3. znanstveno-stručni simpozij Hrvatskog društva za znanost o laboratorijskim životinjama i
2. zajednički skup CroLASA-e i SLAS-a s međunarodnim sudjelovanjem

POKUSNE ŽIVOTINJE U ZNANSTVENIM ISTRAŽIVANJIMA

LABORATORY ANIMALS IN SCIENTIFIC RESEARCH

3rd Congress of Croatian Laboratory Animal Science Association (CroLASA) and
2nd joint CroLASA and Society for Laboratory Animals of Slovenia (SLAS) Congress with international participation

25. i 26. listopada 2018. / 25th and 26th October 2018
Dvorana Matice hrvatske / Matica hrvatska hall,
Matice hrvatske 2, Zagreb

CroLASA

SLOVENSKO DRUŠTVO ZA LABORATORIJSKE ŽIVALI

KNJIGA SAŽETAKA / BOOK OF ABSTRACTS

3. znanstveno-stručni simpozij Hrvatskog društva za znanost o laboratorijskim životinjama i
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"Pokusne životinje u znanstvenim istraživanjima"

3rd Congress of Croatian Laboratory Animal Science Association (CroLASA)
and
2nd joint Congress of CroLASA and Society for Laboratory Animals of Slovenia (SLAS)

"Laboratory Animals in Scientific Research"

Zagreb, 25. – 26. 10. 2018

Radionica / Workshop

Radionica o klasificiranju i izvješćivanju o težini pokusa pod EU direktivom 2010/63/EU

Workshop on the Severity Classification and Reporting Under EU Directive 2010/63/EU

26. 10. 2018.

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Croatian Society for Laboratory Animal Science (CroLASA)

Organizacijski odbor / Organizing Committee

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Mjesto održavanja / Venue

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Matice hrvatske 2, 10000 Zagreb

Sadržaj / Content

FELASA CELEBRATES ITS 40TH ANNIVERSARY

The Federation of European Laboratory Animal Science Associations (FELASA) was founded in 1978. by the representatives from United Kingdom (LASA), Scandinavian countries (Scand-LAS), Germany and Austria (GV-SOLAS), who at that time recognized the need for integrated, coordinated and professional work in the field of laboratory animal science within Europe.

Over the years, FELASA has grown and become an umbrella organization of all European national associations, acting not only as their strong representative, but also as a single voice of experts in the field of animal welfare and experimental animal science.

In June 2007, Croatian Society for Laboratory Animal Science (CroLASA) joined FELASA as a full member. Today, FELASA consists of 21 associations from 28 countries, encompassing 4,000 experts and scientists seeking to spread and advance the laboratory animal science and responsible research work, with focus on animal welfare and 3R principles.

FELASA as an organization works on a democratic basis. Members of the Governing Board make decisions on current affairs and orientations. Each full-fledged association has representatives in the Management Board, which proposes to the Board of Expertise topical issues in the field of work with experimental animals. Executive Board consisting of elected representatives is responsible for the execution of decisions, the preparation of working materials and reports, and other various tasks.

FELASA promotes and coordinates the development of laboratory animal science and good practices in Europe through participation in numerous activities (legislation, professional, scientific and civil initiatives), as well as networking with other national, international associations (ICLAS, AALAC, AALAS) and legislative bodies (Council of the EU, EU Commission).

FELASA also provides basic and continuous education. Through various working groups, it publishes guidelines, recommendations and reports on current challenges regarding work with experimental animals. Each association can propose an expert from his own country, as its representative in the working group. Every three years, FELASA organizes a congress, which gathers around 2,000 people from all over the world (mostly Europe). In addition, it organizes many other activities, including harmonization, accreditation of courses, workshops, etc.

PROGRAM / PROGRAMME

Četvrtak 25. listopad 2018. / Thursday 25th October 2018.

