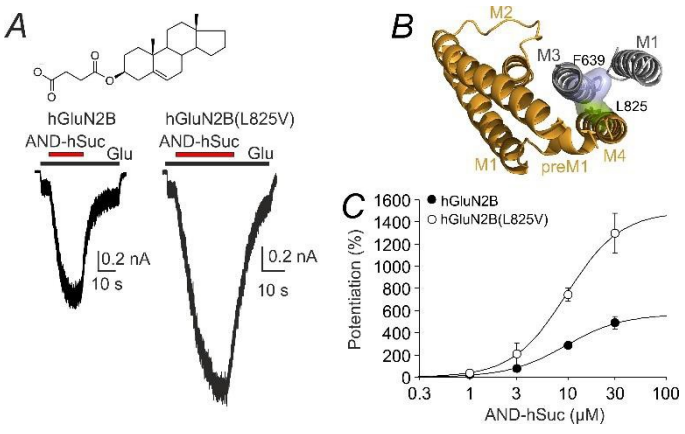
A1. Structural and functional analysis of NMDA receptor channel

1) Structure of the NMDA receptor channel gate. Native NMDA receptors are tetramers composed of two obligatory GluN1 subunits and various combinations of GluN2 and GluN3A-B subunits. Each subunit consists of an amino-terminal, ligand-binding, transmembrane and carboxyl-terminal domain. The ligand-binding and transmembrane domains are interconnected via polypeptide chains (linkers). Upon glutamate and glycine binding, these receptors undergo a series of conformational changes leading to the opening of the Ca^{2+}-permeable ion channel. Here, using electrophysiological examination including single-channel recording of receptors with introduced mutations in the linker region we have identified residues (GluN1(L657) and GluN2B(I655)), the LILI motif, that are part of the extracellular channel gate.

- Ladislav M, Cerny J, Krusek J, Horak M, Balik A, Vyklicky L (2018) The LILI Motif of M3-S2 Linkers Is a Component of the NMDA Receptor Channel Gate. **Front Mol Neurosci** 11:113. IF = 3.720

Team's contribution: 6/6. All authors are members of the LCN.

2) Consequences of de novo mutations. Recent sequencing data from neurological and psychiatric patients have indicated numerous mutations in genes encoding for NMDAR subunits. Here, we present surface expression, functional, and pharmacological analysis of 11 de novo missense mutations of the human hGluN2B subunit (P553L, V558I, W607C, N615I, V618G, S628F, E657G, G820E, G820A, M824R, L825V) located in the pre-M1, M1, M2, M3, and M4 membrane regions. These variants were identified in patients with intellectual disability, developmental delay, epileptic symptomatology, and autism spectrum disorder. Immunofluorescence microscopy indicated reduction in the ratio of surface-to-total NMDAR expression, electrophysiological recordings revealed that agonist potency, receptor desensitization and the probability of channel opening were significantly altered. Further, we have used molecular dynamics simulations to relate the functional consequences of mutations to structural changes (see also point 1 in chapter C1. Steroid modulation of NMDAR).



Mutation hGluN1/hGluN2B(L825V), found in schizophrenia patients and individuals diagnosed with autism, has an enhanced sensitivity to steroids. Responses are shown for *A*. WT and hGluN2B/hGluN2B(L825V) receptors. Androst-5-en-3β-yl hemisuccinate (AND-hSuc) (30 μM) potentiated the mutated receptor responses induced by co-application with 1 μM glutamate in the continuous presence of 30 μM glycine substantially more than mutated receptors. *B*, Membrane domains of

the NMDAR channel viewed from the extracellular side. Structural data indicate that the leucine residue at hGluN2B (L825; the surface is indicated in green) makes a van der Waals contact with the phenylalanine residue at hGluN1 (F639; the surface is indicated in blue). C, Dose response for AND-hSuc-induced potentiation of WT and hGluN2B/hGluN2B(L825V) receptor responses.

- Vyklicky V, Krausova B, Cerny J, Ladislav M, Smejkalova T, Kysilov B, Korinek M, Danacikova S, Horak M, Chodounska H, Kudova E, Vyklicky L (2018) Surface Expression, Function, and Pharmacology of Disease-Associated Mutations in the Membrane Domain of the Human GluN2B Subunit. **Front Mol Neurosci** 11:110. IF = 3.720

Team's contribution: 10/12; LCN members (VV, KB, CJ, LM, ST, KB, KM, DS, HM, VL) designed the project, performed, analyzed and interpreted all the experimental measurements; (CH, KE) synthetized the steroids.

3) Computational model of NMDAR structure. The crystallographic studies provided important insight into the structure of the NMDA receptor, however, the crystallized receptor is quite likely non-functional and it is uncertain which functional state the structure represents. In order to infer on the conformational states of the NMDA receptor associated with closed and open states that could be used for a detailed analysis of steroid binding sites we used an aggregate of nearly 1 μs of unbiased all-atom implicit membrane and solvent molecular dynamics simulations and identified distinct structural states of the NMDA receptor, revealing functionally important residues. The activated and open states of the receptor are structurally similar to the liganded crystal structure, while in the unliganded receptor the extracellular domains perform rearrangements leading to a clockwise rotation of
up to 45 degrees around the longitudinal axis of the receptor, which closes the ion channel. The derived structures of the open and closed states are used in docking experiments to understand the molecular basis of use- and disuse-dependent steroid interaction with the receptor.

- Cerny J, Bozikova P, Balik A, Marques SM, Vyklicky L (2019) NMDA Receptor Opening and Closing-Transitions of a Molecular Machine Revealed by Molecular Dynamics. **Biomolecules** 9(10):546.

Contribution. 3/5; The modeling was done mostly by members of the LCN. BP is a PhD student and MSM helped with details of modeling.

4) Dependence of NMDAR on cholesterol. Using cultured rat cerebellar granule cells, we show that acute and chronic pretreatments resulting in cell cholesterol depletion profoundly diminish NMDAR responses and increase NMDAR desensitization, and also that cholesterol enrichment potentiates NMDAR responses; however, cholesterol manipulation has no effect on the amplitude of AMPA/kainate receptor responses. Diminution of NMDAR responses by cholesterol depletion is caused by a reduction of the ion channel open probability, whereas the increase in receptor desensitization is a result of an increase in the rate constant of entry into the desensitized state.

- Korinek M, Vyklicky V, Borovska J, Lichnerova K, Kaniakova M, Krausova B, Krusek J, Balik A, Smejkalova T, Horak M, Vyklicky L (2015) Cholesterol modulates open probability and desensitization of NMDA receptors. **Journal of physiology** 593:2279-2293. IF = 4.731

Contribution. 11/11. All sample preparation, experimental work and manuscript writing were entirely conducted in the LCN. Multiple affiliations of LK and KB are formal as their work was supported by Charles University student grants. Our paper was selected as a cover story of the issue.

5) Preferential inhibition of tonically activated NMDAR. Using heterologously expressed GluN1/GluN2A and GluN1/GluN2B receptors and rat autaptic hippocampal

microisland cultures, we show that pregnanolone sulfate inhibits NMDAR currents induced by a prolonged glutamate application with a higher potency than the NMDAR component of EPSCs. Novel synthetic analogs of pregnanolone sulfate had no effect on phasically activated receptors while inhibiting tonically activated receptors and had neuroprotective activity without psychomimetic side effects.

- Vyklicky V, Smejkalova T, Krausova B, Balik A, Korinek M, Borovska J, Horak M, Chvojkova M, Kleteckova L, Vales K, Cerny J, Nekardova M, Chodounska H, Kudova E, Vyklicky L (2016) Preferential Inhibition of Tonically over Phasically Activated NMDA Receptors by Pregnane Derivatives. **J Neurosci** 36:2161-2175. IF = 5.988

Contribution. 10/15; Electrophysiological recordings, analysis, design, and writing weredone by members of the LCN (VV, ST, KB, BA, KM, BJ, HM, CJ, NM, LV); behavioral experiments were done by (CM, KL, VK); steroids were synthetized by (CH, EK).

6) Role of N-glycosylation for the NMDAR function. Our results revealed that cerebellar NMDARs associate with 23 different lectins that have unique specificities for glycan structures. Using electrophysiology, we found that specific lectins altered the functional properties of native NMDARs. These data reveal potential targets for the development of novel therapeutic approaches.

- Kaniakova M, Lichnerova K, Skrenkova K, Vyklicky L, Horak M (2016) Biochemical and electrophysiological characterization of N-glycans on NMDA receptor subunits. **J Neurochem** 138:546-556. IF = 4.083

Contribution. 5/5; The project was exclusively performed by LCN members.

7) Identification of the binding site for inhibitory steroids at the NMDAR. Here, we 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. These results, in combination with molecular modeling, characterize structure details of the open configuration of the NMDAR channel.

- Vyklicky V, Krausova B, Cerny J, Balik A, Zapotocky M, Novotny M, Lichnerova K, Smejkalova T, Kaniakova M, Korinek M, Petrovic M, Kacer P, Horak M, Chodounska H, Vyklicky L (2015) Block of NMDA receptor channels by endogenous neurosteroids: implications for the agonist induced conformational states of the channel vestibule. **Scientific reports** 5:10935. IF = 5.228

Contribution. 11/15; Electrophysiological recordings, modeling, analysis, design, and writing were done by members of the LCN (VV, KB, CJ, BA, KM, LK, ST, KM, KM, PM, HM, VL); prediction of the free steroid concentration in the vicinity of the membrane was worked out by (ZM); sequence homology by (NM); steroids were synthetized by (CH).

8) Lectins affect NMDAR function. We aimed to determine whether the presence of specific N-glycans and/or interactions with specific lectins regulates functional properties of GluN1/GluN3A and GluN1/GluN3B receptors. We found that removing putative N-glycosylation sites alters the functional properties of GluN1/GluN3B receptors, but has no effect on GluN1/GluN3A receptors; functional properties of both GluN1/GluN3A and GluN1/GluN3B receptors are modulated by a variety of lectins; and that lectins achieve their effect only when applied to non-activated receptors.

- Hemelikova K, Kolcheva M, Skrenkova K, Kaniakova M, Horak M (2019) Lectins modulate the functional properties of GluN1/GluN3-containing NMDA receptors. **Neuropharmacology** 157:107671. IF = 4.367

Contribution. 5/5; The project was exclusively performed by LCN members.

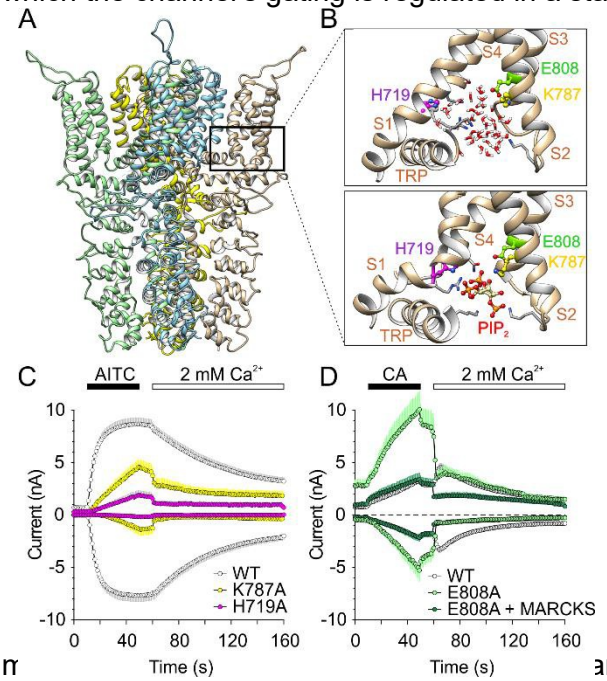
A2. Structural determinants of polymodal activation of TRP ankyrin 1 (TRPA1) channel

1) Proof that TRPA1 acts as a heat- and cold-activated ion channel. We compared the properties of human and mouse TRPA1 and demonstrated that both orthologues are activated by heat and their kinetically distinct components of voltage-dependent gating are differentially modulated by heat and cold. For the first time, we showed that both orthologues can be strongly activated by cold after concurrent application of voltage and heat. We proposed an allosteric mechanism that could account for the variability in TRPA1 temperature responsiveness.

- Sinica V, Zimova L, Barvikova K, Macikova L, Barvik I, Vlachova V (2019) Human and Mouse TRPA1 Are Heat and Cold Sensors Differentially Tuned by Voltage. **Cells** 9. IF = 5.656

Contribution. 5/6; All work on this project was done by team members at the LCN except the part concerning the molecular modeling.

2) Allosteric mechanism of TRPA1 channel gating. We identified conserved polar residues facing the putative lower crevice of the sensor domain that are crucial determinants of the electrophilic, voltage and calcium sensitivity of the TRPA1 channel. This part of the sensor forms a water-accessible cavity that undergoes changes in solvation during channel gating and comprises a domain capable of binding membrane phosphoinositides through which the channel's gating is regulated in a state-dependent manner.



Intracellular cavity of sensor domain controls allosteric gating of TRPA1 channel. (**A**) Structure of tetrameric TRPA1 channel. (**B**) Close-up view of the model of the intracellular cavity (highlighted in A)formed by the S1-S4 sensor domain and the C-terminal TRP helix. The cavity can be both hydrated (above) or it can bind phosphatidylinositol 4,5-bisphosphate (PIP_2) (below, only inositol headgroup of PIP_2 is shown). Mutations of positively (H719, K787) and negatively (E808) charged residues proximal to the phospholipid binding site diversely impact the voltage- and agonist- dependent activation and calcium (Ca^{2+})potentiation of TRPA1. (**C**) Time course of average whole-cell currents induced by 100 μM allyl isothiocyanate (AITC) measured at +80 and −80 mV in HEK293T cells expressing wild-type (WT) or indicated mutants. Mutations E808A and H719 created loss-of-function phenotypes. (**D**) Mutation E808A increased responses to 100 μM cinnamaldehyde (CA) and abolished Ca^{2+}-dependent potentiation. The activating effect of this mutation was reversed by co- transfection of TRPA1 with PIP_2 sequestering agent Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS).

- Zimova L, Sinica V, Kadkova A, Vyklicka L, Zima V, Barvik I, Vlachova V (2018) Intracellular cavity of sensor domain controls allosteric gating of TRPA1 channel**. Sci Signal** 11. IF = 6.481

Contribution. 5/7; All work was done by LCN team members ZL, SV, KA, VL and VV, except the part concerning the molecular modeling.

- ***Identification of phosphoinositide-binding sites regulating TRPA1.*** We demonstrated that the inter-subunit intracellular vestibule of the channel contributes to conformational changes that accompany channel gating and represents a key element of

TRPA1 regulation by membrane voltage. The study is the first demonstration that membrane phosphoinositides interact with TRPA1 to regulate the channel in a state-dependent manner.

- Macikova L, Sinica V, Kadkova A, Villette S, Ciaccafava A, Faherty J, Lecomte S, Alves ID, Vlachova V (2019) Putative interaction site for membrane phospholipids controls activation of TRPA1 channel at physiological membrane potentials**. FEBS J** 286:3664-3683. IF = 4.739
- Witschas K, Jobin ML, Korkut DN, Vladan MM, Salgado G, Lecomte S, Vlachova V, Alves ID (2015) Interaction of a peptide derived from C-terminus of human TRPA1 channel with model membranes mimicking the inner leaflet of the plasma membrane. **Biochimica et Biophysica Acta** 1848:1147-1156. IF = 3.687

Contribution. 4/9 and 2/7 Members of the LCN (WK, ML, SV, KA, VV) initiated, conceptualized and designed the project, performed and analyzed the experimental measurements, and wrote the manuscript.

3) Molecular basis of TRPA1 pain-related channelopathy. We proposed the molecular mechanism underlying the gain-of-function mutation, N855S within the S4-S5 linker of TRPA1, causing episodic pain syndrome in humans. We described the previously unrecognized importance of the inter-subunit interaction for the stability of conformational states associated with chemically and voltage-induced gating of the TRPA1 ion channel.

- Zima V, Witschas K, Hynkova A, Zimova L, Barvik I, Vlachova V (2015) Structural modeling and patch-clamp analysis of pain-related mutation TRPA1-N855S reveal inter-subunit salt bridges stabilizing the channel open state. **Neuropharmacology** 93:294-307. IF = 4.936

Contribution. 4/6; LCN team members conceptualized the project, designed and performed all experimental measurements, analyzed and interpreted data, and wrote the manuscript.

4) Regulation of TRPA1 by CDK5 phosphorylation and N-glycosylation. We have characterized the strictly conserved structural TPLH motifs present in the amino-terminal region of TRPA1 as potential targets for CDK5-dependent phosphorylation. We presented evidence that upon opening, the conformational dynamics of the first extracellular flexible loop, the putative site of N-glycosylation, may govern the voltage-dependence of multimodal gating, thereby serving to stabilize the open state of the TRPA1 channel pore.

- Hynkova A, Marsakova L, Vaskova J, Vlachova V (2016) N-terminal tetrapeptide T/SPLH motifs contribute to multimodal activation of human TRPA1 channel. **Sci Rep** 6:28700. IF = 4.259

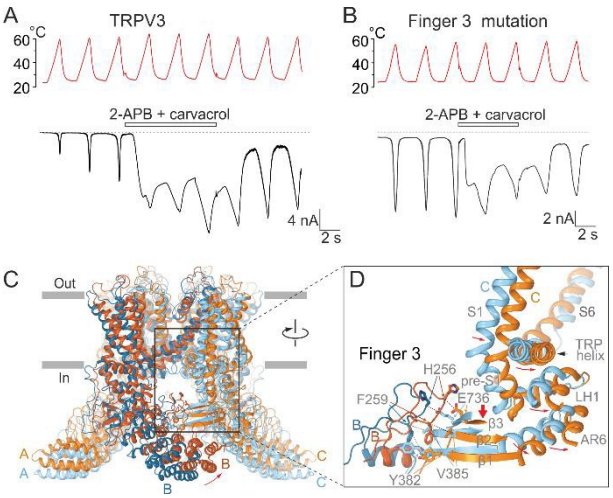
Contribution. 4/4; All the conceptualization, research and actual writing of this paper were entirely conducted in the LCN.

- Marsakova L, Barvik I, Zima V, Zimova L, Vlachova V (2017) The First Extracellular Linker Is Important for Several Aspects of the Gating Mechanism of Human TRPA1 Channel. **Front Mol Neurosci** 10:16. IF = 3.902

Contribution. 3/5; LCN members (ML, ZL, VV) designed the project, performed, analyzed and interpreted all the experimental measurements and wrote the manuscript.

A3. Thermosensitive ion channel regulation in sensory neurons

1) Molecular basis of use-dependent gating of TRPV3. We identified a specific interfacial cytoplasmic region through which repeated stimulation of the human TRPV3channel by high temperatures progressively increases its responses and shifts the activation threshold to physiological temperatures. The results explain how TRPV3 senses temperature and integrates diverse stimuli under physiological and pathophysiological conditions.



Cytoplasmic inter-subunit interface controls use-dependence of thermal activation of TRPV3 channel. (**A**) Representative whole-cell currents evokedby repetitive heat stimuli, applied at -70 mV, recorded from HEK293T cell expressing wild-type human TRPV3 in the absence or presence of mixed agonists (100 μM 2-APB + 100 μM carvacrol). (**B**) Mutation within the Finger 3 loop produced sensitized phenotype. (**C**) Superposition of TRPV3 structures in the closed (PDB ID: 6DVW; subunits A and C are colored cyan, B blue) and open (PDB ID: 6DVZ; subunits A and C are colored orange, B brown) states of TRPV3. (**D**) Close-up view of the interface between subunits B and C. Sliding down motion of the three-stranded β sheet relative to Finger 3 during TRPV3 opening (thick red arrow) is shown. Thin red arrows illustrate movements of the domains that link the β sheet to the gate formed by S6.

- Macikova L, Vyklicka L, Barvik I, Sobolevsky AI, Vlachova V (2019) Cytoplasmic Inter-Subunit Interface Controls Use-Dependence of Thermal Activation of TRPV3 Channel. **Int J Mol Sci** 20. IF = 4.183

Contribution. 3/5; The study was initiated at the LCN and all experimental work on this project was done by members of the LCN except the part concerning the molecular modeling.

2) Phosphorylation of TRPV3 by ERK kinase. In the amino terminus of TRPV3, we have identified phosphorylation sites for extracellular signal-regulated kinase responsible for the modulation of TRPV3 via growth-factor downstream signaling. These results are includedin the PhosphoSitePlus database as, to date, the only known functionally confirmed phosphosites on TRPV3.

- Vyklicka L, Boukalova S, Macikova L, Chvojka S, Vlachova V (2017) The human transient receptor potential vanilloid 3 channel is sensitized via the ERK pathway. **J Biol Chem** 292:21083-21091. IF = 4.010

Contribution. 5/5; All the conceptualization, research and actual writing of this paper were entirely conducted by LCN team members.

3) Polymodal activation of thermosensitive ion channels in model sensory neurons. We provide new evidence that electromagnetic field directly modulates the activity of sensory neurons and highlight the potential of sensory neuron-derived cell line as a tool for studying the underlying mechanisms at the cellular and molecular level. Furthermore, we show that the exceptional capacity of skin C-fiber nociceptors to encode noxiously hot temperatures depends on two tetrodotoxin-resistant sodium channel alpha subunits: NaV1.8 and NaV1.9.

- Prucha J, Krusek J, Dittert I, Sinica V, Kadkova A, Vlachova V (2018) Acute exposure to high-induction electromagnetic field affects activity of model peripheral sensory neurons. **J Cell Mol Med** 22:1355-1362. IF = 4.658

Contribution. 5/6; Members of the LCN conceptualized and designed the project, performed and analyzed the experimental measurements and wrote the manuscript.

- Touska F, Turnquist B, Vlachova V, Reeh PW, Leffler A, Zimmermann K (2018) Heat- resistant action potentials require TTX-resistant sodium channels NaV1.8 and NaV1.9. **JGen Physiol** 150:1125-1144. IF = 4.258

Contribution. 2/6; The contribution of LCN team members (TF and VV) was (i) planning, performing and analyzing the patch-clamp and behavioral experiments and (ii) conceptualization and help with the supervision of the project.

B. Glutamate receptor surface expression and trafficking

1) Kainate receptor-dependent LTP. Induction of classical LTP involves an NMDA receptor-and calcium-dependent increase in functional synaptic AMPA receptors. Here we report a physiologically relevant NMDA receptor-independent mechanism of LTP induction requiring the metabotropic action of kainate receptors and activation of G protein, protein kinase C and phospholipase C. Like classical LTP, kainate receptor-dependent LTP recruits recycling endosomes to spines and increases AMPA receptor surface expression.

- Petrovic MM, Viana da Silva S, Clement JP, Vyklicky L, Mulle C, Gonzalez-Gonzalez IM, Henley JM (2017) Metabotropic action of postsynaptic kainate receptors triggers hippocampal long-term potentiation. **Nat Neurosci** 20:529-539. IF = 19.912

Contribution. 3/7; PM, G-GIM and VL were members of the LCN. PM performed electrophysiological experiments, analyzed data, and wrote the manuscript; G-GIMperformed the trafficking experiments using confocal microscopy; VL contributed to the design of experiments and data analysis.

2) Mutation in the glycine-binding sites affects NMDAR surface delivery. We examined the surface delivery and functional properties of NMDARs containing mutations in the glycine-binding sites in GluN1 and GluN3A subunits. We found that structural features of the glycine-binding sites in both GluN1 and GluN3A subunits are correlated with receptor forward trafficking to the cell surface.

- Skrenkova K, Hemelikova K, Kolcheva M, Kortus S, Kaniakova M, Krausova B, Horak M (2019) Structural features in the glycine-binding sites of the GluN1 and GluN3A subunits regulate the surface delivery of NMDA receptors. **Scientific reports** 9:12303. IF = 4.011

Contribution. 7/7; The project was exclusively performed in the LCN.

3) N-glycosylation affects NMDAR surface expression and trafficking. We identified *N*-glycosylation sites in the GluN1 and GluN3A subunits that are essential for releasing NMDARs from the endoplasmic reticulum and for its surface mobility.

- Lichnerova K, Kaniakova M, Park SP, Skrenkova K, Wang YX, Petralia RS, Suh YH, Horak M (2015) Two N-glycosylation Sites in the GluN1 Subunit Are Essential for Releasing N-methyl-d-aspartate (NMDA) Receptors from the Endoplasmic Reticulum. **J Biol Chem** 290:18379-18390. IF = 4.258

Contribution. 4/8; The first and the last (co-corresponding) authors were from the LCN. This study was highly international as we collaborated with the scientists from Korea and the USA.

- Skrenkova K, Lee S, Lichnerova K, Kaniakova M, Hansikova H, Zapotocky M, Suh YH, Horak M (2018) N-Glycosylation Regulates the Trafficking and Surface Mobility of GluN3A-Containing NMDA Receptors. **Front Mol Neurosci** 11:188. IF = 3.720

Contribution. 4/9; The molecular biology and microscopy was done in the LCN (SK, LK, KM, HH, HM); (LS; SYH) were foreign collaborators; (ZM) analyzed diffusion.

4) Surface expression of human disease-associated NMDAR. Immunofluorescence microscopy indicated that the ratio of surface-to-total NMDAR expression was reduced for hGluN1/hGluN2B(S628F) receptors and increased for hGluN1/hGluN2B(G820E)receptors (see also point A1 in the chapter “Structural and functional analysis of NMDA receptor channel” for details).

- Vyklicky et al. (2018) **Front Mol Neurosci** 11:110 **(&)**

6) Phosphorylation as a factor controlling synaptic localization of NMDARs. Phosphorylation of the PDZ ligand of the GluN2B subunit of NMDARs (at S1480) has been identified as a critical determinant in dynamically controlling NMDAR synaptic content. Phosphorylation of GIuN2B(S1480) maintains NMDARs at extrasynaptic membranes and dephosphorylation of GluN2B(S1480) promotes an increase in synaptic NMDAR content.

- Chiu AM, Wang J, Fiske MP, Hubalkova P, Barse L, Gray JA, Sanz-Clemente A (2019) NMDAR-Activated PP1 Dephosphorylates GluN2B to Modulate NMDAR Synaptic Content. **Cell Rep** 28:332-341 e335. IF = 7.815
- Chiu AM, Barse L, Hubalkova P, Sanz-Clemente A (2019) An Antibody Feeding Approach to Study Glutamate Receptor Trafficking in Dissociated Primary Hippocampal Cultures. **J Vis Exp**. IF = 1.108

Contribution. 1/7. HP performed microscopy and some electrophysiological experiments. She was as a Fulbright Visiting Student Researcher at Northwestern University in Chicago (IL, USA).

C. Pharmacology of NMDAR

C1. Steroid modulation of NMDAR

(&) In this section we list papers in which we explored steroid structure-activity relationship for their modulation of NMDARs. This project was carried out jointly with the research group of dr. Kudova at IOCB who have synthesized the steroids. The LCN provided electrophysiological and pharmacological examination of recombinant and native receptors. Design of new molecules was done based upon experimental results and discussion of prof. Vyklicky and dr. Kudova. This joint project exists for about 20 years and high-quality papers have been published as a results, as well as parents submitted/or accepted (see the section on Patents). For clarity the members of the LCN are underlined.

1) Sensitivity of human disease-associated NMDARs to steroids. The sensitivity of *de-novo* missense mutant hGluN2B receptors to positive allosteric modulators of steroid origin showed that glutamate responses induced in human variants hGluN1/hGluN2B(V558I, W607C, V618G, and G820A) were potentiated by 59-96% and 406-685% when recorded in the presence of 20-oxo-pregn-5-en-3 beta-yl sulfate (PE-S) and androst-5-en-3 beta-yl hemisuccinate (AND-hSuc), respectively. Surprisingly, hGluN1/hGluN2B(L825V) receptors were strongly potentiated, by 197 and 1647%, respectively, by PE-S and AND-hSuc (see also point A1 in the chapter “Structural and functional analysis of NMDA receptor channel” for details).

- Vyklicky V, Krausova B, Cerny J, Ladislav M, Smejkalova T, Kysilov B, Korinek M, Danacikova S, Horak M, Chodounska H, Kudova E, Vyklicky L (2018) **Front Mol Neurosci** 11:110 **(&)**

2) Positive steroid modulators of NMDARs. Here, we report the synthesis of pregn-5-ene and androst-5-ene dicarboxylic acid esters and explore the structure-activity relationship for their modulation of NMDARs. All compounds were positive modulators of recombinant GluN1/GluN2B receptors (EC_{50} varying from 1.8 to 151.4 μM and E_{max} varying from 48% to 452%). A selected compound, 20-oxo-pregnenolone hemiadipate, potentiated nativeNMDARs to a similar extent as GluN1/GluN2A-D receptors and inhibited AMPAR and $GABA_AR$ responses.

- Krausova B, Slavikova B, Nekardova M, Hubalkova P, Vyklicky V, Chodounska H, Vyklicky L, Kudova E (2018) Positive Modulators of N-Methyl-D-Aspartate Receptor: Structure-Activity Relationship Study on Steroidal 3-Hemiesters **J Med Chem** 61:4505- 4516. IF = 6.054 ***(&)***

3) Negative steroid modulators of NMDARs. In this study, we describe a new class of neurosteroid analogues which possess structural modifications in the steroid D-ring region. These analogues were tested on recombinant GluN1/GluN2B receptors to evaluate the structure-activity relationship. The tested compounds were potent NMDAR inhibitors (IC50 values varying from 90 nM to 5.4 μM).

- Kudova E, Chodounska H, Slavikova B, Budesinsky M, Nekardova M, Vyklicky V, Krausova B, Svehla P, Vyklicky L (2015) A New Class of Potent N-Methyl-D-Aspartate Receptor Inhibitors: Sulfated Neuroactive Steroids with Lipophilic D-Ring Modifications. **J Med Chem** 58:5950-5966. IF = 5.589 ***(&)***

4) Perhydrophenanthrene analogues as negative modulators of NMDAR. We performed a structure-activity relationship study for perhydrophenanthrene analogues possessing a framework that mimics the steroidal ring system. This study comprises the design, synthesis, and assessment of the biological activity of a library of perhydrophenanthrene 2-sulfates and 2-hemisuccinates. Their ability to modulate NMDA-induced currents was tested on recombinant GluN1/GluN2B receptors.

- Slavikova B, Chodounska H, Nekardova M, Vyklicky V, Ladislav M, Hubalkova P, Krausova B, Vyklicky L, Kudova E (2016) Neurosteroid-like Inhibitors of N-Methyl-d-aspartate Receptor: Substituted 2-Sulfates and 2-Hemisuccinates of Perhydrophenanthrene. **J Med Chem** 59:4724-4739. IF = 6.259 ***(&)***

5) Amide-based steroidal inhibitors of NMDA receptors. Here we report the synthesis, structure-activity relationship study, and biological evaluation of neurosteroid inhibitors of NMDA receptors that contain an amide structural motif, related to pregnanolone glutamate (PAG), a compound with neuroprotective properties.

- Adla SK, Slavikova B, Chodounska H, Vyklicky V, Ladislav M, Hubalkova P, Krausova B, Smejkalova T, Nekardova M, Smidkova M, Monincova L, Soucek R, Vyklicky L, Kudova E (2018) Strong Inhibitory Effect, Low Cytotoxicity and High Plasma Stability of Steroidal Inhibitors of N-Methyl-D-Aspartate Receptors With C-3 Amide Structural Motif. **Front Pharmacol** 9:1299. IF = 3.845
- Adla SK, Slavikova B, Smidkova M, Tloustova E, Svoboda M, Vyklicky V, Krausova B, Hubalkova P, Nekardova M, Holubova K, Vales K, Budesinsky M, Vyklicky L, Chodounska H, Kudova E (2016) Physicochemical and biological properties of novel amide-based steroidal inhibitors of NMDA receptors. **Steroids**. IF = 2.523

6) ent-Pregnanolone sulfate is an inhibitor of NMDARs. A unique asymmetric total synthesis of the unnatural enantiomer of pregnanolone sulfate, as well as a study of its biological activity at the NMDA receptor, was carried out. Surprisingly, ent-pregnanolone sulfate retained its biological activity at the NMDAR.

- Kapras V, Vyklicky V, Budesinsky M, Cisarova I, Vyklicky L, Chodounska H, Jahn U (2018) Total Synthesis of ent-Pregnanolone Sulfate and Its Biological Investigation at the NMDA Receptor. **Org Lett** 20:946-949. IF = 6.555

C2. Tacrine derivatives as inhibitors of NMDARs

(§) In this section we list papers in which we exploredstructure-activity relationship for the inhibition of NMDARs by tacrine derivatives. This project was carried out jointly with the research group of Prof. K. Kuča, and dr. O. Soukup at the University of Hradec Králové who have synthesized the compounds. The LCN provided electrophysiological andpharmacological examination on recombinant and native receptors. This is a newly established cooperation.

1) MEOTA is an NMDAR inhibitor. We show that 7-MEOTA is a potent “foot-in-the- door” open-channel blocker of NMDA receptors. This compound inhibits GluN1/GluN2A- M817V receptors with a pathogenic mutation and exhibits neuroprotective activity in rats with NMDA-induced lesions.