VRIJEME / TIME	TEMA / TOPIC	PREDAVAČ / SPEAKER
8:30 – 9:15 Predvorje / Entrance hall	REGISTRACIJA / REGISTRATION	
9:15 – 9:30 Velika dvorana / Large hall	Otvaranje Simpozija / Opening ceremony	
9:30 – 10:15 Velika dvorana / Large hall	Nova uloga starog igrača: IFNɣ posredovana komunikacija imunološkog i endokrinog sustava kod virusnih infekcija / A new role of an old player: IFNɣ-mediated crosstalk between the immune and endocrine systems in viral infections	Bojan Polić
10:15 – 10:35 Velika dvorana / Large hall	Multimodalno oslikavanje kao važan alat za poboljšanje prekliničkih studija ishemijskog moždanog udara kod miša / Multimodal imaging as an important tool for improving mouse preclinical studies of ischemic stroke	Srećko Gajović
10:35 – 10:55 Velika dvorana / Large hall	PET oslikavanje u istraživanju animalnih modela / PET imaging in small animal models	Vladimir Farkaš
10:55 – 11:15 Mala dvorana / Small hall	PAUZA ZA KAVU / COFFEE BREAK	
11:15 – 11:35 Velika dvorana / Large hall	*In vivo* i *in vitro* modeli u istraživanju šećerne bolesti / *In vivo* and *in vitro* models in diabetes research	Marijana Popović Hadžija
11:35 – 11:55 Velika dvorana / Large hall	Eksperimentalni modeli traumatske ozljede mozga / Experimental models of traumatic brain injury	Kristina Pilipović

11:55 – 12:15 Velika dvorana / Large hall	Genska terapija u modelu animalnih tumora / Gene therapy in animal tumour models	Simona Kranjc
12:15 – 12:35 Velika dvorana / Large hall	Terapija matičnim stanicama u životinjskom modelu ozljede bubrega - pretklinička obećanja i izazovi za transplantaciju / Stem cell therapy in animal model of kidney injury-preclinical promises and challenges for translation	Željka Večerić Haler
12:35 – 12:55 Velika dvorana / Large hall	Metabolički sindrom, lipidna fiziologija – animalni i alternativni modeli / Metabolic syndrome, lipid physiology - animal and alternative models	Domagoj Đikić
12:55 - 13:15 Velika dvorana / Large hall	Napredna rješenja za nastambe laboratorijskih životinja /Advanced solutions for the lab animal facilities	Hrvoje Komac MMK trgovina Carlo Demalde Tecniplast & IWT
13:15 – 14:15 Mala dvorana / Small hall	RUČAK / LUNCH	
14:15 – 14:45 Velika dvorana / Large hall	3R – Tehnologija životinjskih stanica – dosezi i izazovi / 3R – Animal cell technology – Achievements and challenges	Igor Slivac
14:45 – 15:05 Velika dvorana / Large hall	Primjer 3R pristupa na modelu *D. melanogaster:* ispitivanje neurogenetike ovisnosti o psihostimulansima / Example of 3R approach in *D. melanogaster*: Investigating neurogenetics of addiction to psychostimulants	Rozi Andretić Waldowski
15:05 – 16:45 Mala dvorana / Small hall	POSTER SEKCIJA / POSTER SECTION PAUZA ZA KAVU / COFFEE BREAK	

16:45 – 17:05 Velika dvorana / Large hall	Standardi u znanstvenim istraživanjima / The standards in animal research	Martina Perše
17:05 – 17:25 Velika dvorana / Large hall	Odabrani primjeri spontane patologije i patologije starenja kod GEMs, NSG i NOD miševa / Selected examples of spontaneous pathology and pathology of aging in GEMs, NSG and NOD mice	Andrea Gudan Kurilj
17:25 – 17:45 Velika dvorana / Large hall	Unapređenje standarda u držanju laboratorijskih životinja na primjeru glodavaca / Evolving rodent housing. Improvement of standards in holding laboratory animals	Peter Hepburn Animal Care Systems
17:45 – 18:00 Mala dvorana / Small hall	Dodjela nagrade za najbolju postersku prezentaciju / The best poster presentation award Zatvaranje prvog dana / Closure of the first day	
18:00 - 19:00 Mala dvorana / Small hall	DRUŽENJE UZ VINO I SIR/ WINE & CHEESE PARTY	

Petak 26. listopad 2018. / Friday 26th October 2018.