● Kaniakova M, Kleteckova L, Lichnerova K, Holubova K, Skrenkova K, Korinek M, Krusek J, Smejkalova T, Korabecny J, Vales K, Soukup O, Horak M (2018) 7- Methoxyderivative of tacrine is a 'foot-in-the-door' open-channel blocker of GluN1/GluN2 and GluN1/GluN3 NMDA receptors with neuroprotective activity in vivo. **Neuropharmacology** 140:217-232. IF = 4.367 ***(§)***

2) Multi-target-directed therapeutics. Here, we investigated 7-methoxytacrine-memantine heterodimers developed with respect to the multi-target-directed ligand theory. The spectroscopic, microscopic and cell-culture methods were used for systematic investigation of the interference of the heterodimers with beta-secretase activity, Aβ peptide amyloid fibrillization (amyloid theory) and interaction with the M1 subtype of muscarinic, and with nicotinic acetylcholine receptors (cholinergic theory), as well as with NMDA receptors (glutamatergic theory). We have found that 7-methoxytacrine-memantine heterodimers are able to hit multiple targets associated with Alzheimer's disease and thus have clinical potential for slowing or blocking the neurodegenerative process related to this disease.

● Gazova Z, Soukup O, Sepsova V, Siposova K, Drtinova L, Jost P, Spilovska K, Korabecny J, Nepovimova E, Fedunova D, Horak M, Kaniakova M, Wang ZJ, Hamouda AK, Kuca K (2017) Multi-target-directed therapeutic potential of 7-methoxytacrine-adamantylamine heterodimers in the Alzheimer's disease treatment. **Biochim Biophys Acta Mol Basis Dis** 1863:607-619. IF = 5.108 ***(§)***

D. Patents

Some newly synthetized steroids were patented JP 6437636 (2018); EP 3186267 (2019); EP 3260462 (2019); CZ 305733 (2016); CZ 307648 (2018); EP 2675821 (2018); (see Chapter “Participation in large collaborations” for details)

E. Book chapters

Ionotropic Glutamate Receptor Technologies, editor: Gabriela K Popescu; Analysis of Whole-Cell NMDA Receptor Currents - Vyklicky V., Korinek M., Balik A., Smejkalova T., Krausova B. Vyklicky L., *Springer* ● 205-219 (2016).

F. Miscellaneous publications with minor contribution of LCN members

Members of the LCN are underlined.

● Franco, R. - Casadó-Anguera, V. - Muñoz, A. - Petrovič, Miloš - Navarro, G. - Moreno, E. - Lanciego, J. L. - Labandeira-García, J. L. - Cortés, A. - Casadó, V. *Hints on the Lateralization of Dopamine Binding to D-1 Receptors in Rat Striatum*. **Mol Neurobiol 2016, 53(8):5436-5445**. IF = 6.190

● Bohuslavova R, Dodd N, Macova I, Chumak T, Horak M, Syka J, Fritzsch B, Pavlinkova G (2017) Pax2-Islet1 Transgenic Mice Are Hyperactive and Have Altered Cerebellar Foliation. **Mol Neurobiol** 54:1352-1368. IF = 5.076

● Hubálková, P. *G-kvadruplexy v oblasti lidských telomer a jejich terapeutický potenciál* . **Chemické listy. 2015, 109(12): 918-922**. IF = 0.279

● Šepsová, V. - Karasová, J. Z. - Tobin, G. - Jun, D. - Korábečný, J. - Cabelová, P. - Jánská, K. - Krůšek, Jan - Skřenková, K. - Kuča, M. - Valko, M. - Soukup, O. *Cholinergic properties of new 7-methoxytacrine-donepezil derivatives*. **General Physiology and Biophysics. 2015, 34(2): 189-200**. IF = 0.892

● A review on the effect of tacrine and its derivatives on the NMDA receptors (NMDAR) with a focus on the mechanism of action and biological consequences related to the Alzheimer's disease treatment. Horák, M. - Holubová, K. - Nepovímová, E. - Krůšek J. - Kaniaková, M. - Korábečný, J. - Vyklicky, L. - Kuča, K. – Stuchlík A. - Říčný, J. - Valeš, K. - Soukup, O. *The pharmacology of tacrine at N-methyl-D-aspartate receptors*. **Progress in**

Neuro-Psychopharmacology & Biological Psychiatry. 2017, 75, 54-62. IF = 4.185

● Krůšek, J. - Dittert, I. - Smejkalová, T.- Kořínek, M. - Gottfriedová, K. - Freislebenová, H. - Neuhöferová, E. - Klimša, L. - Sedláková, S. - Taylor, A. - Mortet, V. - Petrák, V. - Benson, V. - Petráková, V. *Molecular Functionalization of Planar Nanocrystalline and Porous Nanostructured Diamond to Form an Interface with Newborn and Adult Neurons*. **Physica Status Solidi B-Basic Solid State Physics 2019, 256(3), 1800424**. IF = 1.454

Research activity and characterisation of the main scientific results

The laboratory has long-standing experience in testing behavioural, mnemonic, and cognitive functions in laboratory animals. The lab has focused on neural mechanisms of spatial navigation as a model of declarative memory, roles of specific neuronal circuits and brain structures in navigation in dynamic worlds, neurochemistry of spatial navigation, and cognitive deficits in brain disorders in animal models and human patients. We continue to design and develop innovative behavioural tasks. We also develop real-space and virtual-reality behavioural tests for humans, which can be useful for early diagnostics of neuropsychiatric disorders, and combine these tests with intracranial recordings in epilepsy patients.

To these aims, we employ a plethora of behavioural, neuroanatomical, pharmacological, cellular, molecular, imaging, and computational approaches. These methods include behavioural testing in both standard and custom-designed spatial mazes and non-spatial tasks, stereotaxic delivery of ion channel blockers, receptor ligands, and viral vectors to specific brain areas, fluorescent *in situ* hybridization and immunohistochemistry, confocal imaging, and chronic multiple single unit electrophysiology from behaving animals. These techniques span across different levels of resolution and understanding and provide complementary explanatory values enabling us to test our experimental hypotheses in highdetail and fidelity. Advanced transgenic methods using photo- or chemo-sensitive tools to manipulate neuronal circuit activity and intravital two-photon imaging via chronic cranial windows are the most recent aquisitions to our portfolio.

Generally, the focus of the team is quite compact, but for descriptive purposes, we can identify two basic branches with complementary points of view and using different model organisms: experimental research on animals and translational research involving humans.We will deal with these two branches separately at first and discuss their integration later.

Biology of behaviour and memory traces in laboratory rodents

We have achieved significant findings in this field between 2015 and 2019. The most important results are stated below and their significance is explained. The studies ranged from basic mechanisms underlying learning and memory to animal models of schizophrenia and experimental psychosis, Alzheimer´s disease (AD), obsessive-compulsive disorder (OCD), neuroprotection, and narcolepsy. Behavioural and memory trace functioning in health and disease is a leitmotif of our studies.

Examination of basic mechanisms of memory involved testing the functional role of adult neurogenesis, the role of the hippocampus in discrimination of inaccessible objects, and the role of the anterior cingulate cortex, an important cognitive brain hub, in navigation relative to a moving robot. The role of adult neurogenesis in learning, memory, and behavioural flexibility is a pressing, yet unresolved topic. In a study by Brozka et al. (2017), we have shown that ablation of the adult neurogenesis in rats by cytostatic treatment with temozolomide unexpectedly facilitated cognitive coordination and behavioural flexibility on a rotating arena, a dynamic navigation task originally custom-designed in our lab. The results provide a new perspective on the role of the adult-born neurons in cognition and suggested that their role may be species-specific, which is a novel concept in the field. A study by Svoboda et al., (2017) elucidated the organization of spatial behaviour relative to relevant moving objects, which is a yet underappreciated area of cognitive research and its underlying neuronal substrates remain elusive. Using transient silencing with GABAA receptor agonist muscimol, we were the first to show that the rat anterior cingulate cortex (ACC) was crucial for effective preparation and execution of avoidance of a fast-moving robot. If the robot was slow, ACC contribution was unnecessary. This result, together with previous studies, suggests that communication between the dorsal hippocampus and ACC mediates the dynamic navigation relative to fast-moving visible objects. The role of the rodent dorsal hippocampus in object discrimination remains controversial. We developed a novel operant task based on virtual reality stimuli and combined it with transient inactivation by muscimol toexamine the limits of the hippocampus involvement. This study was the first to show that the role of the hippocampus in

visual object discrimination depends on the abundance of object's features and their spatial character (Levcik et al., 2018). In summary, we brought several important pieces of evidence on the spatially- and task-specific involvements of specific brainareas, namely the hippocampus, the anterior cingulate and the retrosplenial cortices, and neuronal circuits and cell populations therein in coding the cognitive aspects of behaviour.

Non-competitive NMDA-receptor antagonists such as MK-801 are used to model psychosis related to schizophrenia. Our laboratory has a long-standing tradition in their investigation. They are known to impair spatial coordination in rats. The study by Buchtova et al. (2017) shows the circuit-specific effects of MK-801 application on immediate-early gene expression in neuronal ensembles in rats exploring two environments of the same or different identity. Spatial context-specificity was eliminated in the hippocampal region CA1, but not in CA3, indicating functional uncoupling between these two hippocampal areas. Ensembles in the retrosplenial cortex were not specific to spatial context during exploration, but ensemble similarity was increased in home cage controls. These results support the discoordination hypothesis of schizophrenia. In a complementary test of the hypothesis, we examined the electrophysiological activity of hippocampal neurons in anesthetized rats after systemic injection of MK-801. MK-801 increased co-activation specifically between previously uncorrelated neurons (hypersynchrony), which resulted in disorganized ensemble activity despite preserved firing properties of individual neurons, lending further support to the discoordination hypothesis (Sczurowska et al., 2018). These results bring evidence on
neuronal substrates of acute psychosis (as a set of symptoms) induced by MK-801, but schizophrenia is a chronic and complex disease that typically manifests after adolescence during early adulthood. Therefore, we also aimed at uncovering the long-term consequences of sub-chronic NMDA receptor antagonism during these periods in the rat model using two different outbred strains of laboratory rats. We have shown that a two-week treatment with MK-801 did elicit delayed working memory deficits in Long-Evans hooded rats, but not in albino Wistar rats, with no alteration in subunit composition of NMDA receptors found by Western blot (Uttl et al., 2018). These results support the face (phenomenological) but not the construct validity of this sub-chronic MK-801 model of schizophrenia, leaving open the possibility that it reflects the rough similarity of symptoms rather than pathophysiological hallmarks of the disease. We have thoroughly discussed the state of the art on the discoordination and disconnection aspects of schizophrenia in a review by Krajcovic et al (2019). In this broad review, we have thoroughly examined multiple biological levels in a search of a common neurophysiological mechanism of schizophrenia's cognitive symptoms.

Fig. 1: A combined setup for electrophysiological recordings and moving robot avoidance of the Laboratory of the Neurophysiology of Memory, operated by Nikhil Ahuja, a Ph.D. student whose work focuses on electrophysiology correlates of avoidance of a moving object in the hippocampus and the lateral entorhinal cortex in rats.

We also tested rat models of AD to describe the nature of the neuronal and behavioural deficits in these models. First, we investigated the Samaritan™ rat model of AD (Taconic Pharmaceuticals, USA) based on chronic concurrent administration of amyloid-beta and pro-oxidative substances. Our results showed that Samaritan rats™ exhibited marked impairment of spatial learning and memory as well as significant changes in expression of the NR2A subunit of NMDA receptor and choline transporter CHT1 activity compared to controls rats (Petrasek et al., 2016), mimicking the situation in patients with early-stage of AD and supporting the face and construct validities of the model. Next, we probed the McGill-R- Thy1-APP transgenic rat model of AD. We observed many symptoms described in human patients including problems with locomotion, altered social behaviour and communication, and disturbed circadian cycle. We also confirmed a previously reported deficit of spatial cognition. Our findings demonstrated that the model is feasible for testing interventions aimed at both cognitive and non-cognitive impairments associated with AD (Petrasek et al., 2018). Both studies were done in cooperation with other laboratories. We also critically reviewed the effect of tacrine and its derivatives on NMDA receptors with a focus on the mechanism of action related to neurodegeneration. We predicted that tacrines with dual cholinesterase and NMDA-receptor modulating activity represent a new and promising target in the development of drugs for diseases associated with cognitive dysfunction (Horak et al.,2017). As a potential neuroprotective therapy for AD and related disorders, we further characterized the novel neuro-pharmacological properties of 7-methoxy derivative of tacrine. The results showed that this substance has a specific mechanism of action at NMDA receptors, which can be seen as a 'foot-in-the-door' open-channel blocker of GluN1/GluN2. In follow-up experiments, we demonstrated a neuroprotective effect without serious side effects in an animal model (Kaniakova et al., 2018). This study was done in cooperation with another lab at the IPHYS and with extramural collaboration. We also studied the neurotoxic effects of the intra-hippocampal application of NMDA. This international cooperation (Rambousek et al., 2016) revealed important mechanisms of excitotoxic action of NMDA on hippocampal neurons. We showed that intrahippocampal infusions caused focal neurodegeneration accompanied by progressive neuroinflammation, affecting preferentially principal neurons and sparing GABAergic interneurons. Besides, the NMDA receptor subunit composition was altered in the surviving neurons. In an international collaboration with the Nencki Institute of Experimental Biology in Warsaw, Poland, we examined acute mechanisms of therapeutic effects of an established neuroprotective drug memantine (Wesierska et al. (2019). This study revealed that memantine, an NMDA antagonist, not only shows beneficial effects in various pathological states but also acts pro-cognitively at low doses in an intact rat brain. We demonstrated that administration of memantine improved performance particularly in a coordination-demanding task that required segregation of stimuli into coherent subsets, but this effect disappeared if segregation was not necessary.

Finally, development of novel drugs for treating dementia has proven notoriously difficult and many drugs showing promising effects in rodent models failed late-phase clinical efficacy tests. This suggests that (i) either there is something fundamentally wrong with the models, or (ii) we lack the appropriate and fully translational paradigms of efficacy assessment. To address the second possibility, a translational paper by Laczo et al. (2017), published in a wide international collaboration, aimed at validating place navigation to a hidden goal in translational research, particularly for AD drug screening. This was accomplished by showing that this task yielded corresponding results in the scopolamine-induced model of amnesia in both rats and healthy human volunteers. Also, the detrimental effect of scopolamine was alleviated by co-treatment with donepezil, symptomatic medication for AD, in both humans and rats. This strongly suggests that the hidden-goal task is a suitable test for potential treatments of dementia that provides full translatability from animal models to humans.

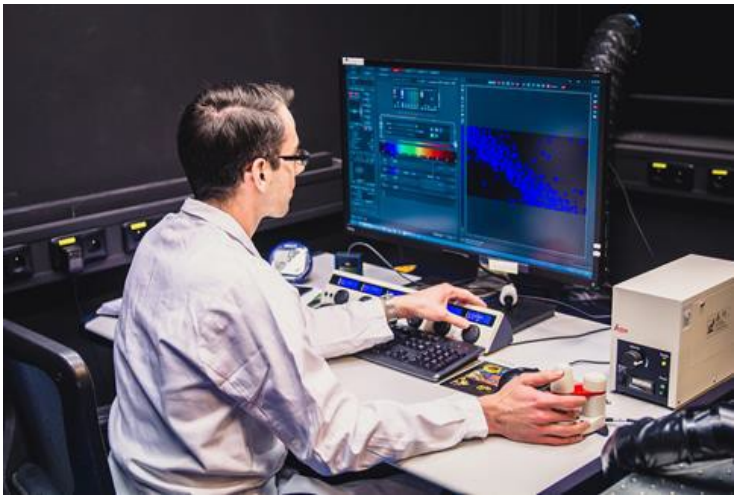


Fig. 2: Acquisition of confocal images for *in-situ* analysis of gene expression, done by Branislav Krajcovic, a Ph.D. student whose project aims at neuronal discoordination in the cortex in animal models of schizophrenia and psychosis.

Loss of neurons producing neuropeptide orexin has been recently discovered in people suffering from narcolepsy, a disorder characterized by excessive daytime sleepiness and sudden intrusions of rapid-eye-movement sleep into periods of wakefulness accompanied byloss of muscle tone and cataplexy. The broad review by Nepovimova et al. (2019) summarized the mechanisms of orexin-mediated control of the sleep/wake cycle, orexin- based therapeutic options, and the potential of orexin receptor agonists as a treatment option for narcolepsy. This review resulted from a collaborative project of the IPHYS with University Hospital in Hradec Kralove.

Experiments with healthy volunteers and neuropsychiatric patients

In the human branch of our research, we focused on translation of rodent model tasks to humans and also on basic research into the electrophysiology of human spatial orientation. In collaboration with the Neurology Department at University Hospital Motol, we focused on early diagnosis of patients with Alzheimer's disease (AD) in the stage of mild cognitive impairment (MCI) using spatial navigation. Besides memory deficits, spatial disorientation is a prominent early sign in AD, and, as we have previously shown, it is detectable also in patients with MCI, prior to progression to dementia. Spatial navigation is a complex cognitive ability arising from several complementary components supported by different brain regions. In two studies, we focused on specific spatial functions and their contribution to spatial navigation in general. In the next study, we focused on the relationship between spatial navigation and other cognitive domains in detecting MCI and AD. In the first study (Markova et al. 2015), we were the first to investigate the ability to imagine a spatial scene from another perspective in MCI and AD patients. For this purpose, we developed a new Arena Perspective Taking Task using an environment of a circular arena on a computer monitor that distinguishes the first-person view from overhead perspective-taking. We found that patients suffering both MCI and AD show deficits of this ability, with women more affected than men. This sex difference was more apparent in the overhead view versions, but the overall discrimination of patients from healthy controls was higher in the first-person view version of the test. Our results show high discrimination accuracy of first-person perspective-taking between the AD, MCI, and healthy control groups. They suggest that this ability, calledspatial perspective taking, is a promising marker in dementia diagnostics and evaluation of cognitive rehabilitation. A line of results from other labs showed an association between optic flow perception impairment and spatial disorientation in AD patients, suggesting that thespatial disorientation may apply selectively to navigation using vision. In the second study (Mokrisova et al. 2016), we were interested if the repeatedly documented spatial disorientation is confined to visual navigation alone. In a newly developed Arena Path Integration Task, we tested subjects suffering from AD and MCI for non-visual navigation to two previously seen goals and back to the start. Both groups

of patients were impaired relative to healthy controls in returning to the start position and their impairment was related to the medial temporal and parietal cortical volumes. Our results demonstrated that non- visual navigation is also impaired in patients with AD and MCI, possibly as a function of medial temporal and parietal cortical neurodegeneration. In the third study (Laczo et al. 2017), we focused on the relationship between visuospatial navigation to a hidden goal and other cognitive functions in characterizing the AD and MCI patients. Scores from a real space analogy of the Morris water maze, previously used in several of our studies, were analysed along with scores from a range of standard neuropsychological tests. Scores from allocentric and egocentric navigation were only marginally associated with scores from other tests, which included verbal, visuospatial, mnemonic, executive, and others. These results confirm indications from previous studies that spatial navigation is an independent cognitive domain. Its assessment, in addition to other cognitive functions, may substantially extend the comprehensive neuropsychology profile.

Schizophrenia is another disease we investigate on a long-term basis in humans. We have previously shown that patients with schizophrenia are impaired in spatial navigation in virtual reality. In a collaborative follow-up study with the National Institute of Health (Rodriguez et al. 2015), we focused on visuospatial abilities and how they influence the patients' quality of life. Patients with first-episode schizophrenia were assessed in a neuro-psychological battery focused on visuospatial and verbal functions. Other tests focused on global functioning and quality of life. The patients were significantly impaired in both verbal and visuospatial abilities, but their quality of life and global functioning scores were influenced only by their verbal scores. This inconsistency suggests that while visuospatial (dis)abilities are an integralpart of schizophrenia, they may be incorrectly represented on the quality of life and global functioning scales. We questioned if these measures are sensitive enough to monitor less noticeable but still limiting deficits.

Intracranial EEG (iEEG) recording from epileptic patients is a new line of research we startedin collaboration with the Neurology Department of the University Hospital Motol during the evaluation period. Patients undergoing intracranial monitoring for surgical treatment of medically refractory focal epilepsy are implanted with a set of deep electrodes allowing monitoring the source and development of epileptic seizures. The iEEG is a developing modern method in human electrophysiology bridging micro- and macroscopic neuroscience with precise temporal and spatial resolution. It can provide data about the accurate timing of cognitive processes and frequency and dependency analysis in precisely localized brain areas. We aimed to investigate the electrophysiology of visuospatial abilities in the brain. Wedeveloped a series of computer tests focusing on the mechanisms of the precise timing of spatial reference frames in dorsal and ventral visual streams. These questions are beyond the limits of functional imaging methods such as fMRI, due to their relatively poor temporal resolution on the order of seconds. The tests address specifically allocentric and egocentric distance estimates, spatial perspective-taking, and functional localization of spatial orientation (spatial scenes and objects), discrimination of location, or spatial scene identity change in specific brain regions. As the available iEEG setup for intracranial patients allows for telemetric recording during free movements in real space, the other two tests focused on free walking at variable speed and allocentric and egocentric estimates during real and virtual space navigation. From 2015 to 2019, we examined 45 patients implanted in various areas of temporal, parietal, and frontal lobes with one or more of these tests. We developed an extensive package in the Matlab environment for analysis of the collected data. The package is available online under an open-source GPL license. It allows synchronization of EEG and behavioural data, positioning of the channel activity in the normalized brain, exclusion of epileptic activity, combining (across multiple patients) channels from similar brain areas and/or with similar response characteristics, time-frequency analysis by different methods, and statistical analysis. Using this iEEG technique, we focused on the role of various areas of the dorsal and ventral visual stream in discrimination of images of spatial scenes and small-scale objects. Previous fMRI studies revealed a spatial-scene processing brain network consisting of occipital place area, parahippocampal area, and retrosplenial complex, and object processing network in the lateral occipital complex area. In a manuscriptnow prepared for submission (Vlcek et al. 2020), we describe much more extensive networksof the scene- and object-selective brain areas. The

scene-selective regions also include the hippocampus, and the object-selective regions also include the anterior temporal lobe, many frontal lobe areas, and areas of the posterior parietal cortex.

Another field of human research developed in our department in collaboration with the National Institute of Health focuses on the role of sleep in spatial memory and navigation. Sleep has been shown to enhance memory consolidation in several behavioural paradigms and in different species. Beyond enhancing consolidation, sleep also makes memories more resistant to interference and facilitates detection of relationships hidden in the structure of a learned task. Several research articles reported more efficient spatial navigation after sleep with shorter travelled distances to goals and superior navigation by shortcuts in a previouslylearned route. One of our experiments tests our hypothesis that night's sleep will enhance integration of spatial representations independently acquired in a complex virtual
environment. In the other experiment, we compare the effect of sleep consolidation on allocentric and egocentric representations in a real-space analogy of Morris water maze and test the hypothesis that sleep positively affects particularly the allocentric memory supportedby medial temporal lobe structures.

Synthesis and current efforts

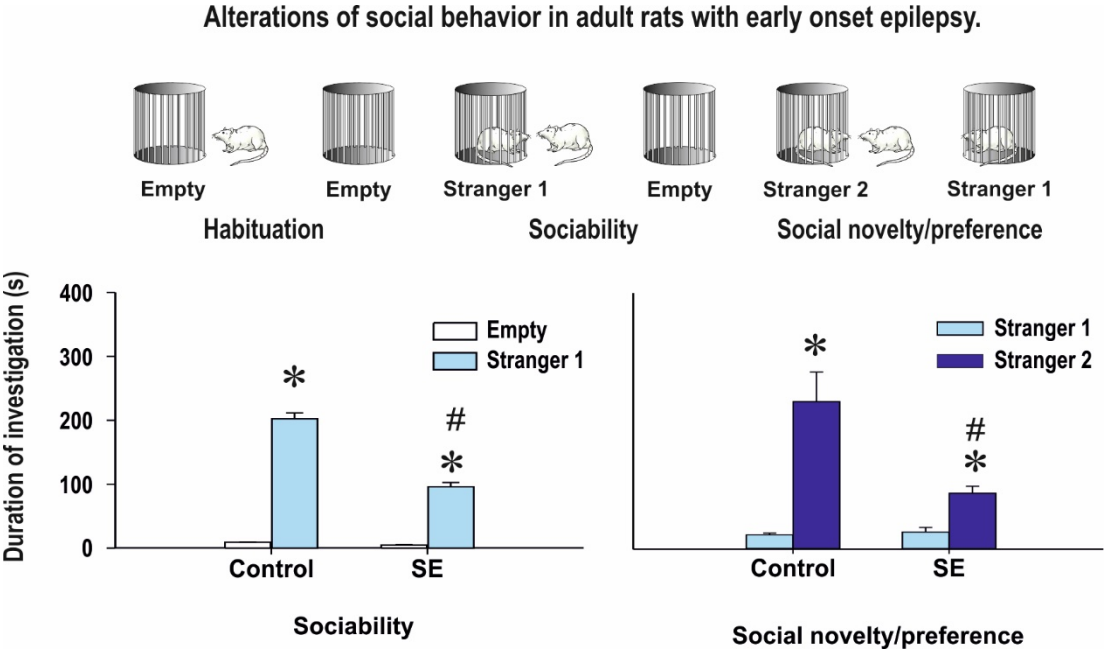
Despite inherently diverse experimental approaches, both animal and human studies converge on understanding basic mechanisms of cognition, both in the healthy and diseasedbrain. Research on animal models and human patients in one laboratory allows us to make more direct comparisons and facilitate translation to clinical practice. An example of this practice is the development of behavioural tests for patients based on their analogues used in animal research. Place avoidance on the rotating arena (Carousel) is a cognitive coordination-demanding task developed in the Laboratory in the late 1990s, which was successfully adapted for testing spatial competencies in humans, in particular for sensitive tests of selective deficits of spatial cognition in patients with MCI and schizophrenia. Another, more recent example is the aforementioned translational paper by Laczo et al. (2017), which validated place navigation to a hidden goal for AD drug screening. We put a great emphasis on the synthesis and integration of animal and human research. This integration is conveniently facilitated by advances in virtual reality techniques available in the lab, as well as the recent acquisition of scalp EEG system for the lab and setting a specialized room for testing healthy human volunteers at the IPHYS. Non-clinical academic institutions are not permitted to carry out studies on patients, hence project involving human patients are carriedout in cooperation with clinical settings including University Hospital Motol and National Institute of Mental Health in Klecany (see the section on collaborations).

Research activity and characterisation of the main scientific results

Mechanisms of ictogenesis, epileptogenesis and epilepsy-related comorbidities in the immature brain.

Comorbidities associated with early onset epilepsy. We have performed studies on pattern and development of behavioral comorbidities in a rodent model of early onset temporal epilepsy. Early onset epilepsy is known to be associated with many neuropsychiatric symptoms – autistic spectrum disorders, depression and cognitive impairment. Using a model of LiCl/pilocarpine-induced status epilepticus in infant (12-day-old) rats we have evaluated an integrative set of behavioral responses, including cognitive/sociocognitive and emotional dimensions. We have found that early onset epilepsy is associated with emotional/anxiety-related behavior and attention deficits, affects sociocognitive memory processing, and alters spatial learning in the MWM. Furthermore, neuropathological analysis performed 24h after the epileptogenic insult has shown selective brain damage that can lead to the remodeling of brain networks and consequently to functional deficits (Mikulecka et al, *Exp. Neurol.* **320**: 113005, 2019).

Support: the Czech Science Foundation 16-04726S and 19-11931S



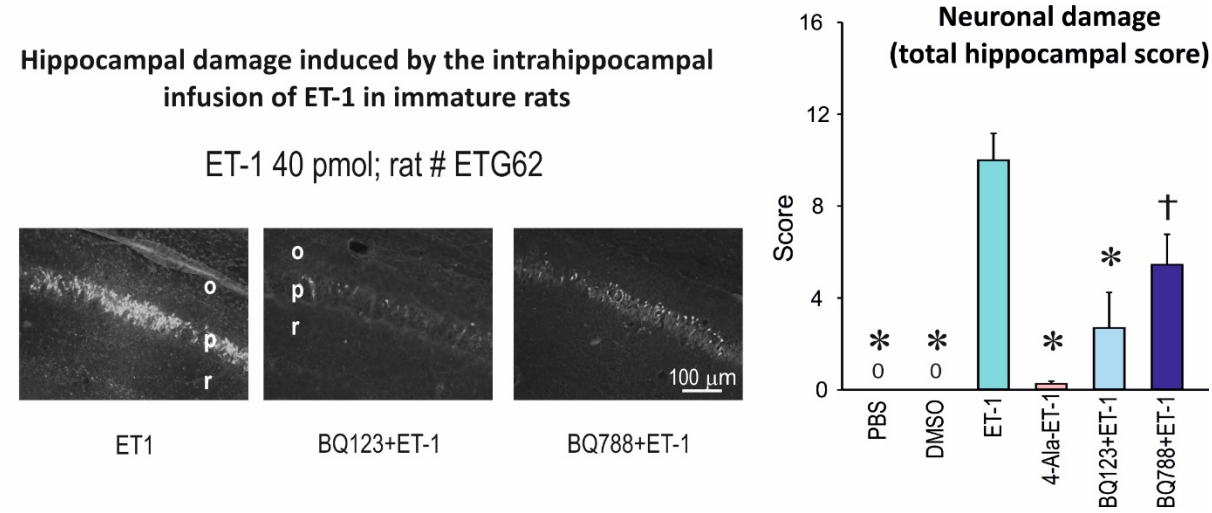
Team's contribution (95%): Experiments were designed, performed and data analyzed in Lab. Developmental Epileptology, Prof. Stuchlik and Prof. Druga participated in result interpretation.

The role of ET-1 and ET-2 receptors in development of seizures. We have examined the role of two endothelin receptors, ETA and ETB in the development of endothelin-1(ET-1)-induced ischemia and symptomatic seizures in infantile rats. Our results indicate that the activation of ETA receptors is crucial for the development of ischemia and neuronal damage,

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). Our recent research brings supporting evidence for neuromodulatory action of ET-1 in the brain and elucidates mechanisms responsible for seizure development after the endothelin receptor activation. The activation of ETB receptors results in development of non-ischemia related seizures associated with an inflammatory process resulting from an excess of leukotrienes (accepted for publication 2020, Tsenov et al, *Exp. Neurol.*).

Support: the Czech Science Foundation P304/12/G069 and P304/14/ 20613S

Team's contribution (95%)*:* Experiments were designed and performed in Lab. Developmental Epileptology. J. Burchfiel, Ph.D (Medical Center, University of Rochester) participated in EEG analysis and data interpretation



Hyperthermia aggravates long-term outcome of status epilepticus. For the first time our study has demonstrated deteriorating effects of hyperthermia (HT) on the outcome of status epilepticus (SE) in infantile rats. We have developed a model which generates the same seizure burden at two different temperatures and which allowed us to isolate the role of HT from other factors. Short-term HT during chemically-induced SE did not affect severity or duration of SE, but greatly increased the epileptogenicity of SE and its neuropathological sequelae later in life. Our results suggest that HT as a ‘‘second hit" to the ictal insult increases vulnerability to harmful effects of SE in infants and neonates. Since HT is easy to treat, a demonstration that correcting it reduces the epileptogenicity of SE might have significant therapeutic implications (Suchomelova et al, *Neuroscience* **305**: 209-224, 2015).

Support: the Czech Science Foundation: P302/10/0971, Ministry of Education, Youths and Sports: LC554

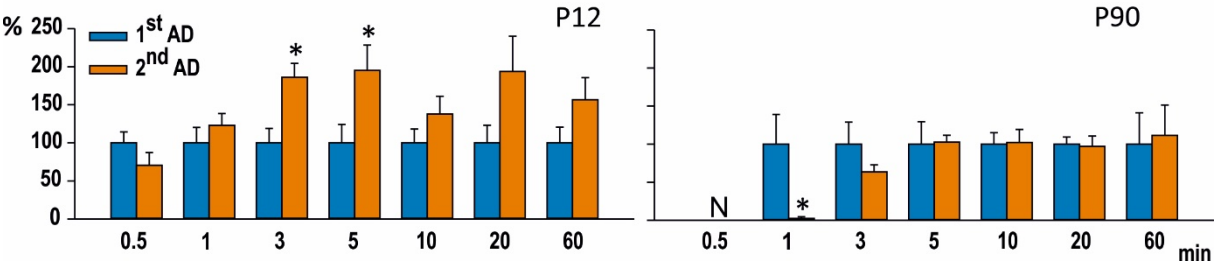
Team's contribution (20%). Member of Lab. Developmental Epileptology participated in study design, manuscript preparation and performed neuropathological analysis.

Age specific characteristics of postictal period in the immature brain. 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 studied the maturation 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). These findings at least partly explain the high incidence of SE in infants. Further, we have shown that refractoriness is due, at least in part, to activation of $GABA_B$ receptors (Mares and Kubova, *Neuropharmacology* **88:** 99-102, 2015). The refractory period could be suppressed by a $GABA_B$ antagonist but not by a $GABA_A$ antagonist. In addition, we have found that $GABA_B$ receptors participate in postictal refracteriness but not in postictal potentiation in very immature brain. Drugs affecting $GABA_B$ receptors might represent possible target for anticonvulsant therapy in developing brain only since a certain stage of maturation (Mares *Eur. J. Pharmacol.*, **818**:26-29, 2018).

Support: Ministry of Education, Youth and Sports US-Czech grant No. LH101, the Czech Science Foundation P302/10/0971, P304/12/G069 and Support: the Czech Science Foundation

Developmental patterns of postictal refractoriness and potentiation. Relative duration of the paired afterdischarges in 12-day-old and adult rats; the first one in the pair is always taken as 100%.

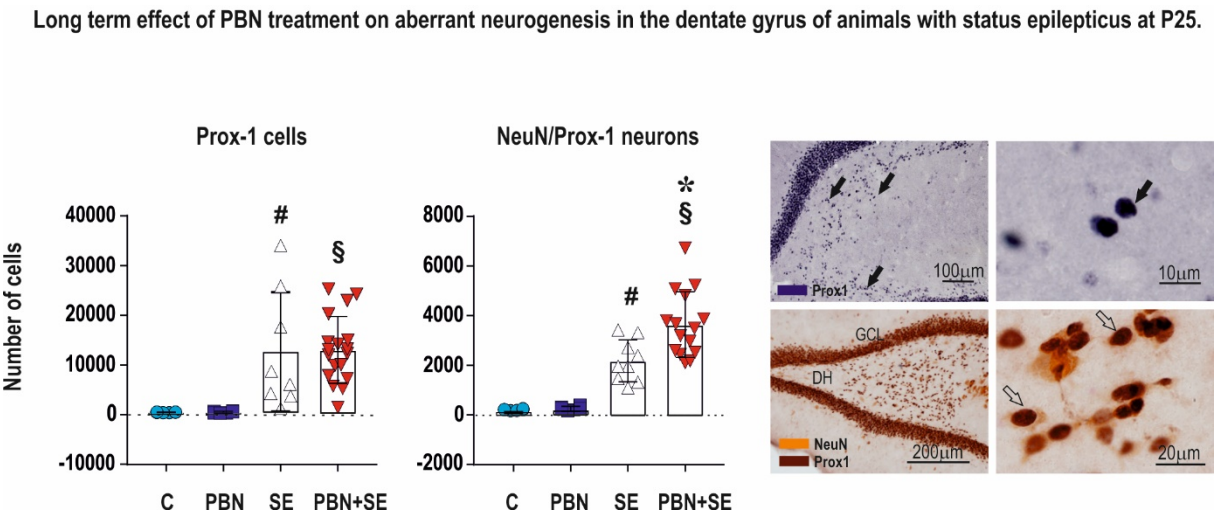


Molecular alterations induced by early status epilepticus (SE). We have demonstrated that in infantile animals (12-day-old) SE results in persisting increase of expression of GRIA2A mRNA and GluA2 protein of AMPA receptors in most of analyzed brain structures except of the ventral hippocampus. Increased expression can participate in the increased brain excitability during epileptogenesis, as has been documented in a model of electrically induced cortical afterdischarges in animals with SE at P12 (Mares and Kubova, *Epilepsia* **57**:e183–e186, 2016). In contrast, absence of GluA2 subunit allows Ca^{2+} permeability of AMPA receptors what might be interpreted as an endogenous anticonvulsant mechanism (Szczurowska et al, *Exp. Neurol.* **283**: 97-109, 2016).

Support: the Charles University Grant Agency No. 200107 and 2120139, the Czech Science Foundation 15-16605S and P304/12/G069

Team's contribution (84%). Study by Szczurowska was designed and performed in Laboratory of Developmental Epileptology, first and last authors are responsible for majority of the study.

Variable effects of free radical scavenging on the outcome of SE. Free radical scavenging is known to have neuroprotective effects. We have found that administration of N-tert-butyl-α-phenylnitrone (PBN) during SE has both beneficial and detrimental effects on its long-term outcome in juvenile animals. Beneficially, PBN protected against severe anatomical damage in the hippocampus and associated spatial memory impairment. Detrimentally, PBN treated animals had more severe seizures and emotionality impairment later in life. In addition, treatment significantly increased the number of ectopic neurons in the dentate gyrus. Aberrant neurogenesis may contribute to development of post-SE epilepsy and epileptic comorbidities. (Kubova et al., *Frontiers Cell. Neurosci.*, 12, 226, 2018).



Support: the Czech Science Foundation P304/12/G069 and Ministry of Education, Youth and Sports US-Czech grant15025

Team's contribution (95%). Study was designed and experiments performed in the Laboratory of Developmental Epileptology. Dr. Burchfiel (University of Rochester) was consulted as EEG expert and participated in manuscript preparation.

Developmental pharmacology of classical and potential anti-seizure drugs

Adenosine A1 receptors as possible therapeutic target in early life seizures. Using the model of epileptic afterdischarges induced by electrical stimulation, we have demonstrated the age-dependent change in anticonvulsant activity of an adenosine A1receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA). CPPA was most effective in animals younger than three weeks and in adults, whereas in animals 25 and 45-days-old drug was either ineffective or less effective. Higher efficacy of CCPA at early stages of development can be associated with increased expression of A1 receptor protein in the hippocampus. (Fabera et al., *Front. Pharmacol.* 14 June, 2019)

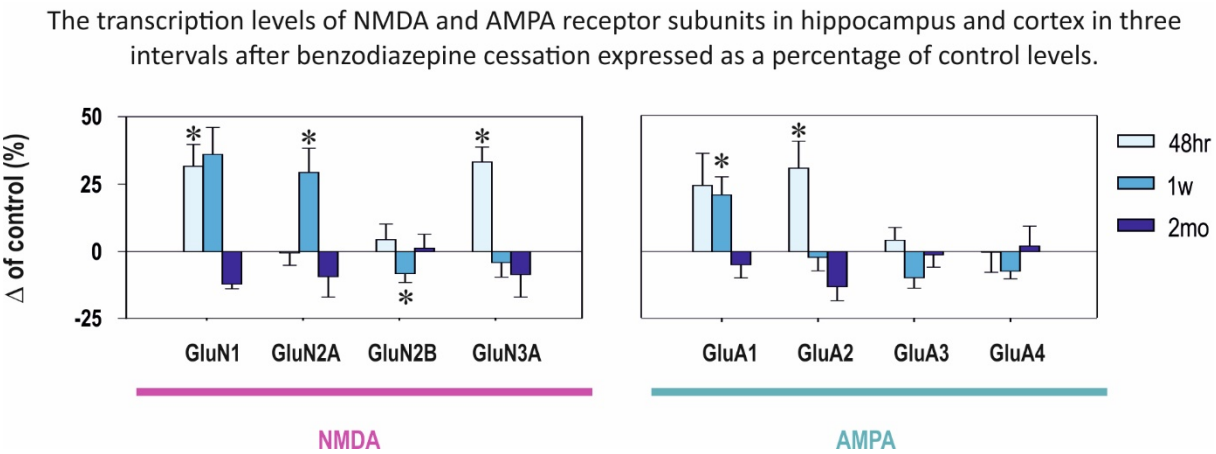
Support: the Grant Agency of the Charles University, Second Faculty of Medicine 2120192/2015 and European Regional Development Fund-Projects "PharmaBrain" No. CZ.CZ.02.1.01/0.0/0.0/16_025/0007444

Antagonists of glutamate receptors as possible antiepileptic drugs in immature brain. NR2B subunit is a major component in NMDA receptors at postnatal development. We have found that Ro 25-6981, highly selective antagonist of NMDA receptors with dominant NR2B subunit, exhibits marked anticonvulsant action in immature animals (Szczurowska and Mares, *Brain Res. Bull.* **111**: 1-8, 2015). We have also shown that IEM1460, a selective antagonist of AMPA receptors lacking GluR2 subunit and thus permeable for calcium ions, exhibits an anticonvulsant action in immature rats. (Szczurowska and Mares, *Epilepsy Res*. **109**: 106, 2015). Our data suggest that both types of glutamatergic receptors might be used as possible molecular target for development of age-specific antiepileptic drugs.

Support: the Czech Science Foundation GBP304/12/G069

Long-term impact of early brain insults on brain development.

Effects of early benzodiazepine exposure on glutamatergic receptors. In early development, GABA plays an important role in neuronal circuitry formation and exposure to GABAergic drugs during this developmental period affects cognition and social behavior later in life. We have found both short-term and persisting changes in NMDA and AMPA receptor subunit composition and receptor binding in response to short-term exposure to clonazepam during neonatal period in rats. Pattern of these changes depended on brain structure and interval after the end of administration (Kubova et al., *Front. Mol. Neurosci.*, **11**:382, 2018).



Support: the European Regional Development Fund-Projects "PharmaBrain" No. CZ.CZ.02.1.01/ 0.0/0.0/16_025/0007444, the Czech Science Foundation P304/12/G069

Team's contribution (35%). Team members were responsible for experimental design, preparation of animals and manuscript preparation. First (corresponding) and last authors are members of the Laboratory of Developmental Epileptology.

Long – term neonatal stress impair responsiveness to exteroceptive stimuli in adult rats. Early life traumatic events strongly alter the physiology and behavior in adult rats and early life stress closely associates with the development of psychological alterations and psychiatric disorders. We have studied effects of long-term neonatal stress on habituation of exploratory behavior and the investigatory response to a stimulus object in adult rats repeatedly separated from their dams between postnatal days 1-14. We have found that long-term neonatal stress affects habituation to experimental environment and impairs an ability to sustain attention to stimuli in adulthood (Holubova et al., *Behav. Processes* **149**: 59-64, 2018).

Support: the European Regional Development Fund-Projects "PharmaBrain" No. CZ.CZ.02.1.01/ 0.0/0.0/16_025/0007444,

Team's contribution (20%). Team member participated in design of experiment, data analysis and manuscript preparation.

Effects of NMDA receptor blockade during adolescence and early adulthood. In this study we have evaluated effects of NMDA receptor blockade with MK 801 on anxiety behavior and cognitive functions on two stages of maturation in two rat strains. Our results have shown no

significant changes in behavior in Wistar rats whereas anxiety-like behavior was observed in Long-Evants exposed to MK 801 during adolescence and impaired working memory in animals treated as young adults. NMDA receptor blockade however did not result in changes in NMDA receptor subunit composition in any experimental group (Uttl et al, *Front. .Pharmacol.*, **9**:42, 2018).

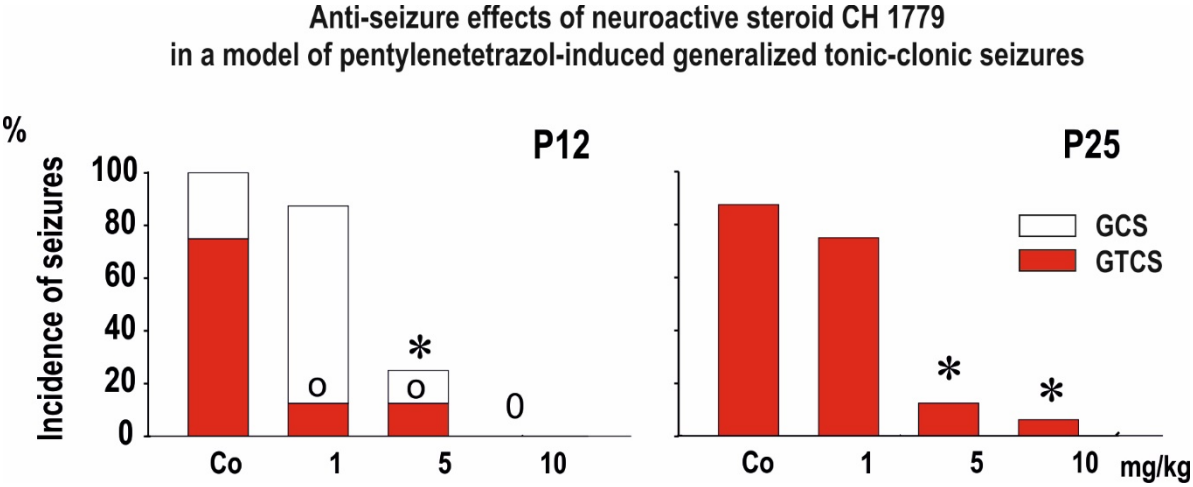
Team's contribution (20%). Team members participated in design of experiment and performed biochemical analysis.

Effects of social stress on glucocorticoid metabolism. In collaboration with *Dpt. Epithelial Physiology* we have shown that in mice microbiota modulates the response of peripheral components of hypothalamus-pituitary-adrenocortical (HPA) axis, colon and mesenteric lymphatic nodes to chronic psychosocial stress, a common aspect of modern life. The main novel finding is that microbiota shapes the stress response not only at the level of brain but also in peripheral tissues (Vodicka et al, *Brain, Behav. Immun.* 73:615-624, 2018). In addition, we have found that repeated social stress increases the local glucocorticoid production in lymphoid organs via corticosterone regeneration and this regeneration partially depends on the strain and organ. Fisher 344 and Lewis rats, which represent two ends of a spectrum of HPA axis responsiveness to stress and vulnerability to immune diseases, respond to repeated social stress, respond differently to repeated social stress (Ergang et al, *Endocr. Connect.* 7:1389-1396).

Team's contribution (15%). Team member designed and performed behavioral part of study.

Applied research – new drug development

Development of new antiepileptic neurosteroids. For several decades, our laboratory collaborates with pharmaceutical companies and provides testing of new antiepileptic drugs in several pediatric models of seizures, which have been developed or adopted in our laboratory for different developmental stages of rodents. At present, we participate in common project with Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences focused development on new neuroactive steroids with anticonvulsant potential. Among many drugs tested CH-1779 is very promising and highly effective in infantile animals. Now it is in the patenting process. In addition this compound was accepted by the National Institutes of Health in Bethesda, MD, for *The Antiepileptic Drug Development Program,* preclinical anticonvulsant screening project supported by US government. This common project will continue.



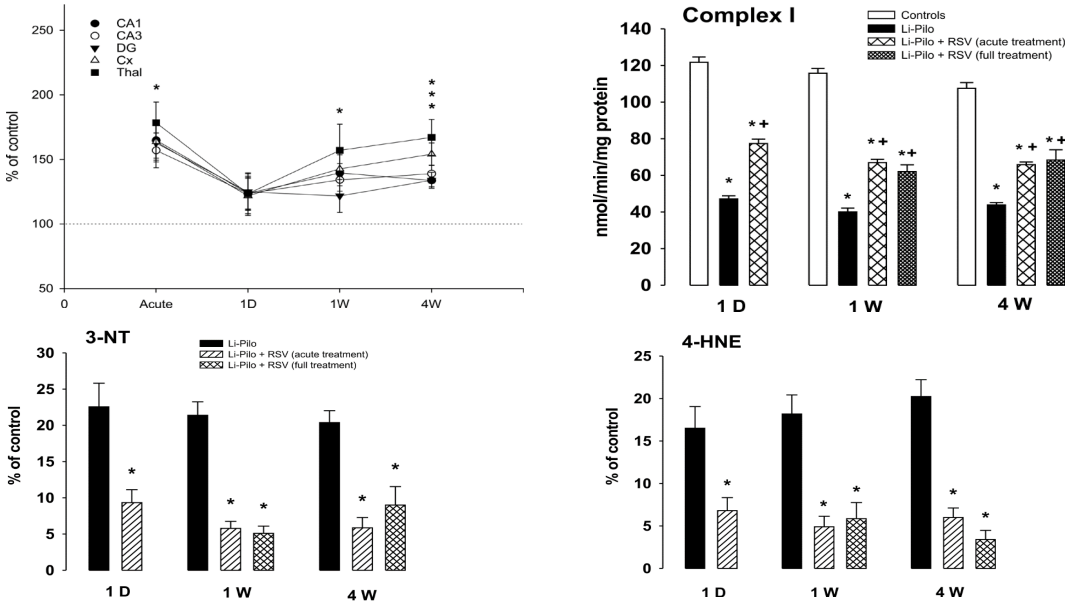
Team's contribution (50%). Team members designed and performed animals experiments.

Development of a new antiepileptic drug for pediatric epilepsies. In collaboration with a UCB we have tested a potential antiepileptic drug *radiprodil* (an antagonist of NMDA receptors containing NR2B subunit) in immature rats. A parallel clinical study was performed in the University Hospital in Paris and efficacy of this drug was demonstrated in both preclinical and clinical tests. (*Annals of Clinical and Translational Neurology,* accepted for publication)

Team's contribution (15%). Team member designed and performed PTZ study.

The role of oxidative stress and metabolic impairment in pathogenesis of epilepsy and seizures during the development

Although, it was concluded earlier by others that oxidative stress is not present in young brain during status epilepticus and thus should not be considered a general phenomenon, we brought evidence in 3 models that oxidative stress is present and plays important role in epileptogenesis (Folbergrová et al. *Front Cell Neurosci***. 10**:136, 2016). Several authors followed our work and designed antioxidant treatment as disease modifying therapy. We have further evaluated that oxidative stress and epileptogenesis itself could be diminished by treatment with a flavonoid resveratrol, a potent SOD mimetics and Nrf2 activator. We have concluded that resveratrol is capable to prevent oxidative damage during and after epileptogenic insult and thus positively interact with epileptogenesis in immature brain (Folbergrová et al. *Mol Neurobiol*. 55:7512-7522, 2018.). This work was awarded as one of the best posters of basic science session on European Congress on Epileptology in Vienna 2018 and reviewed (Kovács R et al, *Front Cell Neurosci*., **12**:335, 2018).

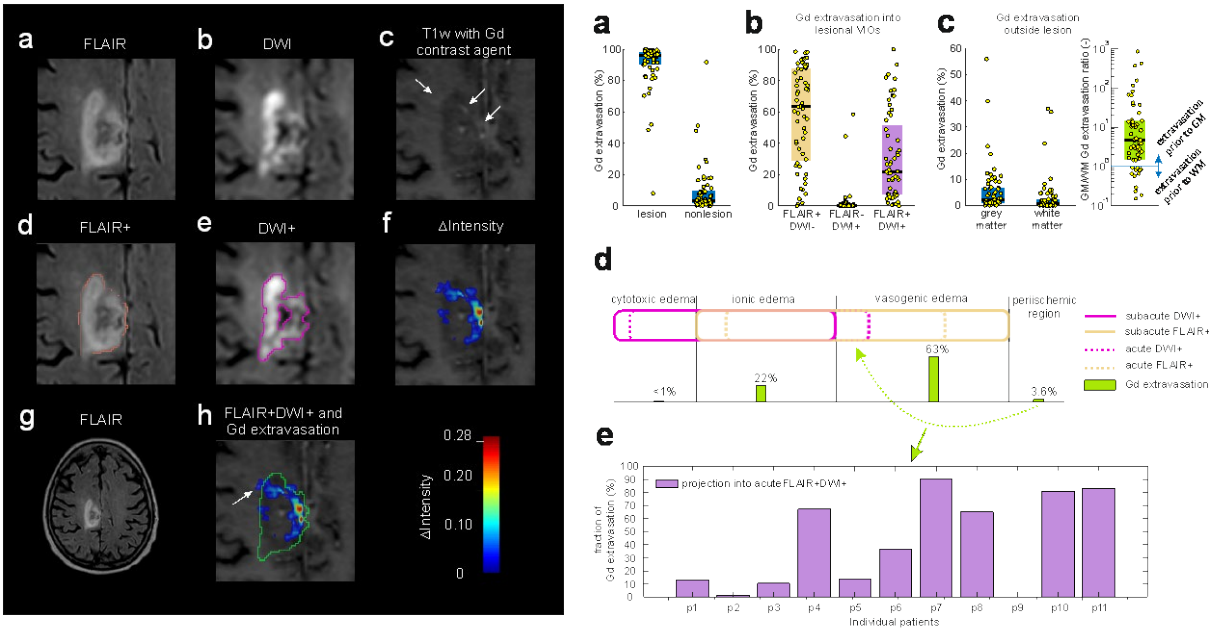


Support: Czech Science Foundation # 15-08565S and # 18-07908S

Team's contribution (95%)**:** Experiments were designed and performed in Dpt. Developmental Epileptology. Dr. Jesina (Dpt. Bioenergetics) performed measurement of the content of mitochondrial enzymes and participated in data interpretation.

Biomarkers of epileptogenesis

Epilepsy develops in ~5% of patients after stroke. We have extensively collaborated with Dpt.of Neurology of University Hospital Motol on seeking for neuroimaging biomarkers of epileptogenesis. We have acquired 300 patients suffering stroke and performed contrast MR to evaluate integrity of blood brain barrier (BBB). Using sophisticated analytical algorithms developed by our team we have shown that BBB is impaired in ischemic lesion but also in perilesional tissue (Kala et al. *Clinician and Technology* **47**:43–48, 2017, Moyer et al. *Epilepsia* **58** (Suppl 4):53-67, 2017). We have shown that 22% of extravasated gadolinium is present in region of ionic edema. Mechanism and temporal profile was experimentally tested with set of optical and radiological methods (Svoboda et al. *PhysRes* **68**:37-48, 2019). Posters of this project were selected as *Best clinical poster on European Congress on Epileptology* in Vienna 2018 and 5th Congress of the European Academy of Neurology in Oslo 2019.



Support: Czech Health Research Council #15-33115A

Team's contribution (95%)**:** The clinical data was obtained in University Hospital Motol and were exclusively analysed by the team of Dpt.of developmental epileptology. Experimental part was performed by our team utilizing two photon microscope in CzechBioImaging facility.

<u>Mechanisms of ictogenesis</u>.

We have performed an extensive interdisciplinary study that combined recording of epileptic activity in vitro in a model of seizures in brain slices, chronic model of temporal lobe epilep[sy and patients with implanted intracranial electrodes. This study brought major breakthroughs about the mechanism of seizure emergence and the role of brief interictal epileptiform discharges (IEDs) in seizure generation. These phenomena and processes are two of the most important unresolved issues in modern epilepsy research. We found that the transition to seizure is not a sudden phenomenon, but is instead a slow process that is characterized by the progressive loss of neuronal network resilience. From a dynamical perspective, the slow transition is governed by the principles of critical slowing, a robust natural phenomenon that is observable in systems characterized by transitions between dynamical regimes. In epilepsy, this process is modulated by synchronous synaptic input from IEDs. IEDs are external perturbations that produce phasic changes in the slow transition process and exert opposing effects on the dynamics of a seizure-generating network, causing either anti-seizure or pro-seizure effects. We found that the multifaceted nature of IEDs is defined by the dynamical state of the network at the moment of the discharge occurrence. Notably, elucidation of the governing dynamical principles of the transition to seizure explained the observed dichotomy of the complex role of IEDs on seizure genesis and allowed unification of antagonizing theories and observations. This study shows that merging complex information about dynamical processes at multiple spatial and temporal scales can bring us much closer to understanding the principles of seizure emergence and initiation, and possibly to a unified theory of ictogenesis in the epileptic brain to explain the existence of the various classes of seizure type and transitions to them.

Chang et al, Nat Neurosci, 21:1742-1752, 2018; Chvojka et al, Epilepsy & Behav, epub, 2019
Our team has designed and lead this research. Members of the team performed

electrophysiological recordings in vitro and in vivo. They developed and applied various techniques of data analysis to recorded data. They significantly contributed to the analysis of long-term intracranial recordings in human patients. Members of the team were involved in computational modeling and verification of theoretical predictions obtained from in silico studies.

Team's contribution Team contribution was 50%.

High-frequency oscillations and organization of epileptic networks

Kudlacek et al. Front Neurol. 2017, 8:687 High-frequency oscillations (HFOs) are a novel marker used to improve the delineation of epileptogenic tissue and, hence, the outcome of epilepsy surgery. Their practical clinical utilization is curtailed by the inability to discriminate them from physiological ones. We have explored whether physiological oscillations can be discriminated from pathological ones using the response to antiepileptic drug treatment. The results showed that physiological HFOs do not respond to drug treatment in contrast to epileptic HFOs. This knowledge increases the diagnostic potential of pathological oscillation opens the prospect for pharmacological discrimination between physiological and pathological oscillation during the presurgical examination.

Team's contribution *The members of the FGU team designed and coordinated the entire study and performed the vast majority of the experimental work and analyses. FGU team contribution was 90%.*

Ferecsko et al., Brain Struct Funct, 2015. We have provided important information about the mechanisms of pathological HFOs. In the tetanus toxin model of temporal lobe epilepsy, we have demonstrated that inhibition is absent in the area of tetanus toxin injection. An electrophysiological study demonstrated that this region is still capable of generating HFOs

Team's contribution *Team's Member of the FGU team has contributed to study design, performed electrophysiological recordings and data analysis. FGU contributions was 15 %.*

Morris et al., Front Neurosci, 2016, 10:519; 220(2):1013-29 We have described the role of interneurons in pathological HFOs and we have described their cellular firing dynamics during the episodes of HFOs. We show that during pathological HFOs with frequency from the ripple band, interneurons actively contribute to HFO genesis. For the purpose of this study, we have developed and tested a new technique for brain slice maintenance.

Team's contribution *Member of the FGU team contributed to study design and performed data analysis. FGU contributions was 15 %.*

Janca et al. Front Neurol. 2018, 23;9 We have explored the organization of the epileptic network in candidates of epilepsy surgery who underwent intracranial recording. We have demonstrated that the epileptic network is composed of multiple independent areas each with a distinct clinical significance. We developed an approach that increases the diagnostic yield of presurgical evaluation by identification of critical components of the epileptic network that must be resected to achieve a good surgical outcome.

Team's contribution *The members of the team designed the whole study and coordinated the interdisciplinary team which involved basic scientists, epileptologists, and bioengineers. Involved members significantly contributed to the data analysis and development of the diagnostic protocol. FGU team contribution was 30%.*

Research activity and characterisation of the main scientific results

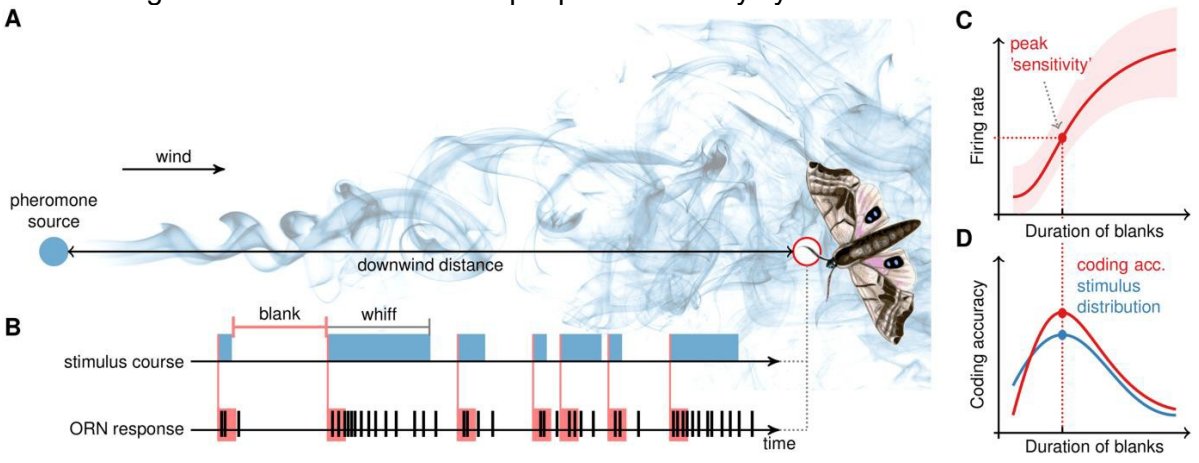
The department employs its own computer cluster (a Supermicro SuperServer rack system) for numerically intensive calculations. We own multiple licenses for MATLAB, PGI compilers, and the mathematical analysis software Mathematica (Wolfram Research). Much of our work is also done using open source computational tools (Linux, R, ImageJ / Fiji, Python). The department does not have its own experimental facilities. Our collaborators rely on the lab equipment in their own departments and on the central facilities provided by IPHYS and by the Krc campus.

The following is the description of the selected results achieved in 2015-19. Note that the team members acted as the first/corresponding authors of all of these results (in some cases the results originate entirely from the Lab), thus highlighting the major contribution of the Lab of Computational Neuroscience. Moreover, the results below were all published in the top-quartile journals (according to the impact factor ranking provided by the Web of Science) and journals PloS Comput. Biol.; eLife and Chaos are in the top decile of their respectivecategories.

The results were achieved thanks to the high success rate of the team in grant applications (GACR), promoting the intensive collaboration and contacts with our foreign colleagues. (2017-2019: Neural coding precision and its adaptation to the stimulus statistics (PI: L. Kostal), 2015-2017: Efficiency of information transfer and the role of energetic constraints in neuronal systems (PI: L. Kostal), 2014-2016: Biophysical modeling of axon fasciculation andtargeting due to adhesive interactions (PI: M. Zapotocky).)

Levakova M, Kostal L, Monsempes C, Jacob V, Lucas P (2018) Moth olfactory receptor neurons adjust their encoding efficiency to temporal statistics of pheromone fluctuations, PLoS Computational Biology, 14, e1006586:

We show that responses of antennal olfactory receptor neurons to pheromone encounters follow the temporal fluctuations in such a way that the most frequent stimulus timescales are encoded with maximum accuracy. The coding accuracy profile and the stimulus-timescale distribution are related in the manner predicted by the information theory for the many- to-one convergence scenario of the moth peripheral sensory system.

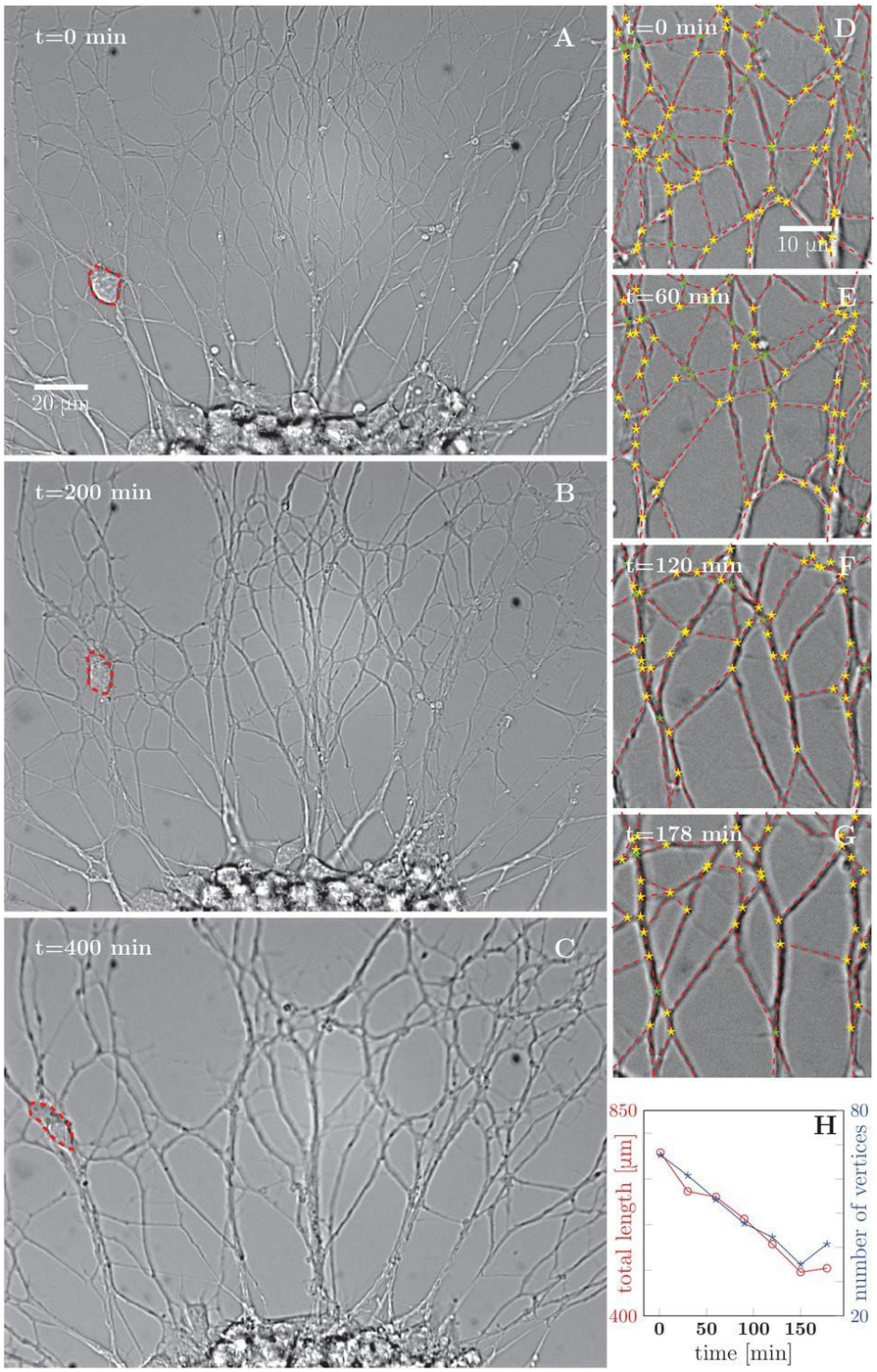


(A) Atmospheric turbulence governs the complicated non-homogeneous dispersion of a pheromone, which is detected by specialized olfactory receptor neurons (ORNs) located on the moth antennae (red circle). (B) A typical time course of the pheromone stimulation at a given distance from the source is intermittent. The signal consists of blanks, intervals of zero local concentration due to the passage of clean-air pockets, and of whiffs, intervals of pheromone presence. The statistics of blanks and whiffs describes the spatio-temporal structure of the turbulent plume. (C) A simple encoding model of a whiff encounter is given by the dependence of the firing rate (measured within a period after the whiff onset) on the

preceding blank duration, the duration-rate relationship. The coding sensitivity of the whiff encounter is determined from the slope of the mean response and the response variability. In order to detect the pheromone optimally, the efficient coding hypothesis predicts the ORN to adjust its encoding sensitivity to the local stimulus conditions by adjusting the duration-rate relationship. (D) We observe that encoding properties of ORNs are adjusted to match the local distribution of blank durations.

D. Smit, C. Fouquet, F. Pincet, M. Zapotocky, A. Trembleau: Axon tension regulates fasciculation/defasciculation through the control of axon shaft zippering, eLife 6:e19907 (2017).