VRIJEME / TIME	TEMA / TOPIC	PREDAVAČ / SPEAKER
8:30 – 9:10 Predvorje / Entrance hall	REGISTRACIJA / REGISTRATION	
9:10 - 9:40 Velika dvorana / Large hall	Opto- i farmakogenetski pristupi u animalnim modelima / Opto- and pharmacogenetic approaches in animal models	Dóra Zelena
9:40 - 10:00 Velika dvorana / Large hall	Retroaktivna procjena pokusa / Retrospective assessment of the project	Branka Bukvić Šošić
10:00 – 10:20 Velika dvorana / Large hall	Razvrstavanje pokusa po bolnosti / Classification of animal pain and distress levels	Daša Ševeljević Jaran
10:20 – 10:40 Velika dvorana / Large hall	Utjecaj različitih načina rukovanja na razvoj anksioznosti kod laboratorijskih miševa / Impact of three handling methods on the anxiety of three different strains of laboratory mice	Tatjana Pirman
10:40 – 11:10 Mala dvorana / Small hall	PAUZA ZA KAVU / COFFEE BREAK	
11:10 – 11:30 Velika dvorana / Large hall	Uloga optineurina u amiotrofičnoj lateralnoj sklerozi / The role of optineurin in Amylotrophic Lateral Sclerosis	Ivana Munitić
11:30 – 11:50 Velika dvorana / Large hall	Istraživanje na životinjama koje koriste pristupe sintetičke biologije / Animal research using approaches of synthetic biology	Duško Lainšček

11:50 – 12:10 Velika dvorana / Large hall	Modeli transgeničnih miševa i alternativni pristup za proučavanje nealkoholne bolesti masnih i hepatocelularnog karcinoma / Transgenic mice models and alternative approach for the study of non-alcoholic fatty liver disease and hepatacellular carcinoma	Kaja Blagotinšek Cokan
12:10 – 13:10 Mala dvorana / Small hall	RUČAK / LUNCH	
13:10 – 13:30 Velika dvorana / Large hall	Photoacoustic and Microultrasound Imaging in preclinical research with the Vevo LAZR System: Principles and Applications	Milan Kopeček FUJIFILM VisualSonics, Inc
13:30 - 15:00 Mala dvorana / Small hall	**WORKSHOP ON THE SEVERITY CLASSIFICATION AND REPORTING UNDER EU DIRECTIVE 2010/63/EU (1st GROUP)**	David Smith David Anderson FELASA
15:00 – 15:15 Mala dvorana / Small hall	PAUZA ZA KAVU / COFFEE BREAK	
15:15 – 16:45 Mala dvorana / Small hall	**WORKSHOP ON THE SEVERITY CLASSIFICATION AND REPORTING UNDER EU DIRECTIVE 2010/63/EU (2nd GROUP)**	David Smith David Anderson FELASA
16:45 - 17:00 Mala dvorana / Small hall	Zatvaranje Simpozija / Closing ceremony	

SAŽETCI IZLAGANJA / LECTURE ABSTRACTS

Example of 3R approach in *D. melanogaster*: Investigating neurogenetics of addiction to psychostimulants

Rozi Andretić Waldowski

Laboratory for Behavioural Genetics, Department of Biotechnology, University of Rijeka, Rijeka, Croatia

Because of the significant genetic homology to humans and similar organisation and function of various organs fruit fly *Drosophila melanogaster* has been used as a model organism for over a century. Basic principles of inheritance and mechanism of early embryonic development have been defined using *D. melanogaster*, while during the last two decades work on *D. melanogaster* contributed significantly to the understanding of genetic mechanisms of human disorders and diseases. These characteristics and discoveries show that *D. melanogaster* is a laboratory organism that satisfies recommendations of 3R approach (Replace, Reduce, Refine). Here we will present the use of *D. melanogaster* in studies that aim to define neurogenetic mechanisms underlying development of addiction to psychostimulant drugs, cocaine and methamphetamine. Oral or volatilized administration of cocaine or methamphetamine leads to arousing, activating and rewarding effects, which can be objectively quantified as a change in the behaviour. Repeated exposures indicate activation of mechanisms of neuronal plasticity measurable as appearance of locomotor sensitization. Rewarding aspect of psychostimulants can be quantified as preferential consumption of food with addition of cocaine or methamphetamine in comparison with food without addition of psychostimulants. These tests objectively quantify change in the behaviour induced by psychostimulants and are used as methods to study genetic influence on behaviour in approaches such as: genetic screen, selective breeding or transgenic approaches. There is a significant similarity in genetic mechanisms underlying development of addiction between *D. melanogaster* and mammals. Therefore, studies directed at defining how do genes influence behavioural change after psychostimulant exposure in *D. melanogaster* are a good example that the application of 3R principles does not restrict types or outcomes of studies that contribute to understanding of complex behavioural disorders in humans.