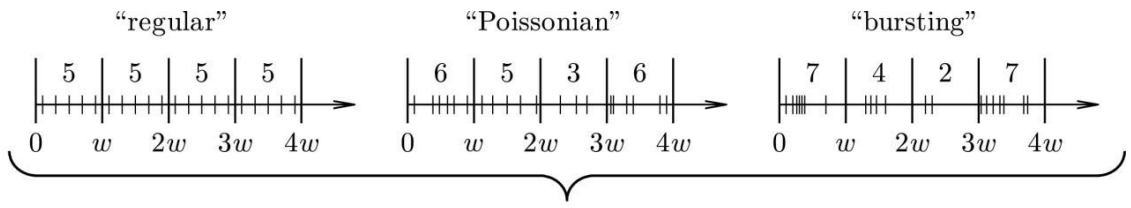
In this project, a wide range of approaches and techniques was used to analyze the adhesion-mediated biophysical mechanisms that regulate axon fasciculation and network formation. This work was achieved by the Prague team in collaboration with the laboratory of prof. Alain Trembleau in Paris. A key strength of the project was the close integration of theoretical and experimental approaches. Experiments were performed using explants of mouse embryonic olfactory epithelium. This type of neuronal cell culture turned out to providea convenient system in which we were able to analyze the dynamics of axon shaft interactions and the consequences for network evolution. Using mechanical micromanipulations, pharmacological approaches, and mathematical modeling, we showed that axon zippering / unzippering can be regulated by changes in the mechanical tension force within the axons. We also revealed how such zippering processes influence the structure and dynamics of fasciculated networks of axons, and we connected our framework to older literature in which indirect experimental evidence for axon zippering was obtained in vivo.

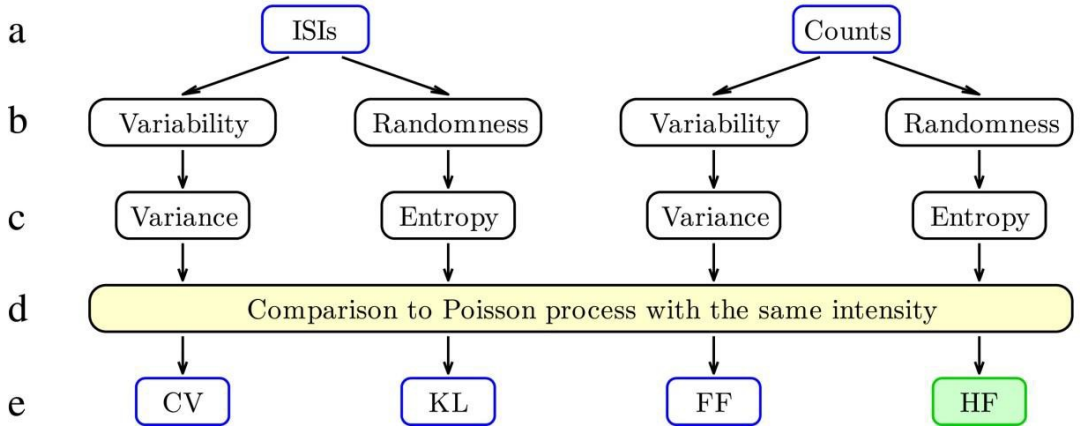


(A–C) Evolution of the axonal network growing from an explant during 400-min time lapse recording, after 2 days of incubation; the red dashed outline delineates a travelling ensheating cell. Progressive coarsening of the network and decrease of total length and density can be seen. (D–G) Red dashed lines outline the edges of the network, while yellow stars indicate junctions between axons or axon bundles, and green stars indicate crossings.
(H) Quantification of total length and number of vertices of the network area depicted in panels (D–G), as a function of time (based on seven manually segmented video frames).

Rajdl K, Lansky P, Kostal L (2017) Entropy factor for randomness quantification in neuronal data, Neural Networks, 95, 57-65

A novel measure of neural spike train randomness, an entropy factor, is proposed. It is based on the Shannon entropy of the number of spikes in a time window and can be seen as an analogy to the Fano factor. Theoretical properties of the new measure are studied for equilibrium renewal processes and further illustrated on gamma and inverse Gaussian probability distributions of interspike intervals. Finally, the entropy factor is evaluated from the experimental records of spontaneous activity in macaque primary visual cortex and compared to its theoretical behavior deduced for the renewal process models. Both theoretical and experimental results show substantial differences between the Fano and entropy factors. Rather paradoxically, an increase in the variability of spike count is often accompanied by an increase of its predictability, as evidenced by the entropy factor

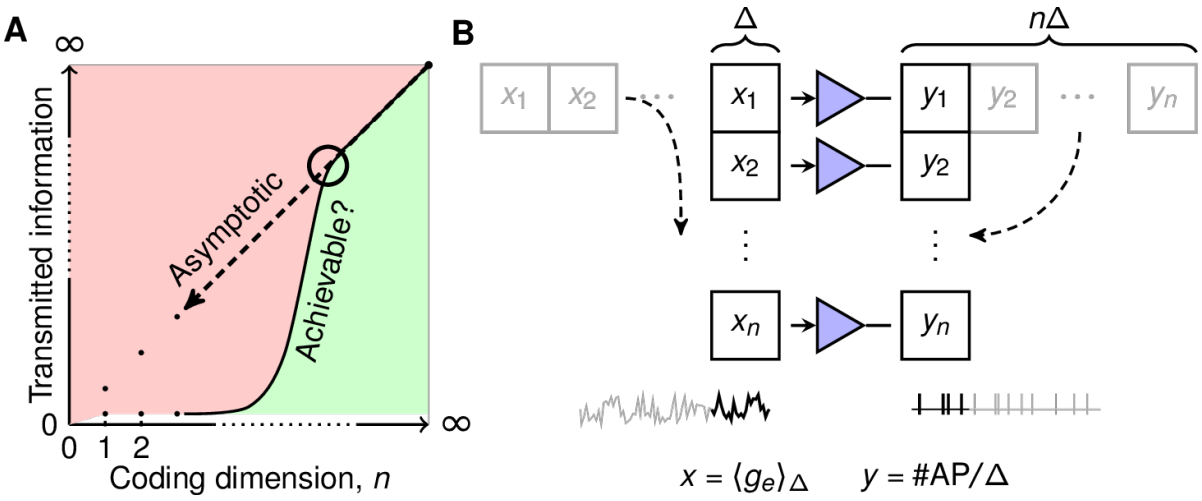




An overview of selected measures of intensity independent behavior of spike trains. (a) Two main ways how to describe spike trains—using ISIs or counts of spikes in a time window of length . (b) Two concepts how to understand variability and randomness. (c) Specific characteristics representing variability and randomness—variance and (Shannon) entropy. (d) Relating of given characteristics to a Poisson process of the same intensity. (e) Resulting measures—coefficient of variation (CV), Kullback–Leibler distance (KL), Fano factor (FF) and entropy factor (HF).

Kostal L, Kobayashi R (2019) Critical size of neural population for reliable information transmission, Physical Review E Rapid Communications, 100, 050401(R):

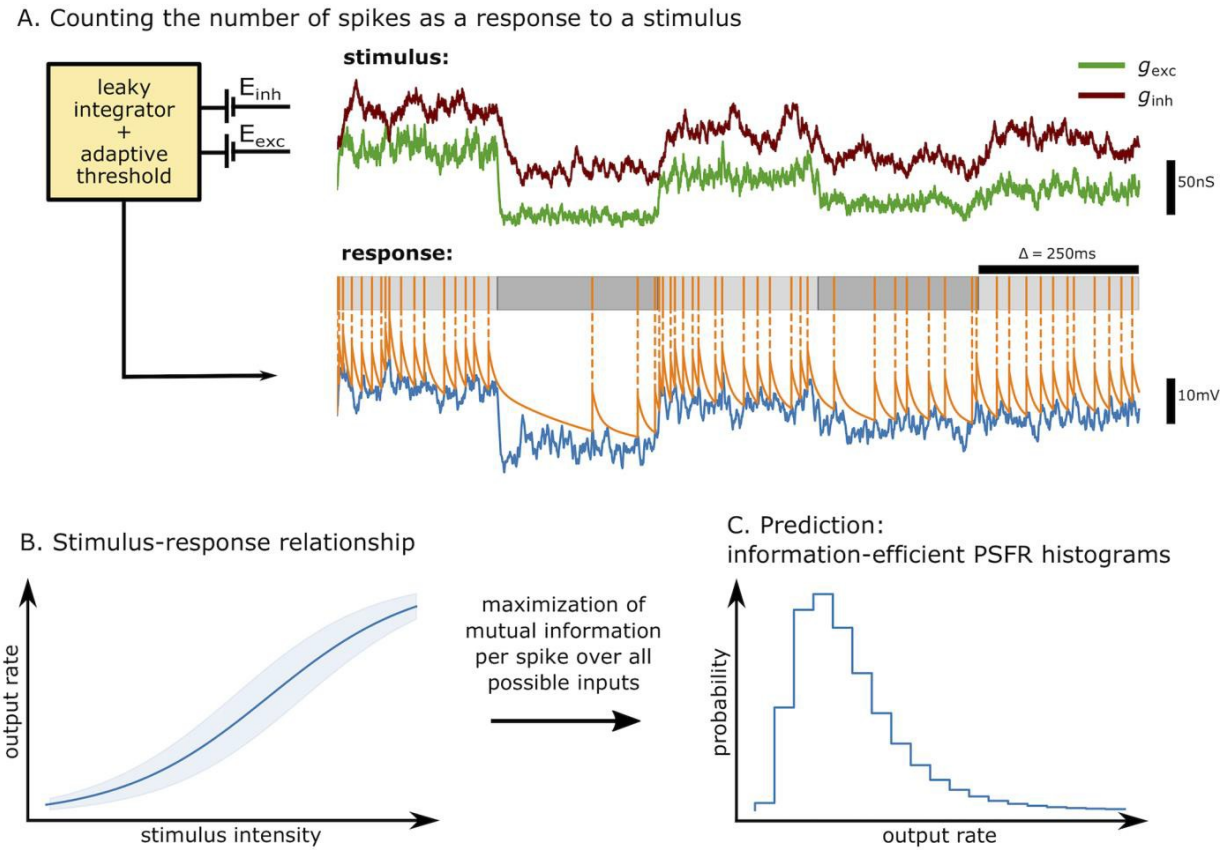
It is known that the probability of decoding error has a phase transition at the information rate equal to the channel capacity. The corresponding thermodynamic limit requires infinite coding dimension, hence making the actual decoding practically impossible. We analyse finite-size effects that occur in limited neural populations and report that the achievable rate approaches the asymptote in a remarkably nonlinear manner.



Framework and neural population setup. (a) Typical results of information theory are asymptotic in the coding dimension n. As n → ∞ it is possible to transmit information reliably up to rates equal to channel capacity per dimension (dashed line). However, the asymptotic limit is unlikely to be achieved with restricted n (schematic illustration). We report that there exists a critical n below which the performance deviates rapidly from the bound (circle). (b) Neuronal population model employed in this Rapid Communication. The input (x i) andoutput (y i) are discretized with time step Δ. For single neurons, Shannon's coding theorem assumes processing sequences of length n (coding dimension), resulting in delays (n Δ) during the sequence formation and readout. Instead we equivalently employ n identical neurons in parallel, using the Hodgkin-Huxley type model with point conductance input. Stimulus is the n vector of average excitatory conductance components; response is the vector of individual firing frequencies.

Barta T, Kostal L (2019) The effect of inhibition on rate code efficiency indicators, PLoS Computational Biology, 15, e1007545

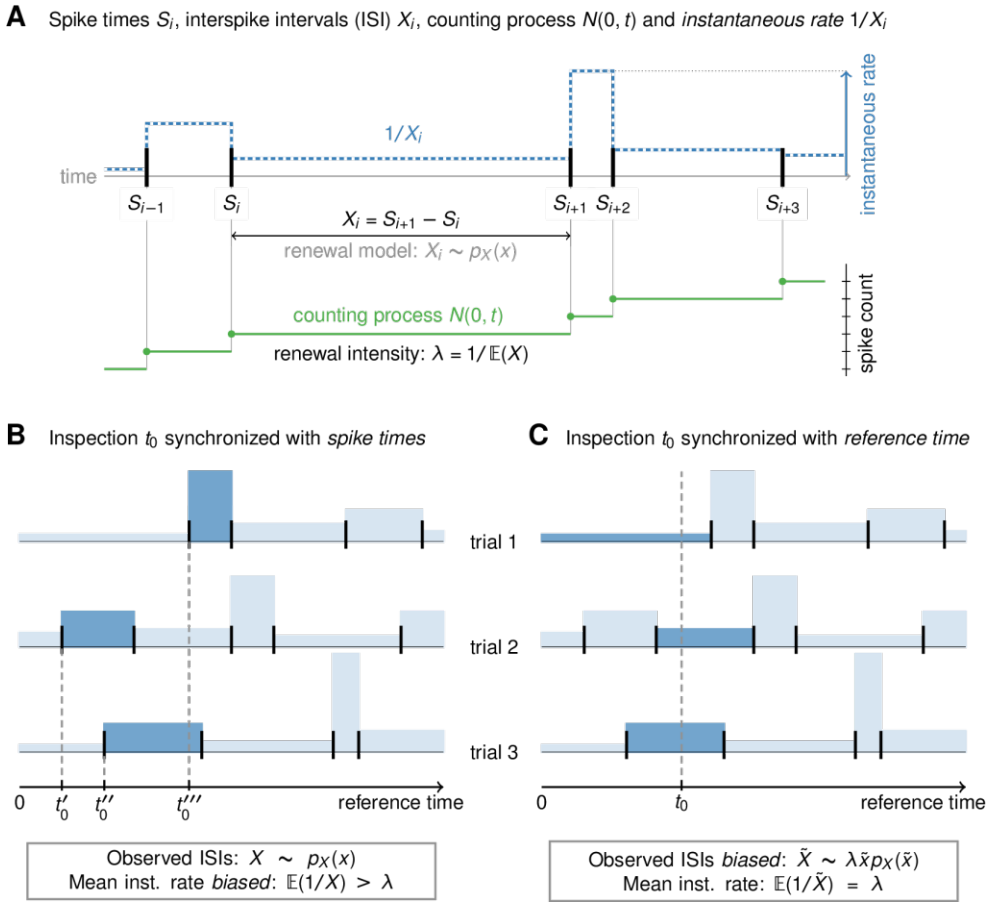
We investigate the rate coding capabilities of neurons described by the conductance-based leaky integrator model with adaptive threshold and parameter sets recreating biologically relevant spiking regimes. We find that, counter-intuitively, increased ratio of inhibition to excitation generally leads to higher signal to noise ratio (SNR). On the other hand, the inhibitory input significantly reduces the dynamic coding range of the neuron. We quantify thejoint effect of SNR and dynamic coding range by computing the metabolic efficiency and we predict the shapes of the post-synaptic firing rate histograms.



(A) Stimulus consisting of excitatory and inhibitory synaptic conductances, generated as shot noise with an exponential envelope, is delivered to the neuronal model, a passive leaky membrane with a dynamic threshold. The measured response is the number of spikes in a specified time window (e.g., 250 ms). (B) For each stimulus intensity the full response distribution is obtained. The mean response (solid) and its standard deviation (shaded) are shown for illustration. (C) We find the probability distribution of inputs that maximizes the mutual information between the stimulus and the response per single spike. The predicted histogram of post-synaptic firing rates (PSFR) can be compared with experimental data.

Kostal L, Lansky P, Stiber M (2018) Statistics of inverse interspike intervals: the instantaneous firing rate revisited, Chaos, 28, 106305

The rate coding hypothesis is the oldest and still one of the most accepted and investigated scenarios in neuronal activity analyses. However, the actual neuronal firing rate, while informally understood, can be mathematically defined in several different ways. These definitions yield distinct results; even their average values may differ dramatically for the simplest neuronal models. Such an inconsistency, together with the importance of ``firing rate'', motivates us to revisit the classical concept of the instantaneous firing rate. We confirmthat different notions of firing rate can in fact be compatible, at least in terms of their averages, by carefully discerning the time instant at which the neuronal activity is observed. Two general cases are distinguished: either the inspection time is synchronised with a reference time or with the neuronal spiking. The statistical properties of the instantaneous firing rate, including parameter estimation, are analysed and compatibility with the intuitively understood concept is demonstrated.



Neuronal spiking activity and the instantaneous firing rate. (A) Spikes arrive at times Si ; the corresponding interspike intervals (ISI) are denoted as Xi and the instantaneous firing rate is the inverse ISI (dashed). Under steady-state conditions, ISIs are assumed to be independent and identically distributed with probability density function (p.d.f). The pdf of possible values of the instantaneous firing rate depends critically on the inspection time t0 (start of observation).

Research activity and characterization of the main scientific results

The role of Pin1 in isoform-specific CRMP2A regulation and in Sema3A-dependent axon guidance.

During nervous system development, axonal growth is tightly regulated by an array of extracellular secreted and membrane-bound cues that interact with their receptors at active growth cones. These interactions trigger signaling cascades that alter microtubule dynamics, resulting in axonal growth, turn, stop, or retraction. While many extracellular cues and their receptors have been discovered within the last two decades, little is known about how the signaling cascades they trigger are integrated into a single unified response.

A key player in translating upstream signaling cascades into axon growth and collapse is collapsin response mediator protein 2 (CRMP2), a tubulin heterodimer-binding protein that promotes microtubule assembly and axon growth. Importantly, upon its CDK5, GSK-3β or Rho kinase-mediated phosphorylation, the affinity of CRMP2 to tubulin is reduced, which shifts the dynamic equilibrium of microtubules toward their disassembly. Consequently, stimulation of growing axons with Sema3A, which activates CDK5 leading to CRMP2 phosphorylation, promotes growth cone collapse.

An alternative splicing of Crmp2 gene has been recently shown to generate two isoforms that differ in their N terminus: CRMP2B and an ∼100-amino-acid-longer CRMP2A. Little is known about CRMP2A, which has been reported to localize in axons rather than dendrites and may be regulated by conformational changes.

Conformational changes may represent an important regulatory mechanism in axon guidance, as they enable a rapid change of protein activity, which is vital to ensure thecorrect response of a growing axon to its changing environment. Importantly, it was demonstrated that a unique prolyl isomerase Pin1, which specifically accelerates conversion pSer/Thr-Pro motifs in certain proteins can regulate signal transduction in multiple cellular cascades and pathological states in neurons linked to neurodegenerative disorders as Alzheimer's disease. But little is known about the function of Pin1 in healthy neurons and during development of the nervous system. Using a proteomics approach, we identified CRMP2A as a major Pin1 target in postnatal neurons and showed that it is the dominant isoform in the distal axons. We demonstrated that Pin1 stabilizes CDK5-phosphorylated CRMP2A and that Pin1 knockdown or knockout reduces CRMP2A levels specifically in the distal axons and inhibits axon growth, which can be fully rescued by Pin1 or CRMP2A expression. Next, we showed that Pin1 knockdown or nockout increases sensitivity to Sema3A-induced growth cone collapse *in vitro* and *in vivo*, leading to developmental abnormalities in axon guidance. Our results thus identified an important isoform-specific function and regulation of CRMP2A in controlling axon growth and uncovered Pin1-catalyzed prolyl isomerization as a novel regulatory mechanism in axon guidance.

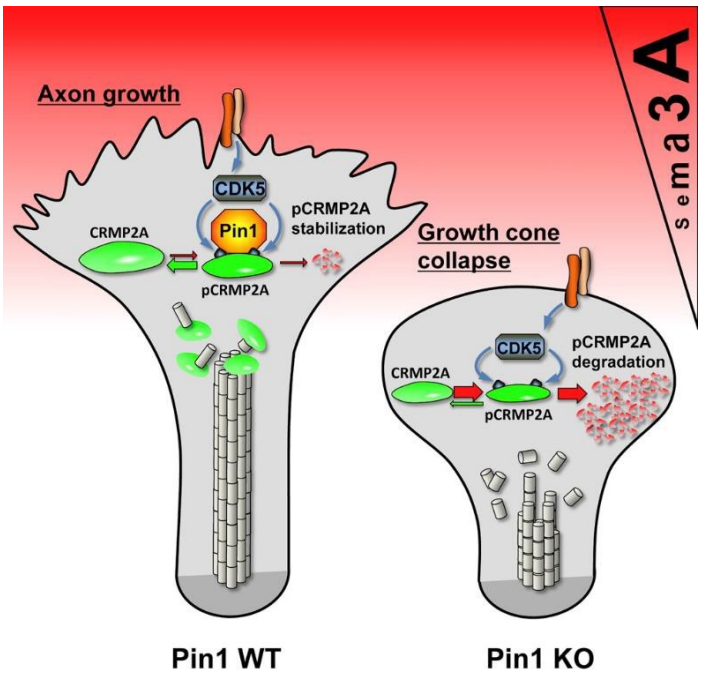


Figure 1. *Pin1 buffers low level of Sema3A stimulation in the WT distal axons by isomerization and stabilization of CDK5-phosphorylated CRMP2A. In the Pin1 KO neurons already low level Sema3A stimulation leads to CRMP2A degradation and collapse of the growth cones.*

The project was initiated during the postdoctoral training of Dr. Balastik in the laboratory of Prof. Kun Ping Lu at the Beth Israel Deaconess Medical Center, Boston and subsequently continued, and concluded in Dr. Balastik's laboratory at IPHYS. Dr. Balastik designed and performed most of the experiments with the contribution of his PhD students R. Weissova and J.Ziak at IPHYS. The work was published in 2015 in Cell Reports and Dr. Balastik is the first and the co-corresponding author of the publication.

CRMP2 as a mediator of Sema3F-dependent axon pruning and dendritic spine remodeling

The pattern of axonal connections is established during pre- and postnatal development by a cascade of multiple events. In embryogenesis, axonal growth cones are guided to their targets and multiple axon branches are formed. Since both correct and incorrect projections are formed, the embryonic brain connectome is only transient and the inaccurate connections are eliminated (pruned) in the early postnatal development. Defects in development and maturation of brain circuits have been linked to several neurodevelopmental disorders including autism spectrum disorder (ASD), schizophrenia, or epilepsy.

Generally, two types of pruning are recognized: (1) small-scale axon pruning, regulated by neural activity or trophic support and (2) large-scale stereotyped axon pruning, which is genetically predetermined. Stereotyped pruning can be further histologically divided into degeneration-like and retraction-like, which has been linked to secreted semaphorins and their coreceptors, e.g., plexin-A4 and plexin-A3. Intracellular mediators that transmit signals from plexins in axon pruning are not completely understood. One of the key molecule downstream of semaphorin 3A (Sema3A) signaling that directly interacts with cytoskeleton components is CRMP2.

Recently, CRMP2 deficiency in conditional knockout mice has been linked to schizophrenia due to changes in dendritic morphology (decreased spine number in CA1 neurons and layer 5 cortical neurons), behavioral changes (hyperactivity and social behavior impairment), and

prepulse inhibition (PPI) deficit. In addition to schizophrenia, deregulation of CRMP2 has been in humans associated with autism spectrum disorder (ASD), mood disorders, epilepsy, or Alzheimer's disease. Among these, ASD and schizophrenia are of special interest for their symptomatic proximity but distinct pathogenesis. The exact role of CRMP2 in the development of these conditions has so far been elusive. Although schizophrenia and ASD share some behavioral characteristics (e.g., decreased cognitive functions, impaired social skills, repetitive behavior), they differ in the timing of their onset (early childhood for ASD, lateadolescence for schizophrenia) and the nature of the underlying neuronal connectivity disorder. Whereas hypoconnectivity (lower number of dendrites, dendritic spines, general decrease of white matter) is often present in schizophrenia, ASD has been associated with local hyperconnectivity caused by either increased synapse formation or their incomplete pruning. Because CRMP2 is downstream of semaphorin signaling, which controls axonal pruning, and it has been linked to both schizophrenia and ASD, it is one of the prime candidates to regulate this process. However, the *in vivo* analysis of both conditional and full CRMP2 knockout mice has mainly focused on the dendritic phenotype and associatedbehavioral aspects. In addition, the role of CRMP2 in class 3 semaphorin signaling (other than Sema3A), which has been previously linked to defects in pruning and ASD (particularly Sema3F), is so far not known.

In order to characterize the function of CRMP2 in axon growth, guidance, and pruning and its role in class 3 semaphorin signaling *in vivo*, we generated CRMP2 full knockout mice (crmp2$^{-/-}$). We showed that CRMP2 participates in regulation of axon guidance in both central and peripheral nervous systems. In peripheral nervous system, deficiency of CRMP2 leads to mild overgrowth and increased branching of ophthalmic branch of trigeminal nerve and other selected peripheral nerves. In the central nervous system, we detected defects in postnatal callosal axon growth and guidance. Both of these systems are regulated by Sema3A, which was previously shown to induce CRMP2 phosphorylation and signaling *in vitro*. Indeed, we confirmed that primary motor and DRG neurons isolated from crmp2$^{-/-}$ micehave defects mediating Sema3A signaling.

Importantly, we showed that CRMP2 is essential also for synaptic refinement as crmp2$^{-/-}$ mice demonstrate defective stereotyped pruning of axons arising from hippocampus and visual cortex and inadequate elimination of dendritic spines in dentate gyrus (DG). Pruning inboth of these systems is dependent on Sema3F rather than Sema3A, and its defect is in accord with ASD rather than schizophrenia-like phenotype. In agreement with this hypothesis, we showed that CRMP2 is essential for Sema3F-induced axon retraction and dendritic spine remodeling in primary hippocampal cultures and that crmp2$^{-/-}$ mice sufferfrom ultrasonic vocalizations defect in early postnatal stages as well as social behavioral changes in adults linked to ASD.

In summary, we provided evidence that in addition to its role in Sema3A-dependent axon guidance, CRMP2 is a key mediator of Sema3F-dependent axon pruning and dendritic spine remodeling. Our data highlighted the importance of CRMP2 in neural circuit formation and refinement *in vivo* and demonstrated that its deficiency leads to defects in neural development associated with neurodevelopmental disorders, in particular ASD andschizophrenia.

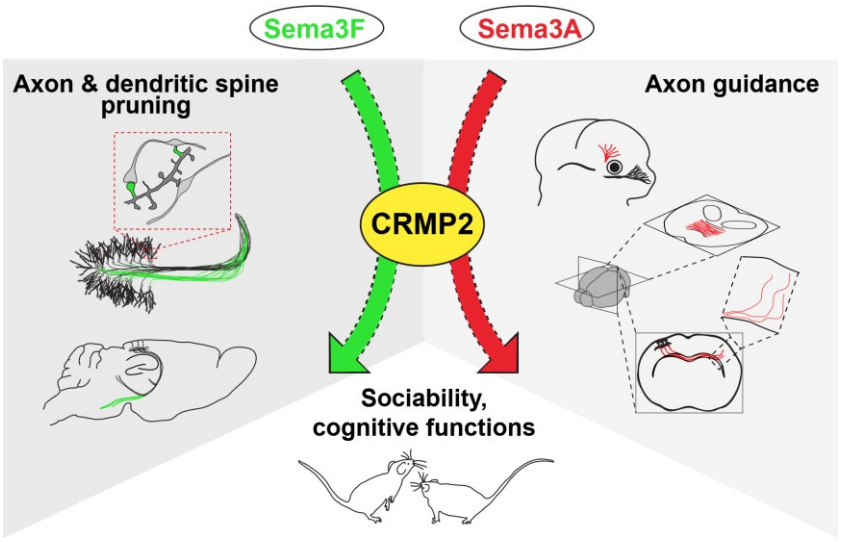


Figure 2. *CRMP2 mediates not only Sema3A-dependent axon guidance, but also Sema3F-dependent stereotyped axon pruning and pruning of dendritic spines. Crmp2$^{-/-}$ mice display behavioral cognitive defects and defects in sociability related to autism spectrum disorder.*

The project was designed and most of the experiments performed in the laboratory of Molecular Neurobiology at IPHYS. The first, second, and the last (corresponding) authors (in total 5 authors) are members of the laboratory.

Posttranslational modifications of tubulin in neurodegeneration

The microtubule cytoskeleton is a key structural component of neurons, where it carries out a multitude of specialized functions. Microtubules are essential for establishing and maintaining neuronal polarity, regulating neuronal morphology, transporting cargo, and scaffolding signaling molecules to form signaling hubs. Consequently, dysfunction of microtubules can lead to neurodevelopmental disorders as well as to neurodegeneration. The role of the microtubule cytoskeleton in neuronal dysfunction has been widely recognized; however, it was usually associated with abnormalities in a variety of microtubule-interacting proteins, the most prominent being the aggregation of the microtubule-associated protein tau in Alzheimer's disease. More recently, the discovery of patients with mutations in genes encoding the building blocks of microtubules—the α- and β-tubulins—revealed that alterations of the microtubules themselves could be causative for a range of neurodevelopmental and neurodegenerative disorders, most likely by subtly but significantly altering microtubule properties. It is thus conceivable that, in a more general manner, mechanisms involved in fine-tuning intrinsic microtubule properties and functions could play causative roles in neuronal dysfunctions.

One such candidate mechanism is dysregulation of tubulin posttranslational modifications (PTMs) that are expected to control many microtubule functions in cells, by either changing the physical properties of microtubules or by regulating their interactions with other cellular components. MTs can carry a large number of PTMs, such as acetylation, polyglutamylation, polyglycylation, and detyrosination. Polyglutamylation, a PTM that is particularly enriched on neuronal microtubules, adds variable numbers of glutamate residues as secondary branches to the main tubulin chain, thus generating a range of graded signals. Polyglutamylation has been expected to regulate intracellular trafficking but little evidence was present to support its role in molecular-motor tuning.

Several groups of tubulin-modifying enzymes have been identified recently. Cytosolic carboxypeptidases (CCPs) catalyze de-glutamylation, that itself was generated by polyglutamylases from the tubulin–tyrosine ligase-like (TTLL) family. Impaired deglutamylase activity was initially linked to neurodegeneration in a mouse model with early loss of the Purkinje cells in the cerebellum, the Purkinje cell degeneration (pcd) mouse. This mouse carries a mutation in the CCP1 gene, which causes accumulation of polyglutamylation in the cerebellum. However, whether deregulation of this PTM directly causes the degeneration of the Purkinje cells, whether impaired deglutamylase activity is universally deleterious to neurons, and which are the underlying molecular mechanisms have remained open questions. In a collaborative effort, we demonstrated that excessive polyglutamylation is a general, cell-autonomous cause of neurodegeneration that induces transport defects in neurons. We further show that by manipulating enzymes catalyzing polyglutamylation, it is possible to protect neurons against hyperglutamylation-induced neurodegeneration.

The project was initiated and most of it performed in the laboratory of Dr. C. Janke (Institute Curie, Orsay, France). Two members or the Laboratory of Molecular Neurobiology, J. Ziak and M. Balastik, performed histological analysis and quantification of cortical neurodegeneration in WT, $Ccp1^{-/-}$, $Ccp6^{-/-}$, and $Ccp1^{-/-}Ccp6^{-/-}$ mice, contributed to the analysis of the degeneration of cerebellar Purkinje cells and to writing of the article. The workhas been published in EMBO Journal, 2018 and the significant contribution of the team members resulted in the third author position and the second senior author position for J.Ziak and M. Balastik, respectively.

Research activity and characterisation of the main scientific results

List of selected projects supported by grant agencies 2015-2019:

Grant #LL1204 (within the ERC CZ program) from the Ministry of Education, Youth and Sports of the Czech Republic in 7/2012-6/2017 Genetic analysis of mitochondrial proteome: Integration of mitochondrial-nuclear epistasis with pathophysiological phenotypes in the rat (PI Pravenec)

Grant 14-36804G from the Grant Agency of the Czech Republic in 2014-2018, Centre of mitochondrial biology and pathology (MITOCENTRE) (PI Houštěk, package leader Pravenec)

Grant 16-04859S from the Grant Agency of the Czech Republic in 2016-2018, Genetic and correlation analyses of lipidome in rat model of metabolic syndrome for identification of disease biomarkers (PI Pravenec)

Grant AP1502 from the Czech Academy of Sciences, Academic Premium (Praemium Academiae) in 2016-2021, Molecular-based hemodynamic mechanisms of salt-dependent hypertension (PI Pravenec)

The above mentioned projects were focused on two major areas, mechanisms regulating hemodynamic and cardiac traits (A) and mechanisms regulating metabolic traits (B)

A. Mechanisms regulating hemodynamic and cardiac traits

Identification of mutant Wars2 gene encoding mitochondrial tryptophanyl-tRNA synthetase as a determinant of cardiac angiogenesis in the SHR

Impaired coronary blood flow causes heart disease, affecting millions of people worldwide. In the absence of coronary artery disease, coronary flow (CF) is largely determined by capillary vessel density. In our previous experiments, we studied the genetic control of CF andidentified a CF locus on rat chromosome 2, which was associated with cardiac angiogenesis. The spontaneously hypertensive rat (SHR) harbors mutant mitochondrial tryptophanyl-tRNA synthetase (*Wars2*) gene, encoding an L53F protein variant within the ATP-binding 'HXGH motif'. To investigate the effects of *Wars2*, we generated a *Wars2* targeted rat on the BN (wild-type *Wars2*) background using zinc finger nucleases (ZFN). Homozygous deletion($Wars2^{-/-}$) was embryonic lethal. In an attempt to avoid embryonic lethality, we crossed BN ($Wars2^{-/+}$) to SHR (*Wars2*L53F/L53F) to generate F1 animals that were genetically identical apart from the *Wars2* locus: F1($Wars2^{+/L53F}$) or F1($Wars2^{-/L53F}$), respectively. Given the loss- of-function associated with the L53F allele, we reasoned that the F1 ($Wars2^{-/L53F}$) would represent a compound hypomorph as compared with the F1($^{Wars2+/L53F}$). Histological analyses of the heart revealed that F1($Wars2^{-/L53F}$) rats had fewer and smaller capillary vessels than the F1($Wars2^{+/L53F}$) controls, in keeping with our data from the F2 mapping and congenic strains. These data confirmed that *Wars2* loss-of-function is causally related to diminished cardiac angiogenesis and reduced CF.
We also measured mitochondrial proteosynthesis on two types of cells primary fibroblast cultures from SHR and SHR-*Wars2* congenic rats and on primary cultures of neonatal cardiomyocytes. In both cases, there was a general increase in mitochondrial proteosynthesis in SHR-*Wars2* congenic rats, as would be expected for moderate ARS2 defect that affects mitochondrial proteosynthesis in general. It has reached significance for different bands in each experimental system (ND4, CYTB in fibroblasts, COX1, COX3 in cardiomyocytes). Presumably, this reflects number of Trp residues in individual protein subunits, as significant differences were observed for proteins with highest number of tryptophanes (17x in COX1, 12x in COX3, 13x in ND4, 10x in CYTB).
In human genome-wide association studies, the WARS2 locus was associated with cardio-

metabolic phenotypes that were also linked to the VEGFA locus. Intriguingly, genetic studies in the mouse implicated *Wars2* in capillary formation in the skin. The WARS2(L53F) variant we identified is associated with reduced enzymatic activity, which can unveil non-canonical effects of ARSs that are often tissue specific. Overall, our data show that WARS2 is a novel determinant of angiogenesis in the heart and other tissues perhaps acting as an integrator of pro-angiogenic signalling, directing cell motility and division to enable endothelial cell migration and proliferation.

Published:
Wang M, Sips P, Khin E, Rotival M, Sun X, Ahmed R, Widjaja AA, Schafer S, Yusoff P, Choksi PK, Ko NS, Singh MK, Epstein D, Guan Y, Houštěk J, Mráček T, Nůsková H, MikellB, Tan J, Pesce F, Kolář F, Bottolo L, Mancini M, Hübner N, Pravenec M, Petretto E,MacRae C, Cook SA. Wars2 is a determinant of angiogenesis. Nat Commun 7:12061, 2016

Validation testing of the Quantitative Cardiovascular Physiology-2005 and HumMod-3.0.4 mathematical models of human integrative physiology

In 1972, Guyton and colleagues published a model of the cardiovascular system that incorporated more than 150 variables. The most evolved derivative of the 1972 Guyton model is a large, multi-scale model called “Quantitative Cardiovascular Physiology-2005 or QCP 2005” and “HumMod” that contains over 8000 independent variables. In reviewing the literature on the 1972 Guyton model and the most evolved derivatives of the Guyton model, we could not find validation studies testing the capacity of the models to accurately predict sodium balance, cardiac output, and vascular resistance responses of normal subjects to increases in dietary salt in humans. Accurate quantitative characterization of the physiologic responses to salt loading in normal control subjects is a prerequisite for accurately determining which physiologic responses to salt loading are abnormal in salt sensitive subjects.
For validation testing, the predictions of the computer models were plotted against 2 human experimental studies. A model was considered to fail validation testing when the salt-induced change predicted by the model fell outside the 95% confidence intervals (Cis) of the mean of the salt-induced changes observed in human studies (Schmidlin et al., Hypertension 58:380-385, 2011; Ishii M et al., Jpn Heart J 24:79-90, 1983).

<u>Contemporary computer models fail to accurately predict sodium balance responses to short-term salt loading in normal humans</u>
With respect to predicting usual sodium balance responses to short-term increases in salt intake in normal humans (normotensive, salt-resistant subjects), both computer models (HumMod 3.0.4 and QCP 2005) failed validation testing. Figure 1 shows that both model simulations vastly underestimate the cumulative amount of sodium that is usually retained in response to switching from a very low NaCl intake of about 30 mmol per 70 kg body weight per day to a high salt intake of about 250 mmol per 70 kg body weight per day in normal subjects.

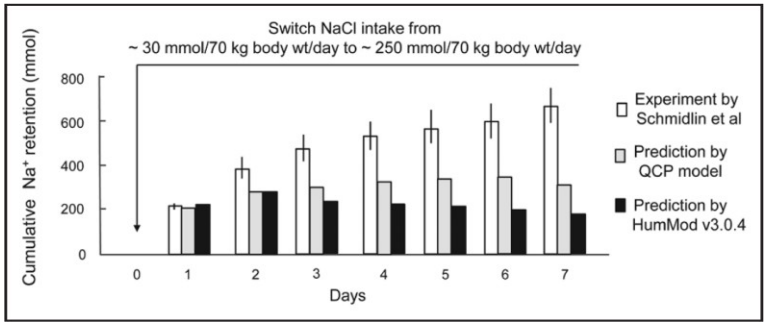


Figure 1 Experimental and predicted short-term effects on cumulative sodium balance induced by switching from a very low-salt diet to a high-salt diet.

Computer model predictions of arterial blood pressure responses to short-term salt loading in normal humans

Neither QCP nor HumMod accurately predicted reduction in mean arterial pressure that is transiently induced on switching normal subjects from a very low-salt diet to a high-salt diet. Both models accurately predicted that in normal subjects, switching from a very low salt diet to a high-salt diet for 5 to 7 days causes minimal increases in blood pressure above baseline.

Contemporary computer models fail to accurately predict cardiac output and vascular resistance responses to short-term salt loading in normal humans

The simulation results from HumMod 3.0.4 markedly overestimate the usual salt-induced increases in cardiac output (Figure 2) and markedly overestimate the usual salt-induced decreases in systemic vascular resistance (Figure 3). The simulation results from the QCP model significantly underestimate the usual salt-induced increases in cardiac output and salt-induced decreases in systemic vascular resistance (Figures 2 and 3). However, both models appropriately reflect the general trends in these variables that normally occur in response to physiological salt loading. Specifically, both models show that in normal subjects, nonextreme salt loading induces relatively little or no increase in blood pressure because normal subjects usually undergo robust vasodilation and reduce systemic vascular resistance sufficiently to offset the potential pressor effects of substantial salt-induced increases in cardiac output. These observations are also consistent with the vasodysfunction theory of salt sensitivity, which holds that in response to salt loading, normal subjectsundergo substantial decreases in systemic vascular resistance that offset potential pressor effects of salt-induced increases in cardiac output, whereas salt-sensitive subjects do not.

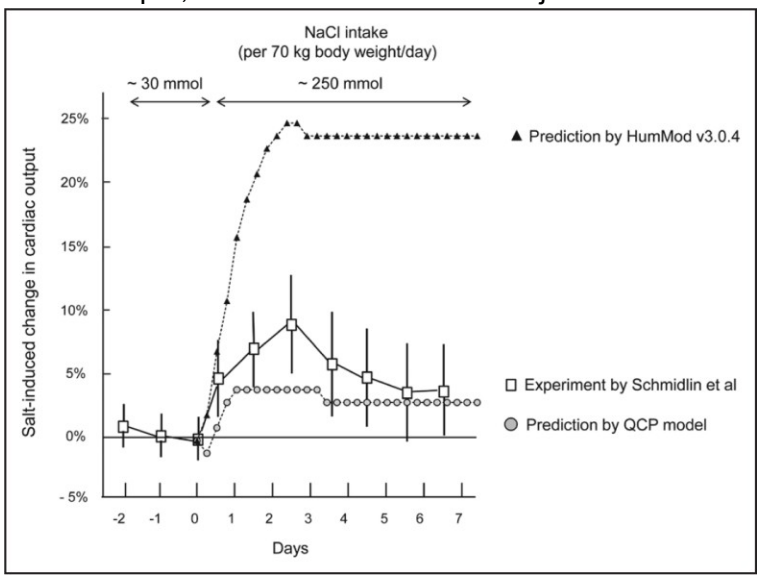


Figure 2 Experimental and predicted short-term changes in cardiac output induced by switching from a very low-salt diet to a high-salt diet.

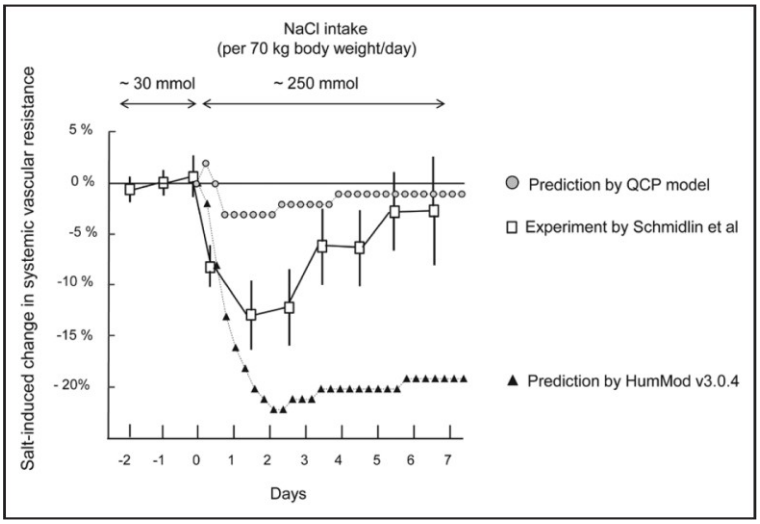


Figure 3 Experimental and predicted short-term changes in systemic vascular resistance induced by switching from a very low-salt diet to a high-salt diet.

Conclusions. The present findings underscore the value of validation testing of mathematical models to assess the accuracy of mechanistic hypotheses inherent in the models. We believe that future models should follow the lead of HumMod 3.0.4 and other models by not incorporating the tautological concept in the early Guyton models, which holds that, for a given level of salt intake, the pressure-natriuresis relationship (curve) determines the chronic level of arterial pressure.

Published:
Kurtz TW, DiCarlo SE, Pravenec M, Ježek F, Šilar J, Kofránek J, Morris RC Jr. Testing computer models predicting human responses to a high-salt diet. Implications for understanding mechanisms of salt-sensitive hypertension. Hypertension 72:1407-1416, 2018 (Editorial commentary: Beard DA. Assessing the validity and utility of the Guyton model of arterial blood pressure control. Hypertension 72:1272-1273, 2018)

The effects of beetroot juice or nitrate supplementation on salt sensitive hypertension

Many theories have been proposed with respect to possible mechanistic pathways involved in the pathogenesis of salt sensitivity. For example, a considerable body of evidence has emergedwhich indicates a role for nitric oxide (NO)-related pathways in determining vascular resistance responses to salt-loading that mediate salt sensitivity and salt resistance. The importance of endothelial dependent increases in nitric oxide activity in determining the normal reductions in renal vascular resistance and systemic vascular resistance that protect against salt-inducedincreases in blood pressure has been suggested. Many factors could cause salt sensitivity by interfering with endothelial dependent increases in nitric oxide activity involved in normal renal vasodilatory responses to increases in salt intake.
An additional source of NO, independent of eNOS (endothelial nitric oxide synthase) activity, is represented by dietary nitrate. Some studies have found that the consumption of flavonoid-richfruit and vegetables, arginine, and nitrate/nitrite supplementation can restore impaired endothelial function. In recent years, the root vegetable *Beta vulgaris rubra*, otherwise known as red beetroot has attracted much attention as a health promoting functional food. The recent interest in beetroothas been primarily driven by the discovery that sources of dietary nitrate may have important implications for managing cardiovascular health. Hypertension in particular has been the target of many therapeutic interventions and there are numerous studies that show beetroot, delivered acutely as a juice supplement, significantly reduces systolic and diastolic blood pressure. Therefore we tested the effects of beetroot supplementation on salt sensitive hypertension in SS (Salt sensitive Dahl) rats which are

extremely sensitive to salt loading.
Figure 4 presents the time course of 24-hour averages of systolic arterial pressure (panel A) and diastolic arterial pressure (panel B) for the 3 experimental groups. Salt loading rapidly induced substantial increases in arterial pressure that were attenuated by treatment with sodium nitrate or beetroot. Administration of sodium nitrate in a molar ratio of added nitrate to added salt of about 1:170 significantly protected against salt-induced increases in systolic arterial pressure as did administration of beetroot and salt which provided a nitrate to salt ratio of about 1:110 (panel C). Administration of sodium nitrate or beetroot also attenuated salt-induced increases in diastolic blood pressure, but after adjustment for multiple comparisons, the effect achieved statistical significance only in the beetroot-treated group (panel D).

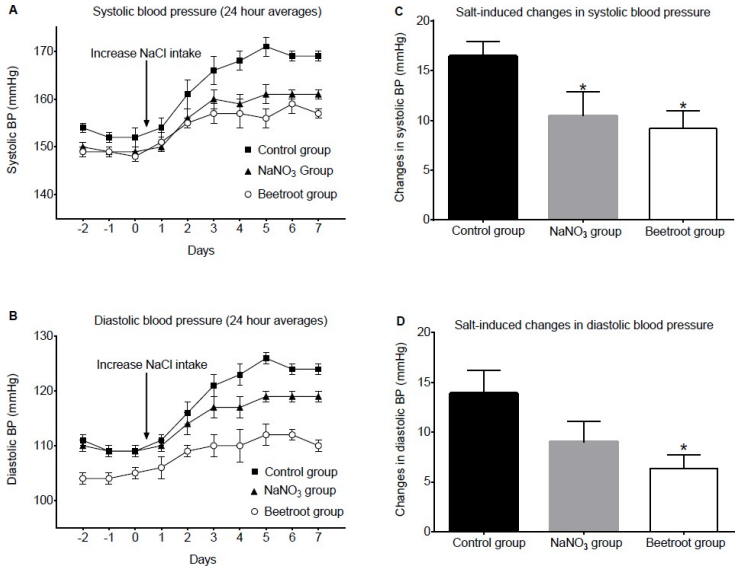


Figure 4 Effects of supplemental sodium nitrate or beetroot on salt-induced increases in blood pressure. A. Time course of 24-hour averages of systolic arterial pressure. B. Time course of 24-hour averages of diastolic arterial pressure. C. Mean changes in systolic arterialpressure induced by salt loading. D. Mean changes in diastolic arterial pressure induced by salt loading.
To test the effects of sodium nitrate supplementation on sodium balance, we conducted a separate metabolic study in which rats treated with salt plus sodium nitrate were compared with rats treated with salt alone. The amount of sodium retained by rats given salt plus sodium nitrate was significantly greater than that retained by rats given salt alone (results not shown). This observation suggests that the capacity of nitrate to attenuate salt-induced increases in blood pressure does not necessarily require the attenuation of salt-induced increases in sodium balance.

Published:

Morris RC Jr, Pravenec M, Šilhavý J, DiCarlo SE, Kurtz TW. Small amounts of inorganic nitrate or beetroot provide substantial protection from salt-induced increases in blood pressure. Hypertension 73(5):1042-1048, 2019

B. Mechanisms regulating metabolic traits

Nrf2-mediated antioxidant defense and peroxiredoxin 6 are linked to biosynthesis of palmitic acid ester of 9-hydroxystearic acid

Fatty acid esters of hydroxy fatty acids (FAHFAs) are lipid mediators with promising anti-diabetic and anti-inflammatory properties that are formed in white adipose tissue (WAT) via *de novo* lipogenesis, but their biosynthetic enzymes are unknown. Using a combination of lipidomics in WAT, QTL mapping and correlation analyses in rat BXH/HXB recombinant inbred strains (derived from SHR and BN progenitors), and response to oxidative stress in murine

models, we elucidated the potential pathway of biosynthesis of several FAHFAs.
Comprehensive analysis of WAT samples identified ~160 regioisomers documenting the complexity of this lipid class. The linkage analysis highlighted several members of Nuclear factor, erythroid 2-like 2 (*Nrf2*)-mediated antioxidant defense system (*Prdx6*, *Mgst1*, *Mgst3*), lipid-handling proteins (*Cd36*, *Scd6*, *Acnat1*, *Acnat2*, *Baat*) and family of Flavin Containing Monooxygenase (*Fmo*) as the positional candidate genes. Transgenic expression of *Nrf2* and deletion of *Prdx6* genes resulted in reduction of palmitic acid ester of 9-hydroxystearic acid (9-PAHSA) and 11-PAHSA levels, while oxidative stress induced by an inhibitor of glutathione synthesis increased PAHSA levels nonspecifically.
Our results indicate that the synthesis of FAHFAs via carbohydrate-responsive element-binding protein (ChREBP)-driven *de novo* lipogenesis depends on the adaptive antioxidant system and suggest that FAHFAs may link activity of this system with insulin sensitivity in peripheral tissues.

Published:
Kuda O, Březinová M, Šilhavý J, Landa V, Zídek V, Dodia C, Kreuchwig F, Vrbacký M, Balas L, Durand T, Hübner N, Fisher AB, Kopecký J, Pravenec M. Nrf2-mediated antioxidant defense and peroxiredoxin 6 are linked to biosynthesis of palmitic acid ester of 9- hydroxystearic acid. Diabetes 67(6):1190-1199, 2018

Downregulation of Plzf gene ameliorates metabolic and cardiac traits in the spontaneously hypertensive rat

The spontaneously hypertensive rat (SHR) is the most widely used model of essential hypertension. Under special environmental conditions, the SHR develops disturbances of lipid and glucose metabolism. In addition, the SHR is predisposed to cardiac hypertrophy and fibrosis. Recently, we identified spontaneous mutation in *Plzf* (promyelocytic leukemia zinc finger) candidate gene for quantitative trait loci (QTL) on chromosome 8 associated with blood pressure, cardiac hypertrophy and fibrosis as well as with metabolic traits usingcongenic mapping in SHR.PD minimal congenic sublines and sequence analyses. In particular, we found an intronic deletion in a putative *Plzf* enhancer associated with the downregulation of *Plzf* expression, amelioration of cardiac hypertrophy, fibrosis and a modestdecrease of blood pressure in the SHR.PD5 minimal congenic subline compared to SHR. The *Plzf* gene was also prioritized because previous studies indicated its possible role in left ventricular hypertrophy and fibrosis. In the current study, we generated a null *Plzf* allele inthe SHR using TALEN (transcription activator-like effector nuclease) mediated gene targetingin an attempt to replicate the previous congenic subline findings and to assess in vivo effects of *Plzf* on metabolic and cardiac traits. Our aim was to identify the cardiac hypertrophy/fibrosis and blood pressure QTL as well as metabolic regulatory QTL at the molecular level. Our results provide new evidence for a significant role of *Plzf* in the regulation of metabolic and cardiac traits in the SHR.

Parameters of glucose and lipid metabolism. When compared to SHR wild type controls, the SHR-*Plzf*$^{+/-}$ rats exhibited reduced body weight and relative weight of epididymal fat. In addition, the SHR-*Plzf*$^{+/-}$ heterozygotes showed lower serum and liver triglycerides, lower serum and liver cholesterol, whereas levels of serum insulin and glucose levels were similar to controls. SHR-*Plzf*$^{+/-}$ rats exhibited significantly increased sensitivity of adipose and muscle tissue to insulin action when compared to wild type controls and were more tolerant to glucose during OGTT (Figure 6).

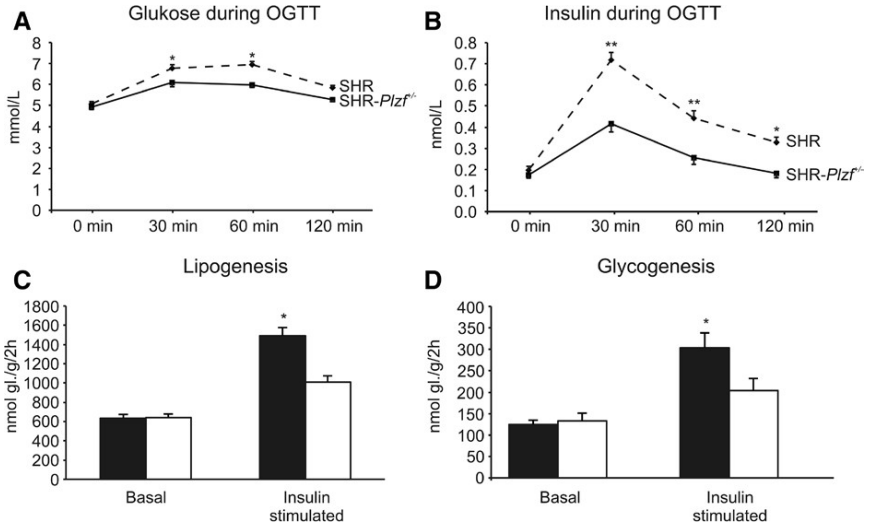


Figure 6 Effects of reduced expression of wild type Plzf on parameters of glucose and lipid metabolism. A. Oral glucose tolerance test (OGTT) in SHR-*Plzf*$^{+/-}$ versus SHR controls(dashed lines). Glucose concentrations after glucose loading were modestly reduced in SHR-*Plzf*$^{+/-}$ compared with control rats at 30 and 60 min after glucose loading. B. Insulin concentrations after glucose loading were prominently reduced in SHR-*Plzf*$^{+/-}$ compared with SHR controls. C. Basal and insulin stimulated lipogenesis. SHR-*Plzf*$^{+/-}$ rats (solid bars)showed significantly higher insulin-stimulated incorporation of radioactively labeled glucose into adipose tissue triglycerides when compared with SHR rats (open bars). D. Basal and insulin-stimulated glycogenesis. SHR-*Plzf*$^{+/-}$ rats (solid bars) showed significantly higher insulin-stimulated incorporation of radioactively labeled glucose into skeletal muscle glycogen when compared with SHR rats (open bars). *P < 0.05, **P < 0.005.

Cardiomyocyte hypertrophy, interstitial fibrosis, and blood pressure. The SHR-*Plzf*$^{+/-}$ heterozygous rats versus wild type controls showed significantly reduced left ventricular mass index (0.207±0.004 vs. 0.244±0.008 mg/100 g body weight, P<0.001), cardiomyocyte hypertrophy and interstitial fibrosis (Figure 7). There were no significant differences in systolic and diastolic blood pressures and heart rates measured by telemetry (data no shown).

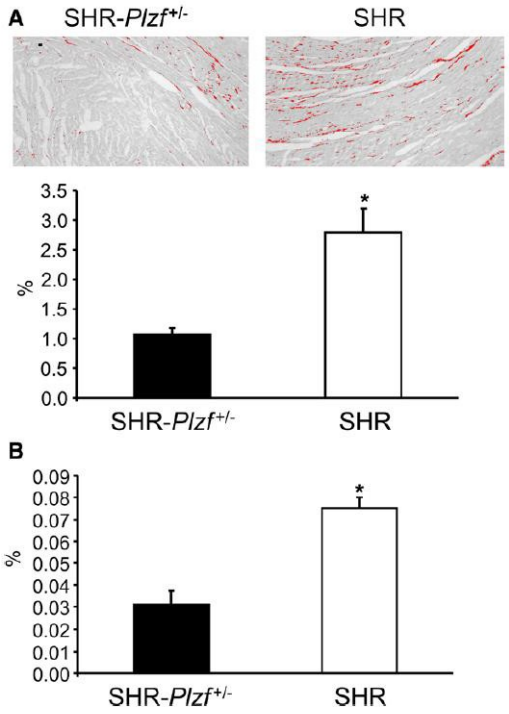


Figure 7 Interstitial fibrosis and cardiomyocyte size reduction in SHR-*Plzf*$^{+/-}$ and SHR rats. A. Interstitial fibrosis (the collagen fraction over myocardial area) is clearly discernible in a section stained with Sirius Red in SHR, much less pronounced in SHR-*Plzf*$^{+/-}$ rats. B. Cardiomyocyte size (the ratio between nuclear area and sarcoplasmic area) was significantlyreduced in SHR-*Plzf*$^{+/-}$ rats when compared to SHR controls. * and ** denote P<0.01 and P<0.0005, respectively.

Published:
Liška F, Landa V, Zídek V, Mlejnek P, Šilhavý J, Šimáková M, Strnad H, Trnovská J, Škop V, Kazdová L, Starker CG, Voytas DF, Izsvák Z, Mancini M, Šeda O, Křen V, Pravenec M. Downregulation of *Plzf* gene ameliorates metabolic and cardiac traits in the spontaneously hypertensive rat. Hypertension 69(6):1084-1091, 2017

Genetic variation in renal expression of folate receptor 1 (Folr1) gene predisposes spontaneously hypertensive rats to metabolic syndrome

Mild hyperhomocysteinemia, a common finding in patients with arteriosclerosis, has been described as a possible component of metabolic syndrome. Because folates and B vitamins modulate metabolism of homocysteine and of other sulfur amino acids (eg, cysteine, methionine, and cystathionine), mild hyperhomocysteinemia may be mediated by nutritional or genetically determined deficiencies of these vitamins. There is a growing body of epidemiological and clinical evidence for an important role of folate deficiency in human metabolic disturbances and hypertension. In this study, in the BXH/HXB recombinant inbred (RI) strains derived from the SHR strain and the normotensive Brown Norway (BN) strain, we used a combination of linkage and correlation analyses of physiological traits and gene expression levels to investigate genetic factors influencing folate and sulfur amino acid metabolism and risk for multiple features of the metabolic syndrome.
We mapped a quantitative trait locus for cysteine concentrations to a region of chromosome 1 that contains a *cis*-acting expression quantitative trait locus regulating mRNA levels of folate receptor 1 (*Folr1)* in the kidney (Figure 8A). Sequence analysis revealed a deletion variant in the *Folr1* promoter region of the SHR. Transfection studies demonstrated that the SHR promoter region of *Folr1* is less effective in driving luciferase reporter gene expression than the Brown Norway promoter region of *Folr1* (Figure 8B). Results in the SHR.BN-chr.1 congenic

strain confirmed that the SHR variant in *Folr1* cosegregates with markedly reduced renal expression of *Folr1* (Figure 8C) and renal folate reabsorption, decreased serum levels of folate, increased serum levels of cysteine and homocysteine, increased adiposity, ectopic fat accumulation in liver and muscle, reduced muscle insulin sensitivity, and increased blood pressure. Transgenic rescue experiments performed by expressing a *Folr1* transgene in the SHR (Figure 8D) ameliorated most of the metabolic disturbances. These findings are consistent with the hypothesis that inherited variation in the expression of *Folr1* in the kidney influences the development of the metabolic syndrome and constitutes a previously unrecognized genetic mechanism that may contribute to increased risk for diabetes mellitus and cardiovascular disease.

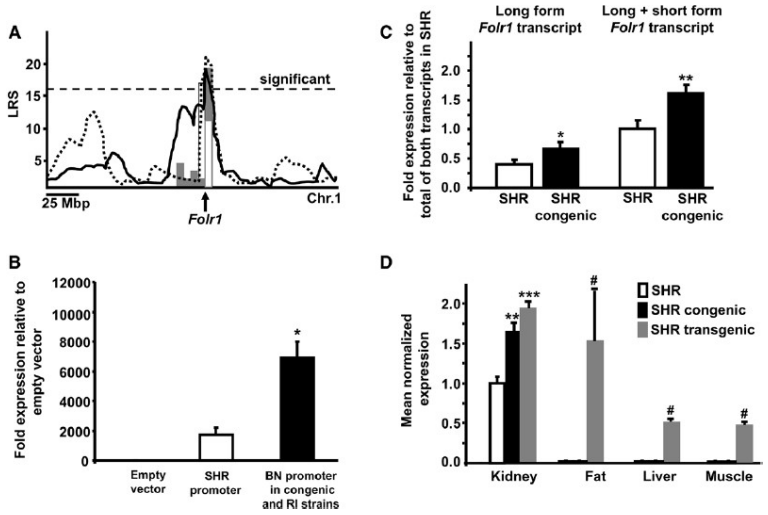


Figure 8 Linkage mapping and gene expression studies. A, Interval mapping of plasma cysteine (solid line) and renal *Folr1* mRNA abundance (dotted line) on chromosome 1 in recombinant inbred (RI) strains. B, *Folr1* promoter analysis. Luciferase reporter gene assay showed lower transcriptional activity of the *Folr1* promoter region of the spontaneously hypertensive rat (SHR) strain compared with the Brown Norway (BN) *Folr1* promoter region present in the SHR congenic strain and the RI strains. C, Renal expression of the *Folr1* gene in the SHR strain (open bars) was significantly lower when compared with the SHR.BN-chr.1 congenic strain (solid bars). D, The expression of the *Folr1* transgene in various organs (depicted by the grey bars).

Published:
Pravenec M, Kožich V, Krijt J, Sokolová J, Zídek V, Landa V, Mlejnek P, Šilhavý J, Šimáková M, Škop V, Trnovská J, Kazdová L, Kajiya T, Wang J, Kurtz TW. Genetic variation in renal expression of folate receptor 1 (Folr1) gene predisposes spontaneously hypertensive rats to metabolic syndrome. Hypertension 67(2):335-41, 2016

Research activity and characterisation of the main scientific results

Studies of Archosaur brain morphology (birds, crocodilians and extinct species).

The studies were performed in project aimed to neuroanatomical interpretation ofpaleontological samples of fossilized archosaurian skulls.

We used geometric morphometrics to compare a comprehensive data set of archosaurian endocasts along the deep evolutionary history of modern birds and found that this lineage experienced progressive elevation of encephalisation through several chapters of increased endocranial doming that we demonstrate to result from progenetic developments. Elevated encephalisation associated with progressive size reduction within Maniraptoriformes was secondarily exapted for flight by stem avialans.

Reference: Beyrand; V. - Voeten; D. F. A. E. - Bureš; S. - Fernandez; V. - Janáček; Jiří - Jirák; D. -Rauhut; O. - Tafforeau; P. Multiphase progenetic development shaped the brain of flying archosaurs . Scientific Reports. 2019; 9(Jul 25)); 10807

The knowledge of the brain/endocast relationship in modern animals is essential for studyingthe endocasts of extinct animals. We obtained the encephalic volume of modern crocodiliansusing ex vivo magnetic resonance imaging to reveal how the endoneurocranial cavity and brain of crocodilians change configuration during ontogeny.

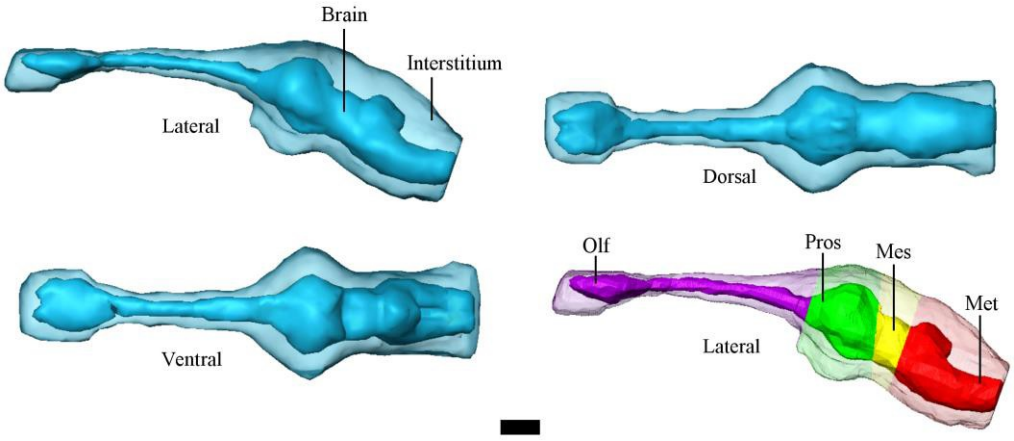


Figure: brain and virtual endocast of adult crocodile divided to main segments.

Reference: Jirák, D. - Janáček, Jiří . Volume of the crocodilian brain and endocast duringontogeny . PLoS ONE. 2017, roč. 12, 6, e0178491

We present a new method based upon coupled magnetic resonance imaging and computed tomography. Our approach utilizes a novel interactive Fakir probe to efficiently estimate total volumes and surface areas of the brain tissue and endoneurocranial space, as well as the discrete cephalic compartments. We visualized brain size differentiation in Ring-necked pheasant. This revealed a trend towards isometric enlargement of the total brain and endoneurocranial space in males versus females.

Reference: Jirák, D. - Janáček, Jiří - Kear, B. P. A combined MR and CT study for precise quantitativeanalysis of the avian brain . Scientific Reports. 2015, Vol. 5, Oct 30, p. 16002

The systematic geometric sampling, i.e. measurements in points of a regular grid, is widelyused in quantitative microscopy and spatial statistics for its high efficiency. We proved theorem relating the variance of the isotropic estimator of the integral of a smooth function

with perimeter of the object of interest in general situation. Extension term in the estimator variance is proportional to the integral of the squared modulus of the function over the objectboundary. Our result generalizes the Kendall-Hlawka-Matheron formula, already proved for smooth convex bodies by Hlawka in 1950, to a sufficiently general setup with arbitrary set with finite perimeter.

Reference: Janáček, J., Jirák, D.: Variance of the isotropic uniform systematic sampling. ImageAnalysis and Stereology 38 (3), 2019, 261-267, IF = 1.778

Effect of nanosecond pulses on tubulin self-assembly and on cytoskeleton

The aim of these studies was to explore the effect of a special non-chemical treatment using electrupulses on the cellular ultrastructure with potential application in biomedicine (e.g. cancer treatment).

We have found that the tubulin (building block of microtubules) can be influenced by nanosecond electropulses (nsEPs), reversibly or irreversibly, and temporarily changed tubulin self-assembles to various structures, depending on nsEPs parameters. The transient conformation changes of tubulin by nsEPs can result in new applications in biomedicine, materials sciences, biophysics and bionanotechnology. Our discovery of an entirely new wayof self-assembly of proteins was published in Advanced Materials (IF=25.8).