Keywords: *Drosophila melanogaster*, addiction, behaviour, psychostimulants

Transgenic mice models and alternative approach for the study of non-alcoholic fatty liver disease and hepatacellular carcinoma

Kaja Blagotinšek Cokan[1], **Martina Perše**[2], **Jera Jeruc**[3], **Uršula Prosenc Zmrzljak**[1], **Peter Juvan**[1], **Damjana Rozman**[1]

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Non-alcoholic fatty liver disease (NAFLD) has emerged as a common pathologic entity that ranges from chronic liver disease, cirrhosis towards hepatocellular carcinoma (HCC). Understanding of trigger metabolic characteristic of NAFLD-induced HCC is possible with the systemic research approach. We have shown previously that disrupted cholesterol synthesis, as one of metabolic dysfunction, leads to severe liver injury with oval cell response and fibrosis noticed in young adults, progressing in adulthood and finally resulting in HCC during aging in the hepatocyte knockout mouse model of lanosterol 14α-demethylase (Cyp51) ($H^{Cyp51-/-}$) from the late part of cholesterol synthesis. From 12 to 24 months old knockout mice we histologically identified the sustained inflammation, ductular proliferation and fibrosis with elevated plasma parameters confirming chronic liver damages. Moreover, the prevalence for tumours was 3-times higher for $H^{Cyp51-/-}$ females. The gene expression profiling of $H^{Cyp51-/-}$ mice clearly indicated the global transcriptome changes in activated pathways in cancer and extracellular matrix interaction while multiple metabolic pathways were severely dampened. Some of enriched genes and transcription factors in females suggest higher susceptibility to carcinogenesis. For hepatocytes specific oriented studies, we used another specific time-dependent Cyp51 ($H^{Cyp51doxy-/doxy-}$) knockout mice model for easier and sufficient hepatocytes isolation with in-vivo collagenase perfusion technique. Considering 3R, the immortalized hepatocyte cell line was designed with CRISPR/Cas9 technology, to deepen the understanding of cell specific molecular mechanisms of complex world liver problem.

Keywords: non-alcoholic fatty liver disease, cholesterol, hepatocellular carcinoma, transgenic mice models, cell lines

Retrospective assessment of the project

Branka Buković Šošić

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Retrospective assessment is formal assessment required by the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals. It is used at the end of the scientific projects with the purposes to determine, amongst other things, whether the objectives have been achieved and whether lessons can be learned to further the implementation of the 3Rs. The purpose of a retrospective assessment is to considered whether the programme of work has been carried out, whether the objectives of the programme of work have been achieved, the amount of harm caused to animals and if any lessons can be learnt from the programme of work which may contribute to the further implementation of the principles of 3Rs. Retrospective assessment of the project should improve animal welfare, ethics, quality of science and project management.

Keywords: retrospective assessment, harm/benefit analysis, principles of 3Rs

Metabolic syndrome, lipid physiology-animal and alternative models

Domagoj Đikić

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The most commonly used definition of the metabolic syndrome is a set criteria described by the WHO defining impaired fasted glucose level, glucose intolerance, insulin resistance, diabetes concomitantly expressed with at least two of the following symptoms: the hypertension (above 130/85 mmHg), hyperlipidaemia, central obesity (above 35 BMI) or microalbuminuria. The incidence of obesity and metabolic disorders such as metabolic syndrome in human population increases worldwide. Development of the biological models of research of the genetic, physiological, behavioural and other causes are imperative in the field of metabolism research. Beside research investigating the causes of metabolic syndrome development, other models study mechanisms by which physiological damage occur within organism after occurrence of metabolic disorders. Alongside elucidation of mechanisms of occurrence of metabolic syndrome, there are also studies of prevention and therapeutic strategies using novel drugs or natural compounds. Preclinical animal models are still a key research tool in the quest to combat obesity. Animal models are well developed over last 30-40 years and can be divided into different categories, such as models induced by diet or physiological factors (light, temperature, energy availability) or genetic (monogenic and polygenic) models. Majority of research on animals relies on premise that a primary causal factor is caused by the interaction of the brain with peripheral tissues (liver, gut, pancreas, white adipose tissue (WAT), brown adipose tissue (BAT)) or hormones such as leptin and insulin, etc. Within this lecture, we will present most common animal models (nutritional, genetic, epigenetic) and examples of possible alternative research models. More specifically, we will present the example of comparison of an *in vivo* and *in vitro* model of synergism of cholesterol accumulation and oxidative stress in the neurodegenerative research.