Reference: Chafai DE, Sulimenko V, Havelka D, Kubínová L, Dráber, P, Cifra, M. Reversible andIrreversible Modulation of Tubulin Self-Assembly by Intense Nanosecond Pulsed Electric Fields. Advanced Materials 31 (39), 2019.

We have developed a chip technology (integrated to structured illumination microscope), which enables delivery of nsEPs to single cells on the chip while imaging them. Such chip- based technological advancement enables the assessment of nsEPs effects on cellular and bionanostructures and observing their effects in real-time. We found that EB3 microtubule end binding protein localization is dramatically affected after treatment of mast cells with intense nsEPs.

Reference: Havelka, D, Chafai, DE, Krivosudsky, O, Klebanovych, A, Vostarek, F, Kubinova, L, Draber, P, Cifra, M. Nanosecond Pulsed Electric Field Lab-on-Chip Integrated in Super-Resolution Microscope for Cytoskeleton Imaging. Advanced Materials Technologies. Article Number: 1900669.

In the next step, we have explored externally triggered microtubule network remodeling by nanosecond electropulses (nsEPs) in cell cultures. We have confirmed that the microtubulesin cells can be engineered by nanosecond electropulses. The nsEPs induced reversible depolymerization or a remodeling of the microtubule network. Depending on the treatment environment, a controllable fate of microtubules could be triggered. This opens a new perspective for microtubule remodeling-based nanobiotechnological applications.

Reference: Chafai, DE,, Vostárek, F, Dráberová, E, Havelka, D, Arnaud-Cormos, D, Leveque, P, Janáček, J, Kubinova, L, Cifra, M, Draber, P, Microtubule Cytoskeleton Remodeling by NanosecondPulsed Electric Fields. Submitted to Advanced Biosystems.

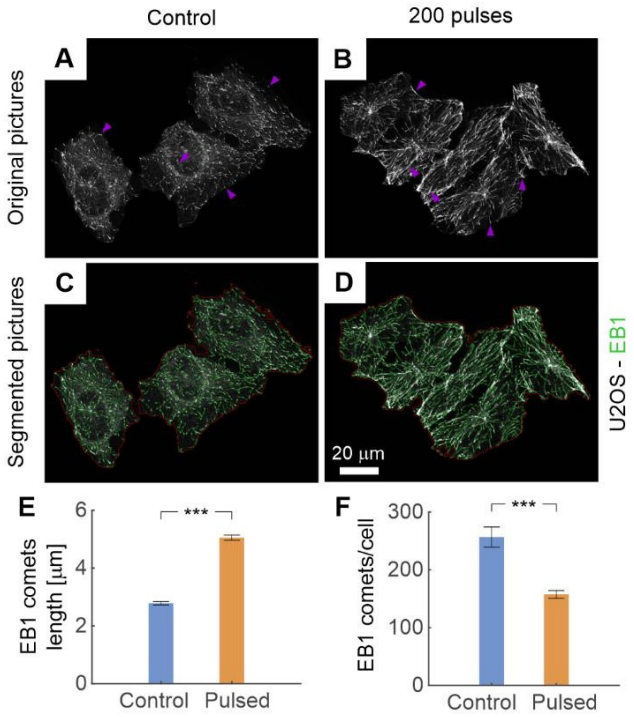


Figure. Remodeling of EB1 binding in U2OS cells after nsEPs treatment in culture media. **A)** Staining of EB1 in control cells and **C)** corresponding segmented image. **B)** Staining of EB1in nsEPs-treated cells and **D)** corresponding segmented image, cells fixed in methanol. **E)** Length of EB1 comets in control and nsEPs-treated cells. **F)** Number of EB1 comets per cell in control and nsEPs-treated cells. Values indicate the mean ± SD (n=310); ***p < 0.001.

Micromorphology of animal and human tissues

Embryonic heart

Mouse embryonic hearts of the Nkx2.5:eGFP strain were cleared and imaged whole mountby confocal microscopy. Activation maps were simulated assuming constant speed of spreading along the trabeculae. The results were compared with experimentally obtained epicardial activation maps. We conclude that in the embryonic pre-septation heart, the geometry of the A-V connections and trabecular network is sufficient to explain impulse propagation and ventricular activation patterns.

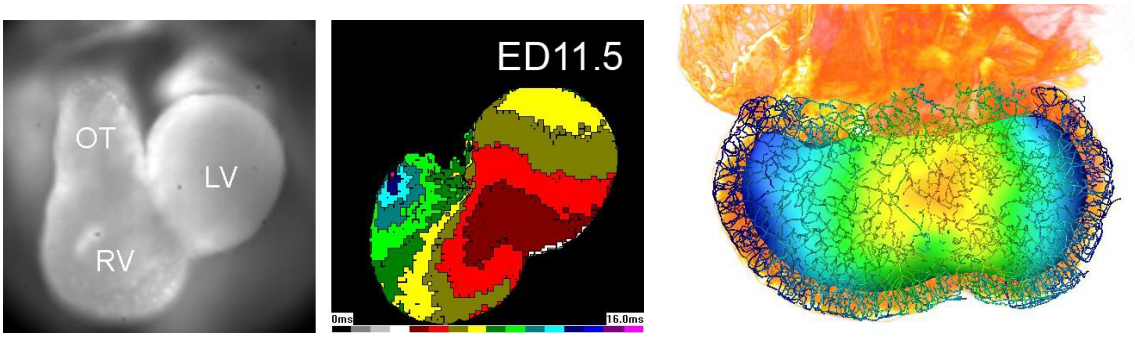


Figure: Activation map and simulated activation map on skeletonized trabecules in embryonic mouse heart.

Reference: Olejníčková, Veronika - Šaňková, Barbora - Sedmera, David - Janáček, Jiří . Trabecular Architecture Determines Impulse Propagation Through the Early Embryonic Mouse Heart . Frontiers in physiology 2019, 9(Jan 8)), 1876

The publication shows an application of modern tissue clearing protocols like SCALE, CLARITY, CUBIC and others to GFP-expressing mouse tissues (embryos, hearts), with theaim to preserve GFP fluorescence signal that easily disappears after treating in chemical compounds. We found that CUBIC works here best and gives possibility to show nicely finestructures, like, e.g., head vasculature, atrium and ventricular trabeculae, by using confocaland lightsheet microscopy.

Reference: Kolesová, H. - Čapek, Martin - Radochová, Barbora - Janáček, Jiří - Sedmera, David. Comparison of different tissue clearing methods and 3D imaging techniques for visualization of GFP- expressing mouse embryos and embryonic hearts . Histochemistry and Cell Biology. 2016, roč. 146, 2,p. 142-152 .

Capillaries

The effect of postmortem changes on the measured parameters must be carefully considered when comparing results from biopsy samples with those from autopsy samples. We performed the first study of its kind to demonstrate changes to the capillary pattern in postmortem skeletal muscle, excised 1 and 24 hr after death. The 3D approach applied to quantify changes in the characteristics of the capillary network detected significant alterationswithin 24 hr of death.

Reference: Eržen, I. - Janáček, Jiří - Kreft, M. - Kubínová, Lucie - Cvetko, E. Capillary NetworkMorphometry of Pig Soleus Muscle Significantly Changes in 24 Hours After Death . Journal of Histochemistry and Cytochemistry 2018, roč. 66, 1, p. 23-31

We simulated blood flow and oxygen transfer in feto-placental capillaries by converting three-dimensional representations of villous and capillary surfaces, reconstructed from confocal laser scanning microscopy, to finite-element meshes, and calculating values of vascular flow resistance and total oxygen transfer. The results could explain the prevalence of fetal hypoxia in cases of delayed villous maturation.

Reference: Pearce, P. - Brownbill, P. - Janáček, Jiří - Jirkovská, M. - Kubínová, Lucie - Chernyavsky, I. L. - Jensen, O. E. Image-Based Modeling of Blood Flow and Oxygen Transfer in Feto-PlacentalCapillaries . PLoS ONE. 2016, roč. 11, 10, článku e0165369

Nerves

We used optical projection tomography to reveal the structure of peripheral nerves and demonstrated suitability of this method for research of inner contents of fascicular nerve groups and their spatial disposition within the nerve including their interconnections. Thiscould became an excellent tool for better interpretation of ultrasound images in clinical practice thus avoiding possible neurological complications.

Reference: Prats-Galino, A. - Čapek, Martin - Reina, M. A. - Cvetko, E. - Radochová, Barbora - Tubbs, R.S. - Damjanovska, M. - Pintaric, T.S. 3D reconstruction of peripheral nerves from opticalprojection tomography images: A method for studying fascicular interconnections and intraneural plexuses . Clinical Anatomy 2018, roč. 31, 3, p. 424-431

The article shows an application of an advanced microscopic technique – Optical Projection Tomography (OPT), installed in IPHYS CAS, Prague, to visualization of piglet nerve microarchitecture in 3D that is studied in medical institutes in Ljubljana and Madrid. OPT is probably the most optimal method for this purpose now, giving also possibility to extract

relevant information like number and diameter of fascicles and to evaluate injuries to nervescreated by intraneural injections.

Reference: Cvetko, E. - Čapek, Martin - Damjanovska, M. - Reina, M. A. - Eržen, I. - Stopar-Pintarič, T. The utility of three-dimensional optical projection tomography in nerve injection injuryimaging . Anaesthesia. 2015, Vol. 70, 8, p. 939-947 .

Tendon

We studied the collagen crimp pattern during the physical growth and development in Achilles tendons from newborn to elderly rabbits by label-free second harmonic generation (SHG) microscopy. Both the crimp amplitude and wavelength increased until the sexual maturation, then the amplitude decreased.

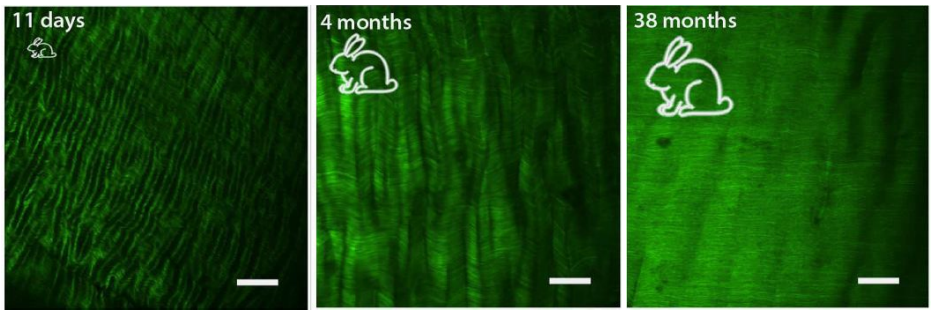


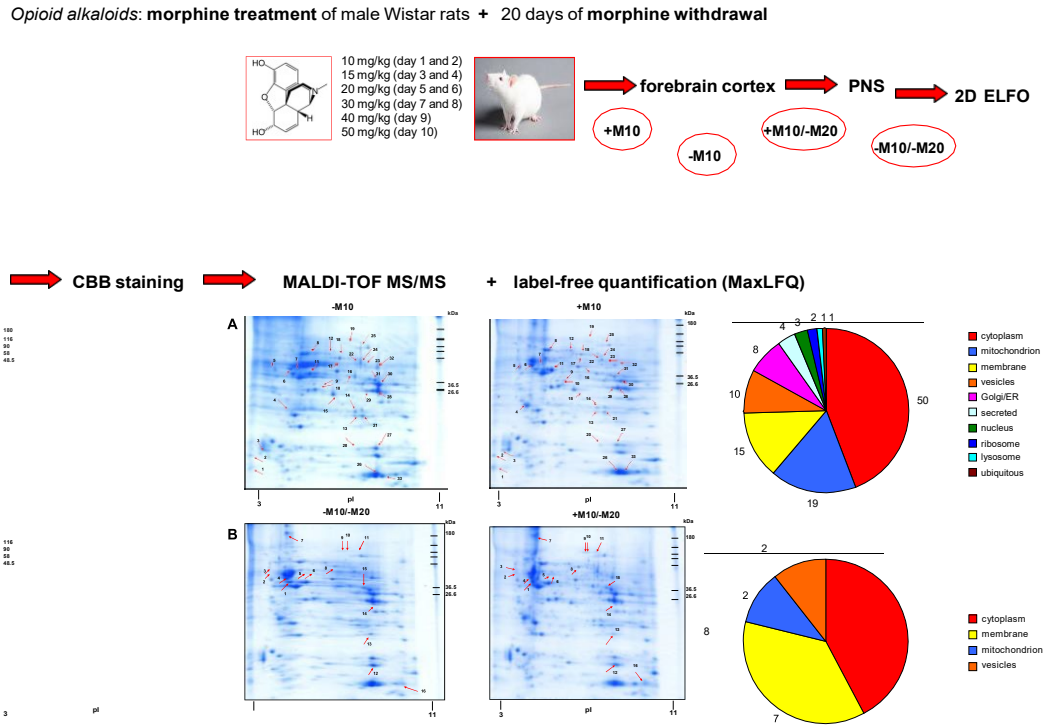
Figure: SHG images of rabbit Achilles tendon.

Reference: Hadraba, Daniel - Janáček, Jiří - Filová, Eva - Lopot, F. - Paesen, R. - Fanta, O. - Jarman, A. - Nečas, A. - Ameloot, M. - Jelen, K. Calcaneal Tendon Collagen Fiber Morphometry andAging . Microscopy and Microanalysis.. 2017, roč. 23, 5, p. 1040-1047

Reversible and non-reversible alterations of brain proteome by morphine

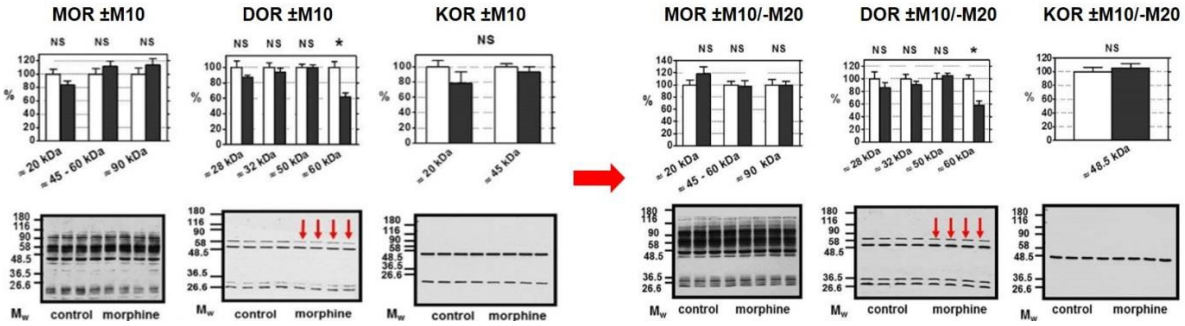
Forebrain cortex of rats exposed to increasing doses of morphine for 10 days is severely altered as far as the overall protein composition is involved. In rats sacrificed 20 days afterthe last dose of morphine, the number of altered proteins was decreased from 28 (±M10) to 14 (±M10/−M20) when determined in CBB-stained 2D gels or from 113 to 19 when determined by LFQ. Our data indicate the high ability of a living organism to oppose the morphine-induced change with the aim to return to physiological norm after withdrawal of the drug.

Reference: Ujčíková, Hana - Vošahlíková, Miroslava - Roubalová, Lenka - Svoboda, Petr. Proteomic analysis of protein composition of rat forebrain cortex exposed to morphine for 10 days; comparison with animals exposed to morphine and subsequently nurtured for 20 days in the absence of this drug . Journal of Proteomics. 2016; 145:11-23



Reversibility of chronic morphine effect on rat forebrain cortex protein composition analyzed by gel-based proteomics and label-free quantification (MaxLFQ).

Chronic exposure of mammalian organism to morphine results in adaption to persistent high opioid tone through homeostatic adjustments. In rat forebrain cortex, chronic morphine treatment for 10 days followed by 20-day drug withdrawal results in minor (δ-OR) or no change (μ- and κ-OR) of opioid receptor content. The reversible increases of caveolin-1 and cholesterol levels suggest the participation of membrane domains in compensatory responses during opioid withdrawal.



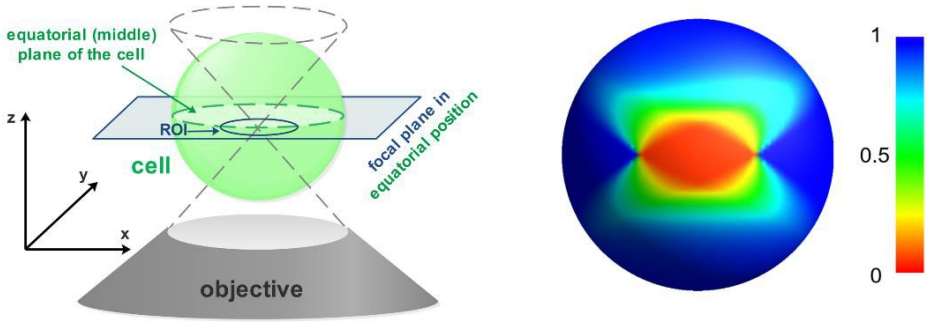
The immunoblot analysis of μ-, δ - and κ-opioid receptors in forebrain cortex of rats exposedto morphine for 10 days (left panels) and subsequent 20-day drug withdrawal (right panels).

Reference: Ujčíková, Hana - Hloušková, Martina - Cechová, Kristina - Stolařová, Kateřina - Roubalová, Lenka - Svoboda, Petr . Determination of μ-, δ- and κ-opioid receptors in forebrain cortexof rats exposed to morphine for 10 days: Comparison with animals after 20 days of morphine withdrawal . PLoS ONE. 2017; 12(10):e0186797

G protein-coupled receptors and the dynamic organization of the cell membrane

With the aim to increase the accuracy of determination of diffusion coefficient and mobile fraction of plasma membrane proteins, we developed the novel method of FRAP analysis in the equatorial plane of the cell. Our method is based on the model of 2D diffusion in the

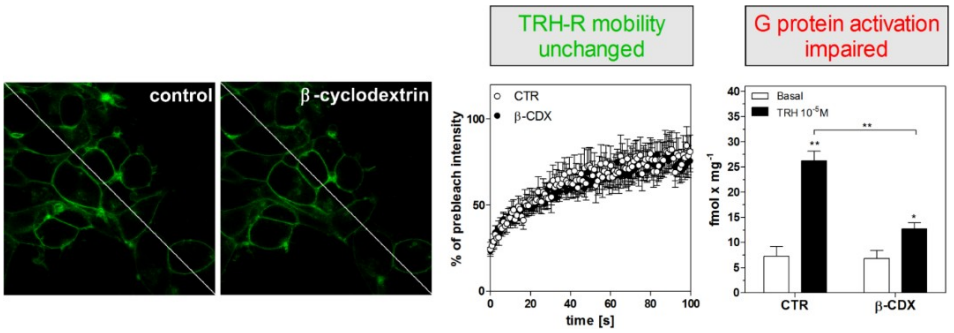
vertical plane of cell membrane and on the hourglass profile of bleaching irradiance. When applied for determination of δ-opioid receptor-eYFP mobility in HEK293 cells, the highly significant decrease of receptor mobility was detected in cholesterol-depleted cells. This decrease was fully reversible by cholesterol replenishment. Our method is generally valid and may be applied for the determination of the mobility parameters of other plasma membrane proteins.



The schematic presentation of basic arrangement of confocal microscope for imaging of plasma membrane region of the cell in the equatorial setup (left) and spatial profile of emitted light immediately after the bleach (right). The cell shape is approximated as a sphere.

Reference: Jiří Janáček, Jana Brejchova and Petr Svoboda (2019) Determination of mobility of δ- opioid receptor molecules in plasma membrane of live cells by novel method of FRAP analysis, BBA Biomembranes, 1861, 1346-1354

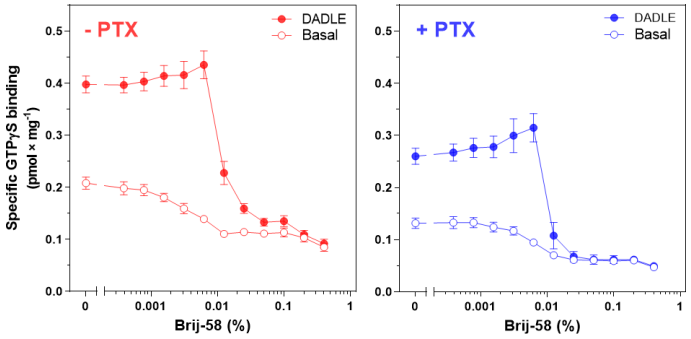
The effect of cholesterol depletion on TRH-R surface mobility determined by FRAP was investigated in live HEK293 cells. Cholesterol depletion did not result in statistically significant alteration of receptor mobile fraction. Decrease of the apparent diffusion coefficient of TRH-R molecules was detectable only under the special conditions of screening and calculation of FRAP data. Hydrophobic interior of plasma membrane became substantially more “fluid” and chaotically organized in cholesterol-depleted cells. The decrease of cholesterol level impaired the functional coupling between the receptor and cognate G proteins of G_q/G_{11} family. The decreased order and increased fluidity of hydrophobic membrane interior suggests an important role of this membrane area in TRH-R- $G_q/G_{11}\alpha$ protein coupling.



Reference: Jana Brejchova, Jan Sykora, Pavel Ostasov, Ladislav Merta, Lenka Roubalova, Jiri Janacek, Martin Hof and Petr Svoboda (2015) TRH-receptor mobility and function in control and cholesterol-depleted plasma membrane of HEK293 cells stably expressing TRH-R-eGFP. BBA- Biomembranes, 1848, 781-796

Limited perturbation of plasma membrane integrity of HEK293 cells stably expressing PTX-insensitive δ-opioid receptor-Gi1α fusion protein by low concentrations of non-ionic detergentBrij-58 results in alteration of δ-OR-G protein coupling. Maximum G protein-response to

agonist stimulation is increased; affinity of response is decreased. Total degradation of plasma membrane structure at high Brij-58 concentrations results in diminution of functional coupling between δ-OR and G proteins. The effect of Brij-58 on δ-OR-G protein coupling wasalso valid for PTX-sensitive G proteins of Gi/Go family endogenously expressed in HEK293 cells.



Direct effect of increasing concentrations of Brij-58 on basal and DADLE-stimulated GTPγSbinding; plasma membranes isolated from PTX treated and untreated δ-OR-Gi1α cells.

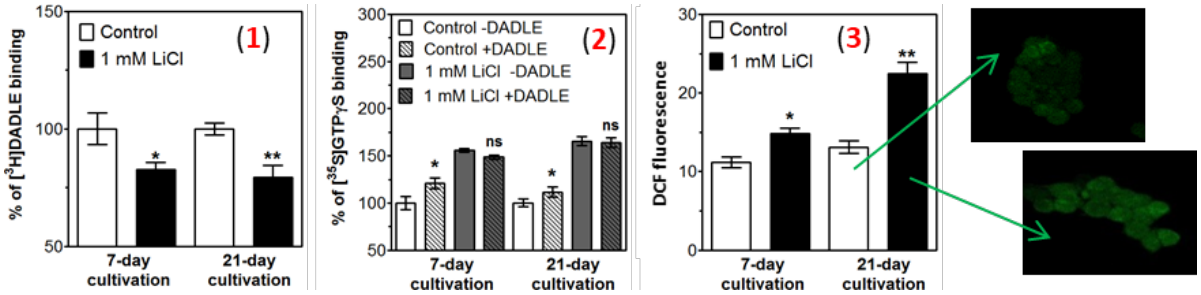
Reference: Roubalová, Lenka - Vošahlíková, Miroslava - Brejchová, Jana - Sýkora, Jan - Rudajev, Vladimír - Svoboda, Petr. High Efficacy but Low Potency of delta-Opioid Receptor-G Protein Couplingin Brij-58-Treated, Low-Density Plasma Membrane Fragments. PLoS ONE. 2015, Vol. 10, 8, e0135664

Analysis of the role of Na+/K+-ATPase and lipid peroxidation in bipolar disorder

Regulation of Na+/K+-ATPase by lithium was investigated. We compared the time- course of Li effect on Na+/K+-ATPase and lipid peroxidation in two widely different cell models - Jurkat and HEK293. Li-induced decrease of lipid peroxidation products was associated with the decrease of Na+/K+-ATPase level and vice versa. We studied the difference between the acute and chronic (long-term) effects of Li on live cells. To accountfor tissue/cell specificity, we used two widely different cell types. These two model cell lines were cultivated with (Li-treated) or without (controls) therapeutic, 1 mM concentration of Li in culture medium for 1, 7, and 28 days. Cultivation of Jurkat cells in 1 mM Li medium resultedin down-regulation of Na+/K+-ATPase (decrease of [3H] ouabain-biding sites and intensity of immunoblot signals) in all Li-groups. In HEK293 cells, the decrease of Na+/K+-ATPase was observed after the acute, 1-day exposure only. The long-term treatment with Li resulted in Na+/K+-ATPase upregulation. MDA and 4-HNE modified proteins were decreased in Jurkat cells in all Li-groups. On the other hand, in HEK293 cells, MDA concentration was decreased after the acute, 1-day Li exposure only; the long-term cultivations, for 7 or 28 days, resultedin a significant increase of lipid peroxidation products. This is the first report of the time- course of therapeutic Li effect on live cells in context with Na+/K+-ATPase expression and products of lipid peroxidation.

Reference: Vošahlíková, Miroslava - Roubalová, Lenka - Ujčíková, Hana - Hloušková, Martina - Musil, Stanislav - Alda, Martin - Svoboda, Petr. Na+/K+-ATPase level and products of lipid peroxidation inlive cells treated with therapeutic lithium for different periods in time (1, 7, and 28 days); studies of Jurkat and HEK293 cells. Naunyn-Schmiedeberg's Archives of Pharmacology 2019, 392(7), 785-799.

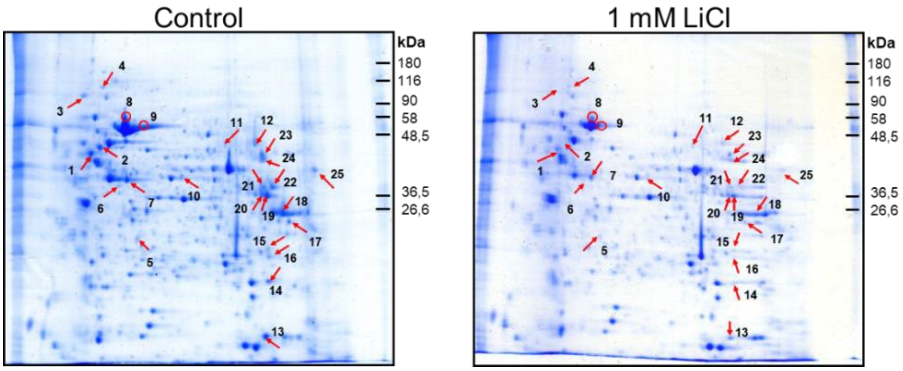
The functional state of δ-opioid receptor signalling cascade in live cells exposed to a therapeutic concentration of lithium for a prolonged period of time (weeks) is not known. Our results indicate that Li results in down-regulation of δ-OR protein level and attenuation of δ-OR function in parallel with increased oxidative stress and increased level of lipid peroxidation products. The aim of this study was to examine the effects of the long-term exposition of live cells to therapeutic, 1 mM concentration of LiCl on δ-OR level and function in a model cell line stably expressing this type of OR. Li treatment resulted in a decrease of binding of specific δ-OR agonist [3H]DADLE and loss of functional coupling between δ-OR and trimeric G proteins measured as DADLE-stimulated [35S]GTPγS binding. In Li-treated cells, the highly increased oxidative stress measured as DCF fluorescence intensity was noticed. Production of 4-HNE-protein adducts and MDA was clearly increased in Li-treated cells.



Decreased expression level of δ-OR (1) and G protein coupling efficiency (2) togetherwith increased DCF fluorescence intensity (3).

Reference: Vošahlíková, Miroslava - Ujčíková, Hana - Hloušková, Martina - Musil, Stanislav - Roubalová, Lenka - Alda, Martin - Svoboda, Petr. Induction of oxidative stress by long-term treatment of live HEK293 cells with therapeutic concentration of lithium is associated with down-regulation of delta-opioid receptor amount and function. Biochemical Pharmacology 2018, 154, 452-463.

Despite abundant clinical use of lithium in pharmacotherapy of bipolar disease, important questions regarding its action remain open. Prolonged exposure of human embryonic kidney cells to 1 mM LiCl results in up-regulation of Na+/K+-ATPase, major reorganization of overall cellular metabolism and decrease of hydration of polar head-group region of plasma membrane lipid bilayer. Up-regulation of Na+/K+-ATPase was evidenced byan increase of immunoblot signal of α subunit as well as in the number of [3H]ouabain binding sites. Identification of Li-altered proteins by two-dimensional gel electrophoresisfollowed by MALDI-TOF MS/MS suggested a change of energy metabolism in mitochondria and cytosol and alteration of cell homeostasis of calcium. Li interaction with plasma membrane was characterized by fluorescent probes DPH, TMA-DPH and Laurdan.



Two-dimensional gel electrophoresis of post-nuclear supernatant prepared from control HEK293 cells and cells exposed to 1 mM LiCl for 21 days.

Reference: Vošahlíková, Miroslava - Ujčíková, Hana - Chernyavskiy, Oleksandr - Brejchová, Jana - Roubalová, Lenka - Alda, Martin - Svoboda, Petr. Effect of therapeutic concentration of lithium on live HEK293 cells; increase of Na+/K+-ATPase, change of overall protein composition and alteration of surface layer of plasma membrane. Biochimica et Biophysica Acta - General Subjects. 2017, 1861(5 Pt A), 1099-1112.

Research activity and characterisation of the main scientific results

1) Discovery of essential role of the mitochondrial phospholipase iPLA2γ in fatty acid-stimulated insulin secretion (*Antioxidants & Redox Signaling =ARS 2015 23(12):958-72*; *explained in ARS 2018 29(7):667-714*) and in *ARS 2019 31(10):722-751*):

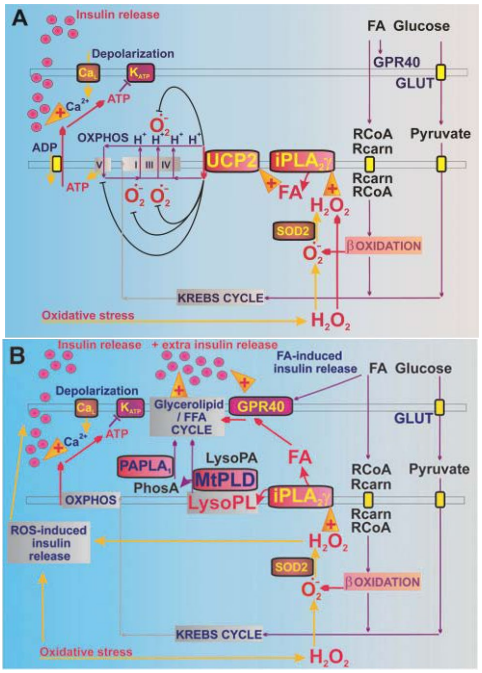


Fig.1A. Cytoprotection by feedback down-regulation of oxidative stress by iPLA2γ and UCP2 synergy

Fig.1B. Mechanism of fatty acid-stimulated insulin secretion (FASIS): FA β-oxidation initiates iPLA2γ cleavage of mitochondrial FAs which spread up to the plasma membrane and activate GPR40 that subsequently initiates glycerolipid/fatty acid cycle

Cytoprotective antioxidant role of iPLA2γ: We revealed that when directly stimulated by H_2O_2, the calcium-independent mitochondrial phospholipase iPLA2γ cleaves fatty acids (FA) to initiate uncoupling mediated by the uncoupling protein UCP2, which then attenuates mitochondrial superoxide formation. The synergy of iPLA2γ and UCP2 provides such antioxidant cytoprotection to pancreatic β-cells on the expense of a slight decrease of glucose-stimulated insulin secretion, due to diminished protonmotive force Δp (Fig.1A).

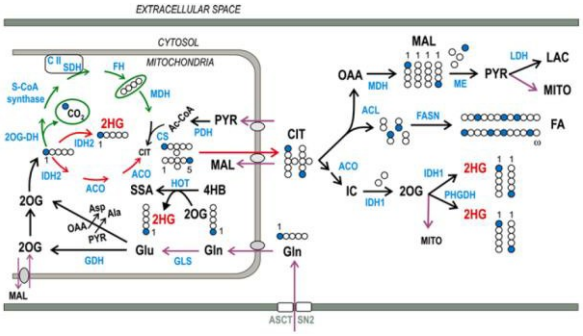
A) *Essential role of iPLA2γ in fatty acid-stimulated insulin secretion*:

Moreover, we revealed that the glucose-independent fatty acid stimulated insulin secretion (FASIS) exists, for which FA β-oxidation stands as one leg in the FASIS mechanism. This provides ATP for such insulin secretion. However, we discovered that the predominant portion of FASIS, the second branch, is initiated due to the **essential intramitochondrial redox signaling**: during FA β-oxidation the ETF:QOR (electron transport flavoprotein :ubiquinone oxido-reductase) forms superoxide which is instantly transformed to H_2O_2 by the matrix superoxide dismutase (MnSOD/SOD2). The resulting H_2O_2 activates iPLA2γ that cleaves FAs from mitochondrial phospholipids (Fig.1B). We have even detected the diffusion of the cleaved mitochondrial FAs toward the plasma membrane using the fluorescent fatty acid binding protein ADIFAB. Consequently, cleaved FAs activate the metabotropic receptor, G-protein coupled receptor-40 (GPR40), at the plasma membrane that subsequently initiates insulin secretion upon so-called glycerolipid/ fatty acid cycle, when the exocytosis-promoting protein Munc13-1 is activated by the released 2-monoacyl glycerol. This pathway is independent of the canonical pathway of insulin secretion, based on closing of the ATP- sensitive K^+ channel.

Thus the revealed mechanism represents an amplifying mechanism for the GPR40 branch of insulin secretion, since it accounts for more than 60% of FASIS. This is deduced from the current work with the own constructed iPLA2γ knockout mouse (unpublished). Nature has developed such a mechanism most probably in order to compensate losses of free fatty acids by β-oxidation, those fatty acids cleaved from incoming chylomicrons into extremely narrow intersticia among β-cells within islets. The revealed self-perpetuating (amplifying) mechanism ensures that GPR40 receptor is exposed to the surplus incoming fatty acid ligands to be activated. This is in turn allowed by the intramitochondrial redox signaling activating iPLA2γ.

Note: *The above discoveries and publications are exclusively provided by our research team.*

2) Discovery of oncometabolite 2-hydroxyglutarate formation by non-mutant isocitrate dehydrogenase 2 (**IDH2**) (*IJBCB 2015 65:125-133*; *ARS 2019, doi. 10.1089/ars.2019.7902*).



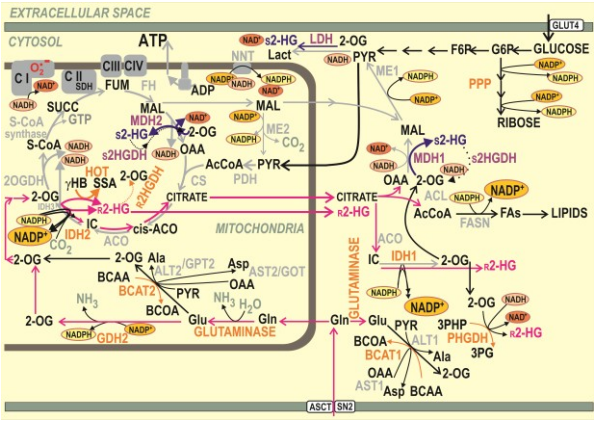


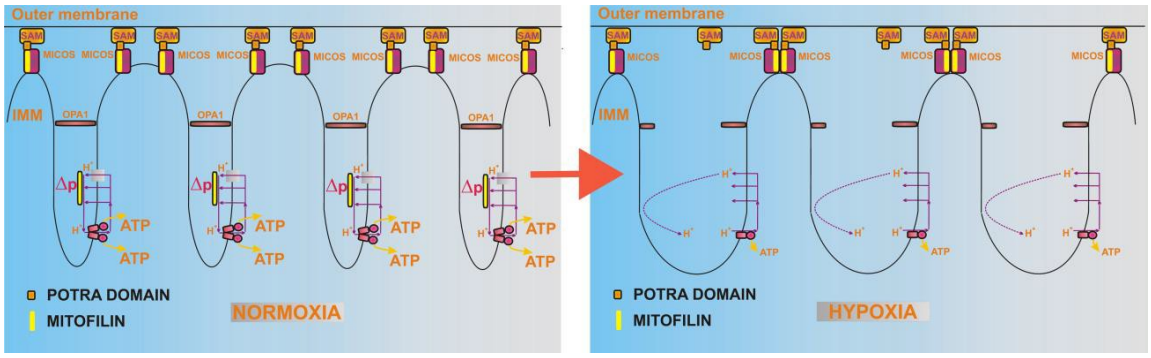
Fig.2A. Our own developed method for accessment reductive carboxylation – using GC/MS quantified ^{13}C-incorporation from 1-^{13}C glutamine into downstream metabolites. Due to the released ^{13}C-CO_2, only counter-Krebs cycle reactions are indicated, such as side-reaction of IDH2, forming oncometabolite 2-hydroxyglutarate (2HG), or reductive carboxylation with subsequent citrate export from the mitochondrial matrix, that leads to ^{13}C labeling of citrate and malate; or else ^{13}C labeling of 2HG formed by the cytosolic IDH1. Silencing of IDH2 thus roughly estimates the 2HG portion synthesized by IDH1 or other enzymes. The surplus in wt vs. silenced cells thanestimates the IDH2 formed 2HG.

Fig.2B. Formation of two 2HG enantiomers: In the (cancer) cell, each of the two 2-hydroxyglutarate enantiomers is formed by the different set of enzymes and similarly is degraded by the specific dehyrdrogenases (2HGDH). *Red*: R-2HG, *dark blue*: s-2HGFA.

Until 2015, it was believed that 2-hydroxyglutarate (2HG) is synthesized exclusively by the Arg mutants of the cytosolic isocitrate dehydrogenase IDH1 or mitochondrial IDH2. 2HG promotes cancerogenesis by inhibiting chromatin-modifying enzymes, namely 2-oxoglutarate-dependent dioxygenases, thus causing DNA and histone hypermethylation; and by interfering with hypoxia-induced factor (HIF) transcriptome reprogramming and also with the mTOR pathway. We have independently indentified (our world priority was canceled by the one year delay in acceptance of publication) that also non-mutant enzymes, IDH2 and IDH1 produce significant amounts of 2HG, albeit reaching ~100 times lower concentrations than the Arg-mutants. Nevertheless, the reached concentrations are still above IC_{50} of 2OG- dependent dioxygenases.

Using the own developed method (Fig.2A), we have documented that in breastcancer HTB-126 cells, ^{13}C-citrate, ^{13}C-malate, and ^{13}C-2-hydroxyglutarate were enriched by 2-, 5-, and 15-fold at 5 mM glucose (2-, 2.5-, and 13-fold at 25 mM glucose), respectively, after 6 hrs. ^{13}C–2HG was formed despite the absence of any *idh2* and *idh1* mutations in HTB-126 cells. Such enrichment decreased by 6% with IDH1 silencing, but by 30–50% upon IDH2 silencing while cell respiration and ATP levels rose up to 150%. Unlike 2HG production the reductive carboxylation (indicated by ^{13}C-citrate or ^{13}C-malate) declined at decreasing CO_2, which is essentially required. At hypoxia (5% O_2), IDH2-related and unrelated ^{13}C- accumulation into citrate and malate increased 1.5–2.5-fold with unchanged IDH2expression; whereas hypoxic 2HG formation did not. ^{13}C–2HG originated by ~50% from other enzymes than IDH2 or IDH1, substantiating remaining activity in IDH1&2-silenced cells.Relatively high basal ^{12}C–2HG levels existed (5-fold higher vs. non-tumor HTB-125 cells).
Since RCG is enhanced at hypoxia (frequent in solid tumors) and 2HG can be formed. We suggested 2HG as an analytic marker (in serum, urine, or biopsies) predicting malignancy of breast cancer in all patients. Subsequently, we have conducted a pilot clinical study with ~30 patients (in collaboration with Dr. Tesařová, General Univ.Hospital), who were indicated by significantly high 2HG in urine *vs*. healthy controls (unpublished).
Note: Prof.L.Vítek (1st Medical Faculty, Charles Univ.) kindly provided the GC/MS instrument.

3) Revealing of hypoxic widening of mitochondrial cristae in cancer cells - as consequence of mitochondrial mitofilin degradation and decreased oligomeric state of the mitochondrial ATP-synthase (*FASEB J 2016 30(5):1941-1957*).



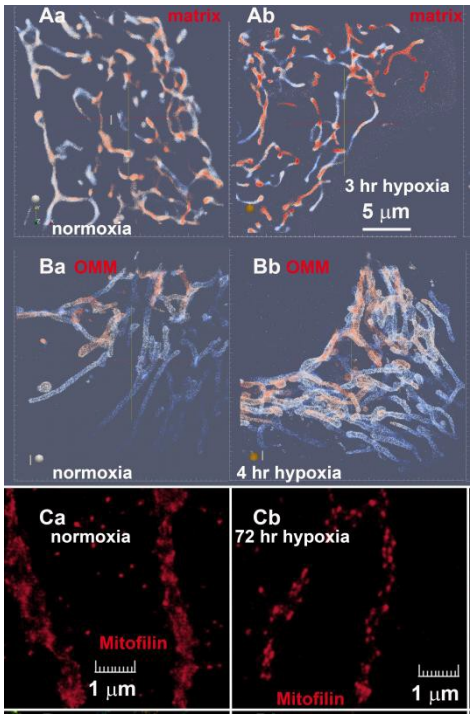


Fig.3. Scheme of the revealed mechanism.

Fig.4. 3D superresolution microscopy (xy projections) visualizing: **A) Mitochondrial matrix**, using matrix-addressed Eos; **B) mitochondrial outer membrane** (OMM), using Eos-conjugated FIS1 protein, truncated so not to cause fragmentation; and **C) mitofilin protein**, i.e. Mic60 subunit of the MICOS complex joinig cristae membranes with OMM cell. Visualization was by the direct stochastic optical reconstitution microscopy (dSTORM), which is the superresolution version of 3D immunocytochemistry.

Panels a) normoxia vs. panels b) 5% O_2 hypoxia clearly indicate the apparent shrinkage of the matrix space at constant OMM; plus clustering of mitofilin. The matrix shrinkage inside the constant OMM means the intermembrnae space expansion. Since the predominant portion of the latter is represented by the cristae, the expansion of the cristae volume is clearly indicated.

Adaptation of HepG2 cells to hypoxia was studied. Using own-developed 3D superresolution microscopy of biplaneFPALM, the distinct mitochondrial compartments were visualized: *i*) with the matrix addressedEos, the matrix volume was visualized; *ii*) using the intracristal space (ICS) marker lactamase β, conjugated with Eos, the ICS was visualized; *iii*) using the Eos-conjugated FIS1 protein, OMM was visualized. We thus demonstrated that the matrix shrunk, ICS expanded, while OMM diameter stayed constant after 1 hr of hypoxia at 5% O_2, Transmission electron microscopy (TEM) confirmed this ICS expansion, showing cristae widening. 3D direct stochastic optical reconstruction microscopy (dSTORM), i.e. immunocytochemistry, then revealed foci of clustered mitofilin/Mic60 (but not Mic19) upon hypoxia, in contrast to its even isotropic normoxic distribution. Mitofilin mRNA and protein decreased by ~20%. ATP synthase dimers vs. monomers andstate-3/state-4 respiration ratios were lower during hypoxia (maximum in glycolytic cells), which was absent in reduced or OMM-detached cristae of OPA1- and mitofilin-silenced cells, respectively. Hypoxic adaptation is interpreted as rounding sharp cristae edges and expanding cristae width (ICS volume) by partial mitofilin/Mic60 down-regulation. Mitofilin-depleted MICOS detaches from the outer membrane SAM while the remaining MICOS with mitofilin redistributes toward the higher interdistances at hypoxia. This phenomenon causes partial OXPHOS dormancy in glycolytic cells via disruption of ATP synthase dimers and higher oligomers. Hence ATP production is decreased in inflated cristae by mitofilin down-regulation concomitant to MICOSclustering. A part of this was regulated by hypoxia-inducible factor.

Note: Dr. J.Bewersdorf and M.Lessard (Yale Univ.) constructed the prototype of our Biplane FPALM microscope, whereas Dr. Malínský and Strádalová ensured cryoelectron microscopy.

4) Participation in studies of **metabolic reprogramming** and redox regulations in **pulmonary hypertension** interstitial fibroblasts and or macrophages (*Circulation 2016 134: 1105-1121; Circulation 2017 136: 2468-2485; Am J.Resp.Cell Mol.Biol. 2016 55:47-57; ARS 2018 28:230-250*). = see further in International Cooperation

5) Revealing drop of transcription factor Nkx6.1 correlating with the mtDNA reduction at constant size of mtDNA nucleoids in β-cells of diabetic Goto Kakizaki rats (*Sci Rep 2017 7:15674*).

Fig.6. Quantification of the β-cell-specifying factor Nkx6.1: **a**) mRNA; **b**) protein in pancreatic islets from diabetic Goto Kakizaki rats as compared to islets from control Wistar rats of the indicated age in weeks ("w").

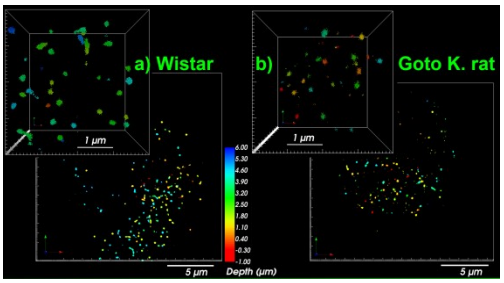
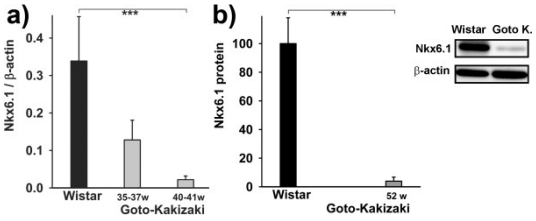


Fig.7. Nucleoids of mtDNA in β-cells of diabetic vs. control rats: 3D BiplaneFPALM images of nucleoids visualized using mitochondrial single-stranded DNA – binding protein mtSSB conjugated with Eos (a,b) 20 × 20 µm xy- projections; depth color-coded in z axis. *Insets*: Detail of 4 × 4 × 4 µm space containing localized points forming nucleoids.

Hypertrophic pancreatic islets (PI) of Goto Kakizaki (GK) diabetic rats contain a lower number of β-cells vs. non-diabetic Wistar rat PI. Remaining β-cells contain reduced mitochondrial (mt) DNA per nucleus (copy number), probably due to declining mtDNA replication machinery, decreased mt biogenesis or enhanced mitophagy. We confirmed mtDNA copy number decrease down to <30% in PI of one-year old GK rats. Studying relations to mt nucleoids sizes, we employed 3D superresolution fluorescent photoactivable localization microscopy (FPALM) with lentivirally transduced Eos conjugate of mt singlestranded-DNA-binding protein (mtSSB) or transcription factor TFAM; or by 3D immunocytochemistry. mtSSB (binding transcription or replication nucleoids) contoured "nucleoids" which were smaller by 25% (less diameters >150 nm) in GK β-cells. Eos-TFAM- visualized nucleoids, composed of 72% localized TFAM, were smaller by 10% (immunochemically by 3%).

A theoretical ~70% decrease in cell nucleoid number (spatial density) was not observed, rejecting model of single mtDNA per nucleoid. The β-cell-specifying transcription factor Nkx6.1 mRNA and protein were declining with age (>12-fold, 10 months) and decreasing with fasting hyperglycemia in GK rats, probably predetermining the impaired mtDNA replication (copy number decrease), while spatial expansion of mtDNA kept nucleoids with only smaller sizes than those containing much higher mtDNA in non-diabetic β-cells.



Note: The work was done in cooperation with Prof. F. Saudek, DSc., the Diabetology director at the Institute of Clinical and Experimental Medicine (IKEM), Prague.

6) Discovery of cristae narrowing upon glucose-stimulated insulin secretion (*BBA 2018 1859: 829-844*). *Note: The work was done entirely by our team.*

Glucose-stimulated insulin secretion was studied in model pancreatic β-cells, INS-1E cells. As we and others have previously observed, a peculiar aspect is revealed for the glucose sensor of pancreatic β-cell, that is its steepness. This means a steepness of the related dose-response, the insulin release dependence vs. glucose concentration. Similarly this is for glucose dependence of phosphorylating respiration rate ratio to the non-phosphorylating rate (quantity ascribing the intensity of ATP synthesis). Their steepness is higher that would correspond to a Hill coefficient of 1.

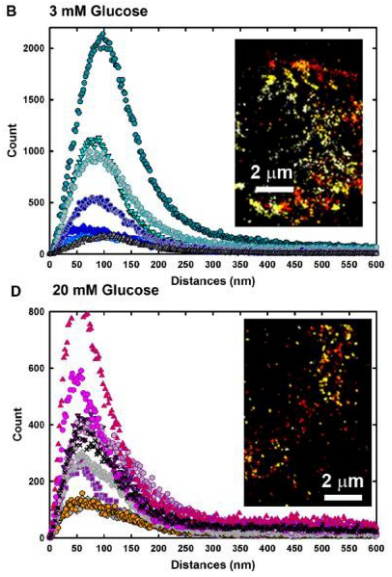


Fig.8. 3D distribution of ATP-synthase molecules upon GSIS in model β-cells, INS-1E cells: 3D dSTORM with Alexa Fluor 647-conjugated primary antibodies against the F1alpha subunit of ATP-synthase was performed and all interdistances between them were displayed in histograms for each image (blue for 3 mM glucose; red/magenta/organge for 20 mM glucose and the most frequent distance (MFD) indicated just the proximal ATP synthase molecules at each edge of the crista. As clearly recognized MFD was shifted from 80 to 55 nm upon GSIS. Histograms were constructed on the basis of the Ripley's K- function using our novel own developed procedure for 3D data treatment.

We have revealed narrowing of mitochondrial cristae upon GSIS and hypothesized that this may contribute to the steepness of the sensor. This can hold true, since if also the intracristal space (ICS) volume would decrease, the proton coupling would be more efficient. Note, according to the chemiosmotic theory such proton coupling transfers the energy of the respiratory chain proton pumping towards the rotation of the c-ring of the ATP-synthase, thus mediating the proton backflow into the matrix. Moreover, the ATP-synthase dimers, tetramers, or speculatively higher oligomers are associated within the rows along the crista rims.

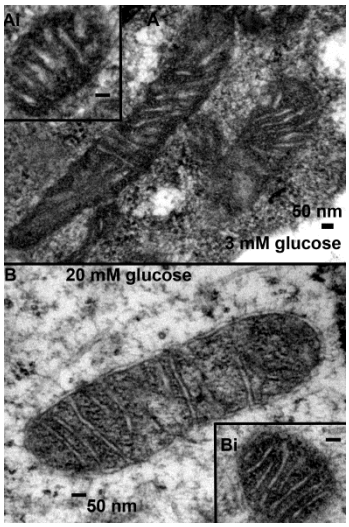
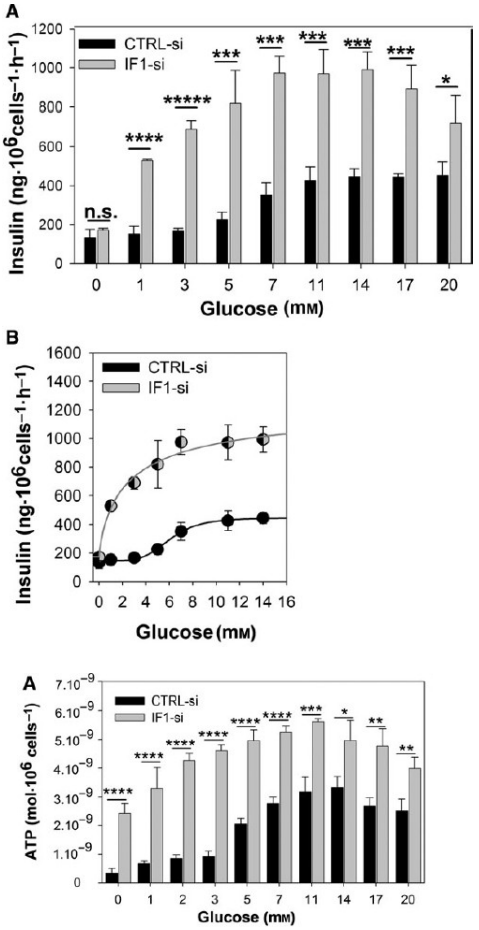


Fig.9. Cristae width as derived from TEM: Note very wide cristae of average 15 nm width at 3 mM glucose (top) vs. narrow (8 nm width) "textbook" cristae at 20 mM glucose in sections of the mitochondrial network in INS-1E cell. When 8 nm (for 2 membranes) plus two lifts of 12 nm are added (F1 lifts above the membrane planes) and two average size of antibodies as 10 nm, one obtains MFD of 48 nm matching the dSTORM.

7) Discovery of the *in vivo* inhibitory ability of ATP-synthase factor IF1 and the lack of glucose stimulation of secretion of insulin upon IF1 silencing in model pancreatic beta cells (*FEBS Lett. 2018 592:999-1009*). *Note: The work was done entirely by our team.*

We found that also the inhibitory factor IF1 of the mitochondrial ATP-synthase belongs to the key proteins, ensuring the physiological range of the glucose sensor. The IF1 slightly inhibits synthesis of ATP, thus setting the range for elevation of phosphorylating to non-phosphorylating respiration ratio vs. glucose and insulin release vs. glucose above 3 mM glucose, with half-activation at ~4 to 5 mM and saturation above 11 mM glucose. When such a slight in vivo inhibition was largely cancelled using the silencing of IF1, the elevation of respiration and OXPHOS occurred atvery low glucose concentration approaching to zero. Simultaneously, the half-activation for the insulin release dose-response was shifted down to the range of 0–2 mM glucose in INS-1E cells. In January 2020 (Kahancová et al. Sci. Rep. 2020; 10, 1551. doi 10.1038/s41598-020-58411-x), we published that, in contrast, the over-expression of IF1 in INS-1E cells substantially blocked GSIS. Thus IF1 is setting the physiological range of insulin release as a response on glucose concentration.



We also revealed that the stimulatory effect of protein kinase A (PKA) on GSIS is diminished in IF1-silenced cells, pointing to a possible role of IF1 in the PKA signaling pathway in pancreatic β-cells. IF1 phosphorylation on serine-39 by PKA can prevent its binding to the ATP synthase. INS-1E cells incubated for 12 hours with 100 IM dibutyryl cAMP (a cell permeable form of cAMP, activating PKA) exhibited 54% increase in ATP cellular levels, whereas in the IF1-silenced cells no significant increase in the already elevated levels was observed. Similarly, 100 microM dbcAMP after 12 hr caused a 71% upregulation of insulin release, whereas in IF1- silenced cells no significant upregulation after thistreatment was found.

Fig.10. Left-shift in insulin release dose-response on glukose upon IF1 silencing: (*Top*, A,B) dose-response for GSIS assayed in KRH buffer containing 0.1% BSA. Insulin secretion was measured in INS-1E cells incubated for 1 h with various glucose concentrations ranging from 0 to 20 mM. *Bottom*: Cellular ATP levels in IF1-silenced (IF1-si) and control cells transfected with scrambled siRNA (CTRL-si) were measured by the biolu-minescence assay.

8) Revealing of the reversal of phenomenon of point 3): i.e. **mitochondrial cristae narrowing** upon a high respiration **substrate load** to the hypoxia-adapted cancer cells (*BBA2019 1860: 659-678*). *Note: The work was done entirely by our team.*

The inner mitochondrial membrane (IMM) is highly invaginated into cristae. We hypothesized that the more flat crista rims (edges) exist, the higher orthogonal dimensions of cristae are established, i.e. observed as larger cristae width in transmission electron microscopy (TEM) images (Fig.11). In contrast, the higher sharpness of cristae edges would be determined by stabilization (strengthening) of the rows of ATP-synthase dimers. Consequently, the assembly of ATP-synthase monomers, their dimerization, and stabilization within the rows at crista rims, all such factors should determine the efficiency of ATP synthesis, similarly as the

organization into tetramers. The simplest destabilization may result from transversal movement of dimers which are not connected by small “glue“ proteins.

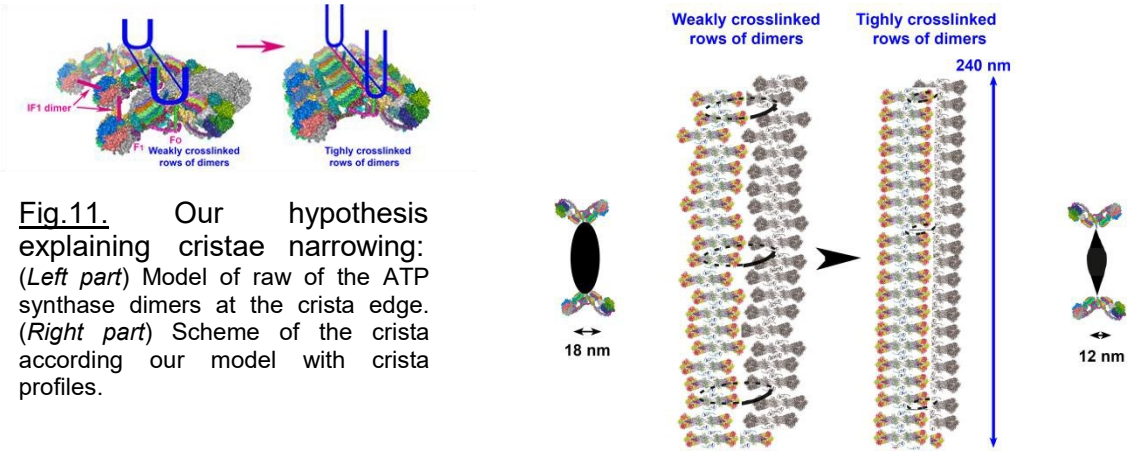
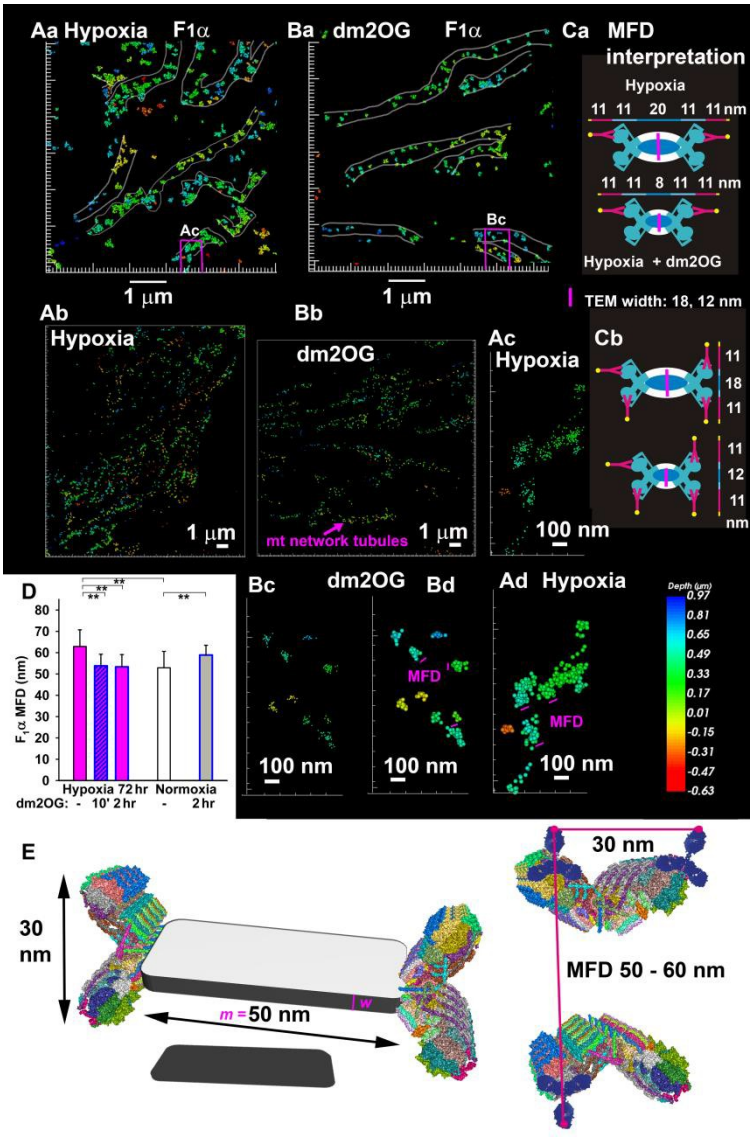


Fig.11. Our hypothesis explaining cristae narrowing: (*Left part*) Model of raw of the ATP synthase dimers at the crista edge. (*Right part*) Scheme of the crista according our model with crista profiles.



In our article, we supported the above hypothesis. Attempting to accelerate metabolism by the addition of membrane-permeant dimethyl-2-oxoglutarate (dm2OG) to HepG2 cells pre-adapted to hypoxia, we found cristae narrowing by transmission electron microscopy. Glycolytic HepG2 cells, which downregulate hypoxic respiration, instantly increased respiration with dm2OG.The spatial relocations of key cristae-shaping proteins were indicated by dSTORM:
i) while analyzing histograms of inter-antibody-distances between F1-α primary Alexa-Fluor-647-conjugated antibodies. Thesedata showed that the ATP- synthase dimers exhibited ahigher fraction of shorter inter- distances after dm2OG dose, indicating cristae narrowing.

Fig.12. Most frequent distances (MFD) between F1α correspond to external size of cristae: ATP-synthase F1α antibody inter-distances and interpretation of MFD. **A**,**B**) 3D dSTORM projections with primary Alexa Fluor 647-conjugated ATP-synthase F1α subunit, after 72h of hypoxia at 5% O_2 (**A**); or after subsequent 10-min incuba- tion with 4mM dm2OG (**B**). Panels **C**,**E**) show interpretation; panel **D**) average MFDs.

ii) similar inter-antibody-distances between Mic60/mitofilin showed ots clusters established upon hypoxia, which decayed after dm2OG, thus restoring isotropic randomMic60/mitofilin distribution (a signature of normoxia). *iii*) outer membrane SAMM50 formed more focused clusters. Moreover, less abundant fractions of higher ATP-synthase oligomers of hypoxic samples on blue-native electrophoresis became more abundant fractions at the high dm2OG load and at normoxia. This indicates more labile ATP-synthase dimeric rows established at crista rims upon hypoxia, strengthened at normoxia or dm2OG-substrate load. In conclusion, we demonstrated that cristae morphology changes even within minutes.

Translational research on development of upconversion nanoparticles for infra-red photodynamic therapy of tumors (*Czech patent No PV 2017-657; PCT/IB2018/057981 and EU patent EP 18822125.3; plus articles in Nanoscale 2015 7(43): 18096-18104;ACS Appl MaterInterfaces. 2016 8(31): 20422-20431*) and numerous other articles.

Photodynamic therapy of malignant tumors by a visible light exhibits light penetration depth of maximum 2 cm into skin or tissues. To overcome this disadvantage, infra red light is required, either 980 nm penetrating to 4 cm or 810 nm penetrating up to 5 cm. Light upconversion nanoparticles having the ability to transfer light of 980 nm to the desired excitation of photosenzitizer for photodynamic therapy can be used in a nanoparticle drug formulation for intravenous application. Hence, we have developed "NanoONKO" nanoparticle drug formulation for the infrared photodynamic therapy of tumors based on a liposome nanoparticle mixture containing hydrophobic hydroxy-aluminum phthalocyanine.

Our work extended our previous patents (EU patents awarded: EP 18822125.3 and EP 07817403.4; cover of five countries to be selected Canadian patent awarded: 2.665.762; Norwegian patent awarded: 20091595; all based on WIPO PCT/CZ2007/ 000107 and CZE patent CZ 298 978). Using a patented procedure of microfluidization for $NaYF_4$-Yb-Er upconversion nanoparticles mixed with a micronized powder of water-insoluble hydroxy-aluminum phthalocyanine microcrystals and with amorphous pharmaceutical grade lecithin in a desired buffer solution. Resulting nanoparticle/liposomal suspension is intended for intravenous application. In preclinical testing on nude mice with xenotransplanted human tumors, our patented preparation intravenously applied to the tail vein exhibited an optimum drug-to light time interval of 10 to
60 min when irradiated by medical 3 mW 980 nm laser directly onto tumors. Resulting efficiency for remission of amelanotic melanoma C32 and colon carcinoma HCT-116 was nearly 100% in certain cases and in remaining mice of testing groups significantly retarded tumor growth. Thus a proof of principle has been achieved for the infra red photodynamic therapy of tumors. Currently, similar procedure is applied to the Nd-containing 800 nm upconversion nanoparticles with liposomes containing the approved drug Foscan. This set-up will help commercialization of the patent.

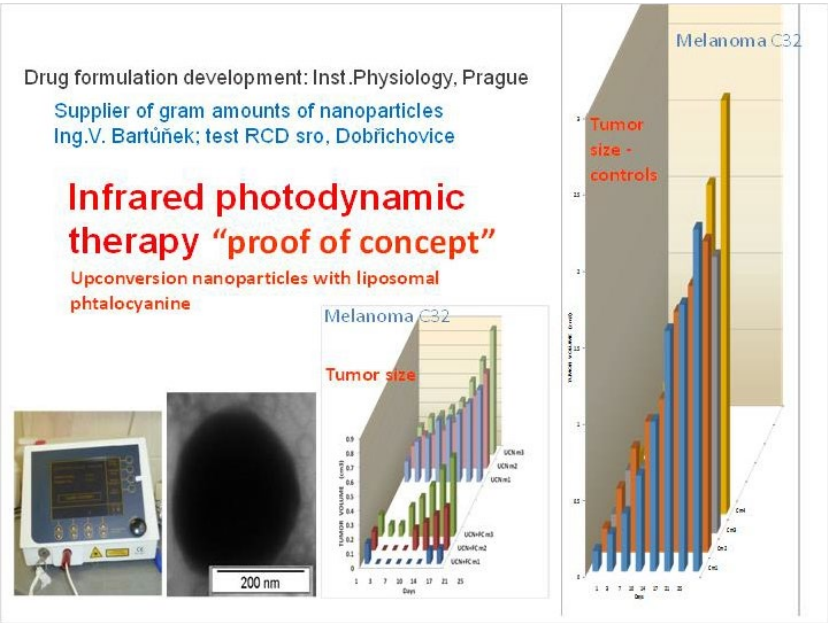


Fig.13. Infra red photodynamic therapy: *Left*: 980 nm laser; *middle*: nanoparticle; right: melanoma size after treatmentvs. in irradiated controls without the photosenzitier. Nude mice were xenotransplanted.