Keywords: obesity, metabolic syndrome, cholesterol, alternative metabolic models

PET imaging of small animal models

Vladimir Farkaš, Robert Bagarić, Alfred Švarc

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Preclinical imaging of small animal models represents indispensable researching tool used for studies of ethiology, pathophysiology and therapy of human diseases. One of the most used preclinical imaging modality is positron emission tomography (PET). PET represents an advanced nuclear imaging method, which allows tracking the distribution and kinetics of radiolabelled molecules *in vivo* and as a function of time. Due to resolution limitation, in the past PET was widely used only in clinical studies and occasionally in big animal models such as nonhuman primates. Development of the PET instrumentation allowed the use of the PET imaging on the small rodents as the most important animal models. PET imaging, as a non-invasive modality, allows that each animal can be used as its own control. This significantly reduces the number of animals required for the study, while at the same time increases the credibility of the study. In this lecture, PET instrumentation, basic principles of PET imaging, the planning of the experiment involving PET imaging, the image processing and the type of data obtained by PET imaging will be briefly described. Examples will be given from the application of PET imaging in animal models we have studied in our imaging facility - diabetes type I, Alzheimer disease, acute myocardial infarction, oxidative stress, lung fibrosis, hypothermia and others.

Keywords: PET imaging, preclinical imaging, small animal models

Multimodal imaging as an important tool for improving mouse preclinical studies of ischemic stroke

Srećko Gajović[1], **Dunja Gorup**[1], **Siniša Škokić**[1], **Marina Dobrivojević Radmilović**[1], **Anton Glasnović**[1], **Jasna Križ**[2]

[1]*Croatian Institute for Brain Research, University of Zagreb, School of Medicine, Zagreb, Croatia*
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The mouse model of the human ischemic stroke includes temporary occlusion of medial cerebral artery (MCAO) followed by the reperfusion. Although model is relevant to the human pathology, i.e. ischemic stroke in humans, there is substantial variability of the lesion and subsequent outcomes. Moreover, although this is a model of acute pathological event, the consequences and repair are long lasting and the outcomes develop during substantial period of time. Subsequently, the *in vivo* imaging represents a solution to long-term monitor and evaluate the consequences of the brain ischemia. Medial cerebral artery occlusion (MCAO) for 60 minutes followed by reperfusion was performed on 3 months old mice. The ischemic lesion was evaluated by magnetic resonance imaging (MRI, Bruker 7T Biospec 70/20 USR) and bioluminescence imaging (BLI, Perkin Elmer IVIS Spectrum). Casp3 and 7, and Gap43 were used as molecular markers. Tlr2 loss of function mice were used as a model for modified neuroinflammation after ischemic lesion. The imaging data were complemented by functional tests and Western blot protein analysis of the brain samples. When multimodal approach was applied, combining bioluminescence and MRI allowed for standardization of the measurements according to the size of ischemic lesion. The multimodal approach revealed the significant increase of Gap43 and caspases activity in the tested group with modified neruoinflammation. The possible therapeutic approaches can target diverse consequences of ischemia being oriented to neuroprotection or repair stimulation. These approaches should be tested by appropriate preclinical settings reflecting the clinical situation.

Keywords: stroke, TLR2, Gap43, neuroinflammation, *in vivo* imaging

Acknowledgements: The study was funded by FP7 GlowBrain, ESF Young Brain, HamagBicro POC6-1-153, EU European Regional Development Fund, Operational Programme Competitiveness and Cohesion, grant agreement No.KK.01.1.1.01.0007, CoRE – Neuro; Croatian Science Foundation project RepairStroke (IP-06-2016-1892). Multimodal imaging was done at Laboratory for Regenerative Neuroscience - GlowLab, University of Zagreb School of Medicine.

Selected examples of spontaneous pathology and pathology of aging in GEMs, NSG and NOD mice

Andrea Gudan Kurilj

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Pathology assessments of morbidity or mortality in GEMs and NOD mice (as well as in other experimental animals) offer quality assurance to the users and aim to identify conditions that can affect production and complicate research by affecting data and conclusions. Such reports can be found mainly as descriptive studies and they contribute to the data on spontaneous or background findings in a strain or "model" and enhance its value in research. In this context, an accurate definition of strain-specific pathology in those inbred mice that are most frequently used in biomedical research (especially for the generation of genetically engineered mice) is crucial to understand whether a phenotype results from the experimental intervention reflects a naturally occurring entity. In the last few years, several studies and review articles dealing with this topic have been published. Therefore, for the purpose of this lecture, selected examples of expected pathology phenotypes and causes or contributors to death will be presented, particularly for strains such as C57BL/6, 129, and FVB/N because of their current relevance and ubiquity in the backgrounds of genetically engineered mice. Also, NSG and NOD mice, which represent the „gold standard" host for xenotransplantation experiments, in recent years has become a popular tool in research. For these strains also will be shown examples of spontaneous infections, spontaneous post-transplant disorders and pathology of aging.

Keywords: mice, GEM, NOD, spontaneous pathology, aging

Gene therapy of animal tumour models

Simona Kranjc

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Gene therapy is a promising treatment for a number of diseases from inherited disorders, and viral infections to cancer and is extensively evaluated in preclinical and clinical studies. Gene therapy enables the replacement or inactivation of mutated gene, introduction a new gene, silencing of a gene, or enhancement of the immune response against cancer. Its efficiency depends on the effective delivery of nucleic acids (DNA, RNA) into cells or tissue and this delivery can be enabled by viral or non-viral delivery methods. One method for the non-viral delivery of genetic material (plasmid DNA, siRNA, miRNA) into cells utilizes the electroporation (gene electrotransfer, GET), in which cells are exposed to external electric field in order to increase the membrane permeability. GET is one of the most efficient non-viral gene delivery approaches for localized gene transfer into tumours. In our lab, we are currently investigating different approaches of GET for the treatment of various superficial solid tumours. We are using electrotransfer of plasmids encoding different cytokines, i.e. interleukin 12 and tumour necrosis factor alpha, and plasmids encoding shRNA for silencing endoglin or melanoma cell adhesion molecule with antivascular actions. Thus, we are testing different immunological approaches of GET; as adjuvant to standard local ablative therapies or as in situ vaccination, and we are also investigating the adjuvant effects of plasmid DNA itself. Likewise, we are testing the feasibility and effectiveness of vascular targeted therapies, using shRNA technology, as an adjunct to the established cytotoxic treatment, i.e. irradiation. In the talk, some recent results using GET in animal tumour models will be presented.

Keywords: gene therapy, gene electrotransfer, immunotherapy, vascular targeted therapies, animal tumour models

Animal research using approaches of synthetic biology

Duško Lainšček[1,2], **Roman Jerala**[1,2]

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Synthetic biology is a relatively new field of science that combines biological, chemical, biochemical and engineering approaches; we can also describe it as the science of bioengineering or biomedical engineering. The goal of synthetic biology is the safe use of mammalian cells as chassis in order to produce our desired final product. The greatest aim in the research, where the principles of synthetic biology are used, is the discovery of new drugs and new therapeutic treatments, in which cells are used as "nanofactories", capable of building practically everything, under appropriate conditions and appropriate information entered. The information for the synthesis of the selected product is simply encoded in the form of a synthetic DNA, which is then assembled and translated into a synthetic mammalian system, which bears no danger for further use and for the environment. Using the principles of synthetic biology, we developed a cellular anti-inflammatory device. In response to an elevated concentration of inflammatory cytokines IL1β and TNFα, the device produces the anti-TNFα antibody, anti-inflammatory cytokine IL10, and anakinra, a specific IL1 receptor antagonist. The effect of the anti-inflammatory device was demonstrated on the model of ulcerative colitis induced by the DSS application and on the CLP (cecel ligation puncture) sepsis model. State-of-the-art equipment and synthetic biology expertise enables us to diversify our work on experimental animals and develop a number of disease and surgical mouse models to show the therapeutic benefit of our discoveries.