Research activity and characterization of the main scientific results

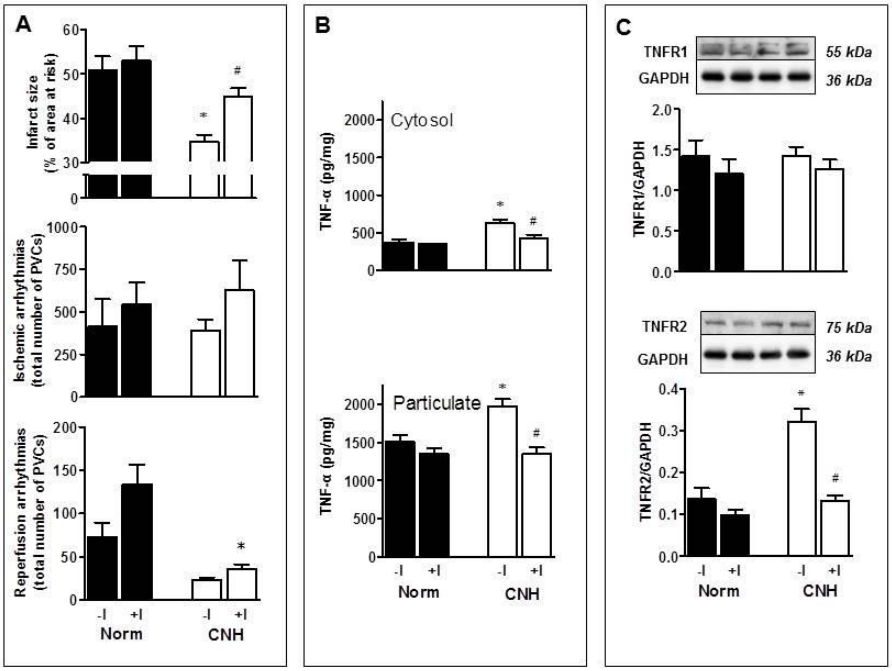
During the evaluated period of 2015-2019, the team members published 68 papers injournals with impact factor and 4 papers in other periodicals. The following is the description of the selected major results.

1. Cardiac ischemic tolerance of chronically hypoxic hearts

Chronic hypoxia (CH) 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. Prolonged exposure to hypoxic environment leads to adaptation which is associated with improved cardiac tolerance to acute oxygen deprivation. We focus on molecular mechanisms underlying the long-lasting cardioprotective effects ofCH that are the subject of 19 papers published during the last 5 years.

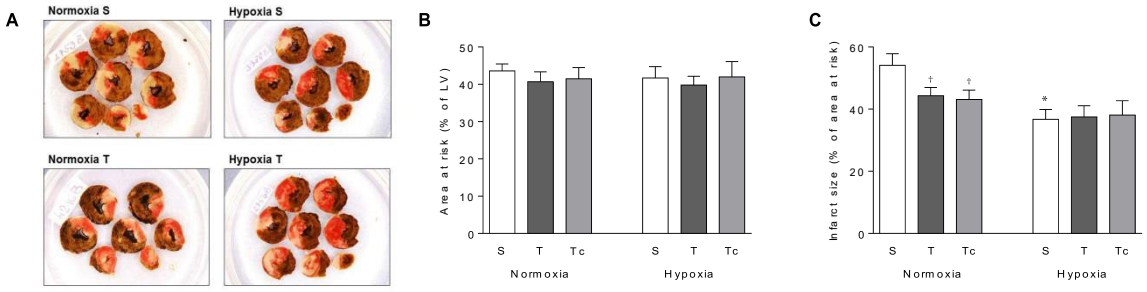
1.1. Cardioprotective mechanisms of chronic hypoxia

Tumor necrosis factor-α (TNF-α) represents a key pro-inflammatory cytokine playing a central role in initiating and sustaining inflammation that is involved in pathophysiology of cardiovascular diseases including myocardial infarction (MI) and heart failure. We show that TNF-α not only exerts detrimental effect on the heart, but also plays a role in the induction of ischemia-resistant cardiac phenotype of CH rats by activating protective redox signaling. Chronic treatment of CH rats by TNF-α antibody infliximab abolished the infarct size (IS)-limiting effect which was dependent on the activation of TNF-α type-2 receptors and antioxidant defense response (Chytilova et al., *Acta Physiol* **214**: 97, 2015). This study was supported by the Czech Science Foundation (grant #303/12/1162, PI: F. Kolar, and #13- 10267S, PI: J. Neckar).



The effect of infliximab (I) on myocardial infarct size, the total number of premature ventricular complexes (PVCs) during 20 min of ischemia and the number of PVCs during the first 3 min of reperfusion (**A**), levels of TNF-α in cytosolic and particulate fractions of left ventricle (**B**), and left ventricle level of TNF-α receptor 1 (TNFR1) and receptor 2 (TNFR2) in rat hearts adapted to continuous normobaric hypoxia (CNH) and normoxiccontrols (Norm). Representative Western blots of TNFR1 and TNFR2 are shown; GAPDH was used as a loading control (**C**). Values are means ± SEM; *P<0.05 vs. corresponding normoxic group; #P<0.05 vs. corresponding untreated group.

Besides CH, regular exercise training represents another natural stimulus that confers sustainable cardioprotection against I/R injury but it is unknown whether they can act in synergy to enhance ischemic resistance. We showed that that rats subjected to regular exercise during continuous exposure to hypoxic atmosphere exhibited the same infarct- sparing effect as their sedentary counterparts. CH led to pro-inflammatory response, increased myocardial expression of several related potentially protective mediators andantioxidant enzymes while none of these effects were observed in the rats exercising at room

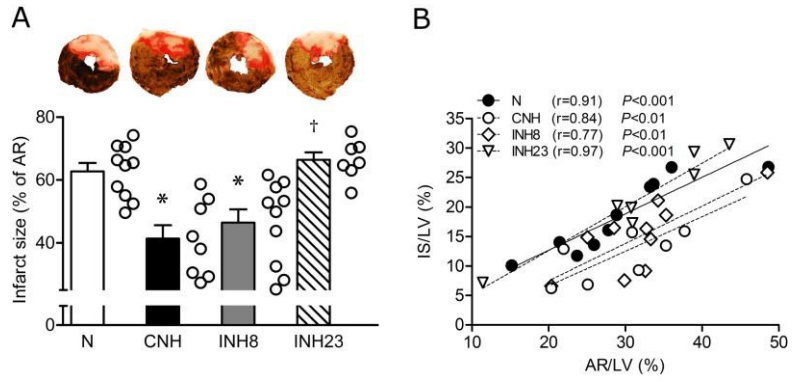


Myocardial infarction induced by coronary artery occlusion and reperfusion in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Typical images **(A)**, the size of area at risk expressed as a percentage of the left ventricle **(B)**, and infarct size expressed as a percentage of the area at risk **(C)**. Tc denotes the subgroups of T rats well compliant to exercise training. Values are means ± SE; *P < 0.05 vs. corresponding normoxic group; †P < 0.05 vs. corresponding sedentary group.

air. The maintenance of ischemia-resistant cardiac phenotype in CNH combined with exercise can be likely attributed to the persisting increase in myocardial antioxidant defense capacity despite attenuating the CNH-induced myocardial inflammatory response and TNF-α-dependent cardioprotective signaling (Alanova et al., *J Appl Physiol* **122**: 1452, 2017). This study was supported by the Czech Science Foundation (grant #303/12/1162, PI: F. Kolar).

Earlier we demonstrated that mitochondria play an essential role in increased cardiac ischemic tolerance conferred by various protective stimuli, including CH. The following study showed that CH decreased myocardial IS in spontaneously hypertensive rats (SHR), the animal model for human essential hypertension of neurogenic origin. The IS-limiting effect was accompanied by decreased sensitivity of cardiac mitochondrial permeability transition pore (MPTP) to opening and increased reserve COX capacity. It shows that CH can improve cardiac ischemic tolerance in high-risk hypertensive rats. More importantly, in the hypoxic SHR-mt^{BN} conplastic strain harboring a mitochondrial genome of the more ischemia-resistantBrown Norway strain, a stronger IS-limiting effect, lower sensitivity of MPTP to opening and improved mitochondrial respiration were observed compared with SHR adapted to . These priority results indicate that mitochondrial DNA can modulate CH-induced cardioprotective response to ischemia (Neckar et al., *Clin Sci* **131**: 865, 2017). Understanding the role of the mitochondrial genome in ischemic heart disease can help to design new cardioprotective strategies and their translation into clinical practice. The study was supported by the Czech Science Foundation (grant #13-10267S to J. Neckar).

We showed earlier that the outcome of CH in terms of cardiac ischemic tolerance strongly depends on a regimen of adaptation andon a signaling role of reactive oxygen species (ROS). To further analyze the underlying mechanism, we compared myocardial redox state in rats adapted to protective and un-protective regimens of CH. The protective regimens of CNH and INH8 decreased myocardial IS and increased the expression ofantioxidant enzymes which maintained unchanged GSH/GSSG ratio. Antioxidants activated under the protective regimens (MnSOD, GSR, TXN2/TXRD2 and PRX2) were mostly located in mitochondria, except for PRX2. Adaptation of rats to a CH regimen which enhanced the expression of only one antioxidant (PRX5) was not sufficient to induce cardioprotection. It can be concluded that increased cytosolic PRX2 and GSR together with mitochondrial MnSOD, TXN2/TXRD2 and GSR likely play an important role in cardioprotection conferred by long-term adaptation to CH (Kasparova et al., *Physiol Genom* **47**: 612, 2015). This study was performed in cooperation with colleagues from the Faculty of Science, Charles University, and supported by the Czech ScienceFoundation (grant #303/12/1162, PI: F. Kolar). The team essential contribution consisted of providing experimental model of CH and performing acute ischemia experiments.



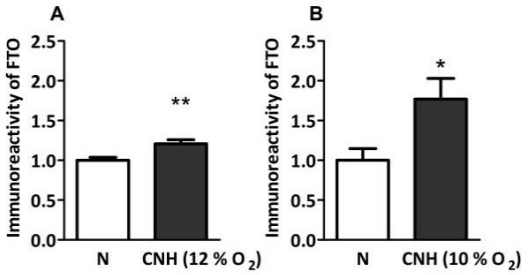
Myocardial infarct size (IS) expressed as a percentage of the area at risk (AR) **(A),** relationship between the area at risk (AR) and infarct size (IS), both expressed as a percentage of the left ventricle (LV) **(B)** in ratsexposed to continuous (CNH) and intermittent (INH8 and INH23) normobaric hypoxia and compared with animals kept at normoxia (N). Values are means ± SEM; **P*<0.05 vs. N; #P<0.05 vs. INH8 and CNH.

Other major results of studies concerning the cardioprotective mechanism of CH, published during the evaluated period, addressed the roles of protein kinase C-ε (Holzerova et al., *Physiol Res* **64**: 191, 2015), protein kinase B/hexokinase-2 pathway (Kolar et al., *Mol Cell*

Biochem **432**: 98, 2017; Waskova et al., *J Appl Physiol* **119**: 1487, 2015), NO/cGMP signaling (Alanova et al., *Physiol Res* **64**: 737, 2015), cytosolic phospholipase A2α/cyclooxygenase-2 pathway (Micova et al., *Mol Cell Biochem* **423**: 151, 2016, *Can J Physiol Pharmacol* **95**: 920, 2017), connexin-43 (Kohutova et al., *Front Endocrinol* **9**: 789, 2019) and opioid receptors (Maslov et al., *Clin Exp Pharmacol Physiol* **42**: 496, 2015;) etc. These studies were performed with a close collaboration with colleagues from the Faculty of Science, Charles University and Institute of Cardiology in Tomsk (the last paper). The team essential contribution consisted of providing experimental model of CH, performing acute ischemia experiments and heart function assessment.

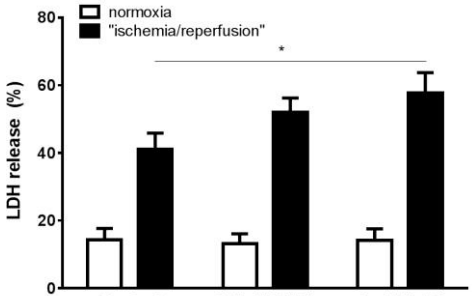
1.2. The role of Fat mass and obesity associated (FTO) protein

Fat mass and obesity associated (FTO) protein is an RNA-based epigenetic regulator (N6-methyladenosine (m6A) demethylase) known to affect energy balance and metabolic health. The presence or absence of m6A in mRNA affects its stability and plays a crucial role in the regulation of gene expression. Previous studies demonstrated that distortion of FTO equipoise might provoke a great number of heart diseases such as hypertrophic cardiomyopathy, ventricular septal and atrioventricular defects and coronary heart disease. In addition, a decrease of the FTO activity disturbs the polarization of ventricular cardiomyocytes and results in proarrhythmic remodeling.



Effect of adaptation to continual normobaric hypoxia (CNH; 3 weeks) (12% O_2) (**A**) and (10% O_2) (**B**) on FTO protein level in adult rat cardiac left ventricle tissue. N – normoxia; values are expressed as means ± SEM; n= 4-6; * P < 0.05; **P < 0.01.

In our study we tested hypothesis that perturbation of FTO activity affects expression of genes, cellular bioenergetics and ischemic tolerance of primary adult rat cardiomyocytes. We found that inhibition of FTO during simulated I/R increased cell injury. Under prolonged hypoxia FTO inhibition decreased cell viability compared to normoxic FTO inhibitor-treated cells. Metabolomic and proteomic analyses showed that FTO inhibition induces significant changes in metabolomic profiles and levels of proteins involved in various metabolic pathways. These findings support our hypothesis that FTO activity is important for ischemic tolerance of cardiomyocytes and plays an important role in regulating energetics in heart cells. Further, we studied the effect of FTO inhibition on cardiac ischemic tolerance in rats adapted to CH by regulating the shift in cellular energy metabolism. Our data show that CH increases myocardial FTO protein level proportionally to the degree of hypoxia. However, inhibition of FTO during CH exposure did not affect the extent of I/R injury. The manuscript has been finalized and prepared for submission). This study was supported by the Czech Science Foundation (grant #16-12420Y, PI: M. Hlavackova).



Effect of FTO inhibition during simulated ischemia/reperfusion (0.1% O2/12h; 4h reoxygenation) on primary adult rat cardiomyocyte injury measured by lactate dehydrogenase (LDH) release. FTOi – FTO inhibitor; values are expressed as means ± SEM; n= 8; * P < 0.05.

2. Cardiac ischemic tolerance and the progression of heart failure in diseased states

Systemic hypertension and diabetes are major risk factors of ischemic heart disease and its acute form, myocardial infarction (MI). These pathological states diversely interfere with mechanisms that protect the heart against acute oxygen deprivation. We study the effects of hypertension of various origin (genetic and renal) on the heart function and ischemic tolerance.

2.1. Cardioprotective action of epoxyeicosatrienoic acids

Current research into pathophysiological mechanisms of cardiovascular diseases provided series of evidences suggesting beneficial actions of soluble epoxide hydrolase (sEH) inhibition in several heart and kidney disorders. sEH is an enzyme responsible for rapid conversion of cytochrome P450 arachidonic acid epoxygenase metabolites, the epoxyeicosatrienoic acids (EETs) to inactive or less active compounds. Inhibitors of sEH (sEHi) represent a potential class of drugs for treating various cardiovascular diseases. As endogenous EETs are short-lived, several synthetic and more stable EET analogues with markedly longer half-life and promising cardioprotective actions have been developed. Therefore, we investigated whether the EET therapy based on EET analogues or sEHi can limit a harmful myocardial injury and remodeling associated with acute MI and the progression of post-MI chronic heart failure (CHF).

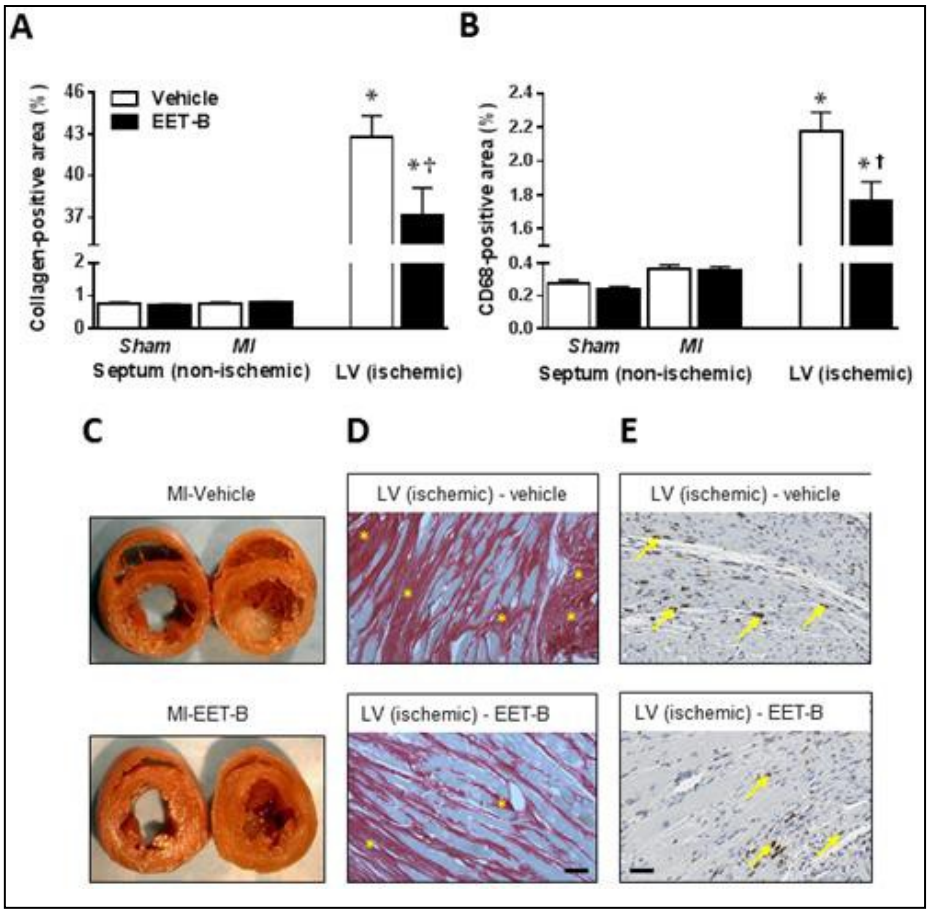
We showed that preventive treatment with sEHi (*c*-AUCB) and EET analogue (EET-A), given alone or combined 2 weeks before MI, decreased blood pressure and cardiac hypertrophy to a similar degree in hypertensive Ren-2 renin transgenic rat (TGR), a unique angiotensin II-dependent rat model of hypertension. With respect to cardiac ischemic tolerance, MI did not differ between untreated TGR and normotensive Sprague-Dawley (SD) controls, but the incidence of ischemia-induced ventricular fibrillations was higher in TGR. EET-based therapies (single or combined) had no IS-limiting effect in both strains but they were all equally effective in reducing life-threatening ventricular fibrillation in TGR. Therefore, preventive chronic treatment with either *c*-AUCB or EET-A exerts distinct antihypertensive and antiarrhythmic actions in hypertensive TGR (Červenka et al., *J Hypertens* **36**: 1326, 2018). This study was supported by the grant of Czech Science Foundation (grant #15- 07544S, Co-PI: J. Neckář). The contribution of team members and the collaborating group from Institute of Clinical and Experimental Medicine in Prague to these results were equal.
In the subsequent study, the same EET-based therapies (*c*-AUCB, EET-A or combination) were administered for 5 weeks starting 24 h after MI in TGR and SD strains. The therapeutic administration of combined EET-based therapy after MI slowed down the progression of post-MI CHF in SD rats, improved left ventricle function and geometry assessed by echocardiography and catheterization. As compared to normotensive SD rats, neither single nor combined treatment by EET analogue and sEHi affected the progression of post-MI CHF in hypertensive TGR (Hrdlička et al., *Front Physiol* **10**: 159, 2019). This study was supported by the Czech Health Research Council (grant #15-27735A, PI: J. Neckář). The contribution of team members to these results was predominant.

The effects of EET analogue EET-A on blood pressure and acute MI were also examined in two-kidney, one-clip (2K1C) Goldblatt hypertensive rats during sustained phase of hypertension. We found that MI was significantly smaller in untreated 2K1C rats as compared with normotensive controls and EET-A reduced it in controls but not in 2K1C rats. Our findings suggest that during the phase of sustained hypertension 2K1C Goldblatt hypertensive rats exhibit increased cardiac tolerance to I/R injury as compared with normotensive rats, and that EET-A treatment does not induce any antihypertensive andcardioprotective actions in this animal model of human renovascular hypertension (Alánováet al., *Vasc Pharmacol* **73**: 45, 2015). This study was supported by the grant of Czech Science Foundation (grant #15-07544S, Co-PI: J. Neckář). The contribution of team

members and the collaborating group from Institute of Clinical and Experimental Medicine were equal.

In another study, cardioprotective effects of structurally different agonistic EET analogue EET-B was examined in another model of hypertension, SHR. We showed that continuous EET-B treatment before and after MI reduced post-MI mortality and systolic dysfunction progression. Cardioprotective actions ofr EET-B treatment were associated with diminished CHF-induced lung edema, reduced myocardial fibrosis, decreased monocytes and macrophages infiltration in the ischemic area, and increased heme oxygenase-1 immunopositivity in viable cardiomyocytes after MI. The data indicate that EET-B attenuates CHF progression without altering blood pressure in SHR with established hypertension(Neckář et al., *Clin Sci* **133**: 939, 2019). This study was supported by the Czech Health Research Council (grant #15-27735A, PI: J. Neckář). The contribution of team members and the collaborating group from Medical College of Wisconsin in Milwaukee to these resultswere equal.

Further, we investigated the cardioprotective action of acute administration of EET-B at



Quantification of myocardial collagen-positive area **(A)** and CD68-positive area **(B)** assessed in non-ischemic septum, and ischemic area of left ventricle (LV) of vehicle- and EET-B-treated SHR subjected to myocardial infarction (MI) and Sham-operated groups. Macroscopic images of heart cross-sections **(C)**, photomicrographs of Picro-Sirius Red staining of collagen positive areas (carmine red marked by stars) **(D),** and immunohistochemical staining depicting macrophage/monocyte (CD68-positive; dark brown dots marked by arrows) **(E)**. Rats were treated by vehicle or EET-B from two weeks before till seven weeks after MI. Scale bars represent 50 μm. Values are means ± SEM from 6-10 rats in each group. **P*<0.05 vs. Sham-Vehicle group. †*P*<0.05 vs. MI-Vehicle group.

reperfusion in normotensive SD rats. Experiments were also carried out to determine the mechanism of cardioprotection afforded by EET-B in relation to a role of hypoxia inducible

factor-1α (HIF-1α) and its degrading enzyme prolyl hydroxylase 3 (PHD3). We showed that EET-B administered before reperfusion markedly reduced myocardial IS. Co-administrationof EET-B with 14,15-EEZE, a 14,15-EET antagonist, abolished the IS-limiting effect afforded by EET-B. These findings confirmed that EET-B is an effective agonistic 14,15-EET analogue. In addition, HIF-1α inhibitors completely abolished the cardioprotective effect of EET-B. In ischemic area, HIF-1α immunoreactivity markedly increased at the end of 30-min ischemia and rapidly decreased during reperfusion. EET-B administration at the start of reperfusion blunted the fast decrease of HIF-1α immunoreactivity and significantly reduced PHD3 immunogenic signal in ischemic tissue following reperfusion. Our priority data indicate that increased HIF-1α levels play an important role in cardioprotection mediated by EET-B at reperfusion likely by mechanism(s) including down regulation of HIF-1α-degrading enzyme PHD3 (Neckář et al., *Am J Physiol Heart Circ Physiol* **315**: H1148, 2018**)**. This study was supported by the grant of Czech Science Foundation (grant #15-07544S, Co-PI: J. Neckář and 18#03207S, PI: J. Neckář). The contribution of team members and the collaborating group from Medical College of Wisconsin in Milwaukee to these results were equal.

2.2. Other major results concerning heart failure

We also contributed to the international project analysing detailed necroptotic signalling in infarcted and non-infarcted myocardial areas and its mechanistic link with main features of CHF. Briefly, it demonstrated that the activation of RIP3 protein in infarcted area proceeds to MLKL (a terminal pro-necroptotic protein) signalling to further terminate in necroptosis and inflammation while in the non-infarcted area the activation of RIP3 can solely mediate pro-inflammatory phenotype without necroptosis execution. (Lichy et al., *J Cell Mol Med* **23**: 6429, 2019). This study was supported by the Czech Health Research Council (grant #15- 27735A, PI: J. Neckář). The team members contributed to these results by performing experimental model and echocardiography analysis of heart function.

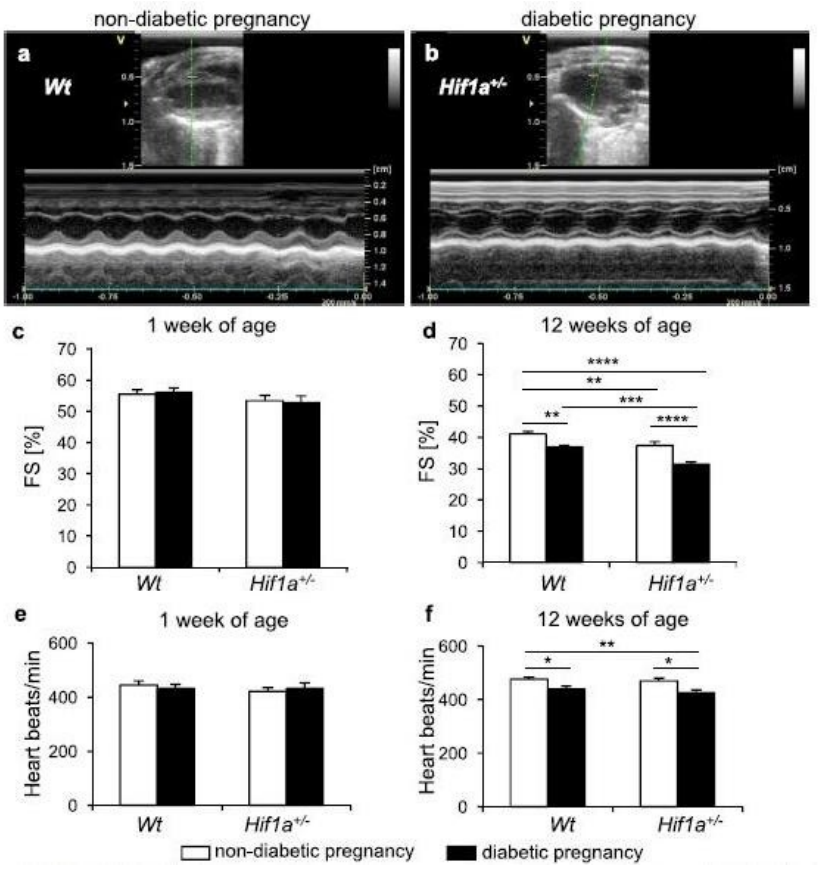
Our team also participated in the study dealing with CHF induced by volume overload due to aorto-caval fistula. Electrophysiological measurements and optical mapping revealed significant alterations of action potential parameters. At the protein level, we confirmed a significant decrease in total and phosphorylated connexin 43 level that was proportional to hypertrophy. Severity of morphological phenotype correlated with progression of molecular and electrophysiological changes, with the most hypertrophied hearts showing the most severe changes that might be related to arrhythmogenesis. (Sedmera et al., *Front Physiol* **7**: 367, 2016). This study was supported by the Czech Health Research Council (grant #15- 27735A, PI: J. Neckář). The contribution of team members and the collaborating groups fromInstitute for Clinical and Experimental Medicine and Charles University to these results were equal.

We contributed by the analyses of the toxicity of clinically used proteasome inhibitors (PIs) alone (bortezomib (BTZ), carfilzomib (CFZ)) as well as their combinations with an anthracycline (ANT (daunorubicin (DAU)) in adult ventricular cardiomyocytes to the study performed at the Faculty of Pharmacy in Hradec Králové, Charles University. PIs used in cancer treatment have been associated with the risk of cardiotoxicity and CHF, and there is even a recent clinical trend to combine them with ANTs with known cardiotoxic potential. Thisexperimental study suggests that unlike immature cardiomyocytes, adult cardiomyocytes are less sensitive *in vitro* to the cardiotoxicity of BTZ or CFZ and, in particular, that neither PI augments the risk of HF induced by ANTs *in vivo* in an animal model. The combination of PIswith ANTs is not accompanied by exaggerated risk of cardiotoxicity and CHF in young adult animal cardiomyocytes and hearts, which may be a promising message for the treatment of refractory types of cancer (Pokorna et al., *Clin Sci* **133**:1827, 2019).

3. Embryonic and early postnatal cardiac development

Hypoxia-inducible factor 1 (Hif-1) is the master regulator of transcriptional responses of cells to decreased oxygen availability. In tight cooperation with the Institute of Biotechnology (G. Pavlinkova) and the First Medical Faculty we participated in a complex study which showed that genetic mutation of the Hif-1α gene suppresses the embryonic development of preganglionic and postganglionic neurons of the sympathetic nerve system and negatively affects sympathetic innervation of the heart that plays a primary role in the regulation of heart rate and contractility. Mice with conditional deletion of Hif-1α gene exhibited coronary artery anomalies and decreased cardiac contractile function. These priority data indicate that deregulation of the transcription factor Hif-1α can result in serious cardiovascular diseases associated with the autonomic nervous system dysbalance and open the way to a development of new therapeutic strategies. This project resulted in the prestigious publication with the 2019 Nobel Prize winner, Greg Semenza (Bohuslavova et al., *PNAS USA* **116**: 13414, 2019). Members of the team performed echocardiographic examination of heart function, micro-CT analysis, detection of coronary artery branching and histochemical analysis of sinoatrial node development.

In another hypoxia-related cooperative study we examined whether Hif-1α, in combination with exposure to a diabetic intrauterine environment, influences the function and molecular structure of the adult offspring heart. In a mouse model of maternal diabetes exposure, HIF-1α heterozygous loss-of-function was associated with impaired cardiac function and structural reprogramming of the heart of offspring, including decreased macrophage migration, increased accumulation of AGEs, and altered Vegfa expression. Since the HIF-1 system is compromised in the diabetic environment, a failure to adequately express and activate HIF-1α regulation provides a molecular mechanism that may contribute to the cardiac dysfunction seen in the offspring. These findings provide compelling evidence that a global reduction in Hif1a gene dosage increases predisposition of the offspring exposed to maternal diabetes to cardiac dysfunction, and also underscore Hif1a as a critical factor in the fetal programming of adult cardiovascular



Representative M-mode recordings of LV structures in long axes viewin **a** *Wt* males from non-diabetic pregnancy and **b** *Hif1a*$^{+/-}$ males from diabetic pregnancy. **c**, **d** The echocardiographic evaluation of LV systolic function, fractional shortening (FS), in the 1- and 12-week-old male offspring. **e**, **f** The heart rate in *Wt* and *Hif1a*$^{+/-}$ males from both diabetic and non-diabetic pregnancies at 1 and 12 weeks of age. The values are mean ± SEM *Significant differences by post hoc Tukey's multiple-comparison test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

disease (Cerychova et al., *Cardiovasc Diabetol* **17**: 68, 2018. The study was supported by the Czech Science Foundation (grant #16-06825S, Co-PI: F. Kolar). Team members performed echocardiographic examination of heart function and characterized myocardial morphology and ultrastructure.

Evidence suggests that FTO may act during the early stages of life when energy homeostasis is first established. The first step in this recently launched project was to determine the expression profile of FTO protein and other proteins responsible for theepigenetic modification, methylation/demethylation of mRNA, in rat heart during early postnatal development and in adulthood. Left ventricles of rat`s offspring were collected on postnatal days (d) 1, 4, 7, 10, 12, 14, 18, 21, 25, 28 and 90. The protein level of FTO displayed a steady decrease from d1 to d90 in both male and female hearts. Interestingly,the level of FTO in female heart tissue was significantly lower compared to males. Both demethylase AlkB Homolog 5 (AlkBH5) and methyltransferase like 3 (METTL3) exhibited a dramatic decline of their levels from d1 to d4 with a subsequent steady decrease from d4 to d90. These results showed that the decrease of both writers and erasers of methyl modification occurs in rat hearts during the early postnatal development. Moreover, we recorded the presence of sex-related differences in levels of proteins responsible for the epigenetic modification during the early postnatal development. This study is supported by the Czech Science Foundation (grant #19-04790Y, PI: M. Hlavackova).

We showed for the first time in developing monitor hearts that two septa, the ‘muscular ridge’ and ‘bulbuslamelle’, express the evolutionarily conserved transcription factors Tbx5, Irx1 and Irx2, orthologues of which mark the mammalian ventricular septum. This correlates with presence of preferential conduction pathway in the septum, a unique feature among squamates (Hanemaaier et al., *Development* **146**: dev177121, 2019). These results suggest, together with our studies on the Crocodylians, that the presence of specialized conduction system in the vertebrate ventricle is linked to a morphological “hardware” – the ventricular septum, rather than homeiothermy, as was previously postulated and believed since 1940s. The study was performed in cooperation with the Amsterdam University Medical Centre (UMC) and our team provided the embryos and performed the functional analysis of their hearts using optical mapping. The study was supported by the Czech Science Foundation (grant #16-02972S, PI: D. Sedmera).