Keywords: synthetic biology, cellular device, inflammation, anakinra

The Role of Optineurin in Amyotrophic Lateral Sclerosis

Ivana Munitić

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Optineurin is a multifunctional poly-ubiquitin-binding adaptor protein implicated in cell signaling, autophagy, cell death and vesicular trafficking. Optineurin mutations have recently been found in patients with amyotrophic lateral sclerosis (ALS), a rapidly progressive neurodegenerative disease of motor neurons. ALS is marked by an unusually large phenotypic and genetic heterogeneity, but no clear link has been established between genetic and phenotypic makeup. Moreover, no disease modifying therapies exist, so ALS almost invariably leads to respiratory muscle paralysis and death within 2-5 years upon diagnosis. Two main hallmarks of ALS are neuroinflammation and intracellular inclusions, which crosstalk and amplify each other. To assess the potential role of optineurin in ALS, we designed an optineurin insufficiency mouse model ($Optn^{470T}$), which mimics patient mutations by lacking the ubiquitin-binding region in the C-terminus. We found that optineurin regulates inflammatory signaling by acting as a scaffold for Tank-binding kinase 1 (TBK1) activation and subsequent interferon (IFN)-ß production. The primary microglia from $Optn^{470T}$ model exhibited diminished expression and activation of several transcription factors that support the amplification loop for IFN-ßproduction including STAT1, IRF7 and IRF9. Notably, expression of both proinflammatory and anti-inflammatory factors distal to IFN-ß was diminished, and could be restored upon IFN-ß supplementation. Our results open up the possibility that disruption of optineurin/TBK1-mediated IFN-ß axis leads to an immune failure, which could predispose to neurodegeneration.

Keywords: neuroinflammation, neurodegeneration, ALS, optineurin, TBK1

The standards in animal research

Martina Perše

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Despite intensive effort and financial stimulation for the development of the methods to replace the use of animals in research, the experiments on animals remain to play an important and yet irreplaceable role. However, animals have an intrinsic value, which cannot be measured. They are sentient creatures, which feel pain, suffering and distress. Therefore, the use of animals in research is allowed only when there is no alternative method that would produce equally valid results, and when ultimately benefits human or animal health or the environment, and when experiment is designed and performed in a way to give a valid and reproducible results. However, recently published papers report remarkably low ability to translate preclinical research to clinical success. In addition, increasing number of publications demonstrated concerning prevalence in the number of studies that cannot be reproduced. Consequently, the irreproducibility of animal research is gaining a big attention in scientific community. The main reasons for such situation were already recognized and some actions and strategies have already been taken. The presentation will summarize recent literature on this subject and highlight the standards in animal research with the aim to encourage scientific audience to pay attention not only to the 3R's principle (replacement, refinement and reduction) but also to factors affecting the reproducibility of studies and transparent reporting in compliance with the ARRIVE guideline or Gold Standards Publication Checklist.

Keywords: animal experimentation, reporting, reproducibility, standardization

Experimental models of traumatic brain injury

Kristina Pilipović, Gordana Župan

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Traumatic brain injury (TBI) is one the major causes of mortality and morbidity worldwide. TBI survivors often experience long-term personality changes and disabilities in cognitive and sensorimotor functions. TBI is not a single pathophysiological, but it is rather a complex process, which includes primary, immediate tissue injury and the secondary injury, which evolves through hours, days and months following the initial trauma. Over the past couple of decades, numerous experimental models have been developed for TBI research by which the scientists intended to reproduce a clinically extremely heterogeneous injury in laboratory conditions. Although there is no one sole TBI model that could adequately mimic all aspects of human brain trauma, their use significantly increased the understanding of molecular and cellular tissue responses as well as neurobehavioral outcomes after brain trauma. Therefore, especially in pharmacological research, the use of several TBI models is necessary. This review talk will provide an up-to-date and critical analysis of the currently most used models of brain trauma, including both *in vivo* and *in vitro* approaches in experimental TBI research. In addition, some recent results of our research in different TBI models will be shown.

Keywords: animal disease models, *in vitro*, *in vivo*, traumatic brain injury

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Impact of three handling methods on the anxiety of three different strains of laboratory mice

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The aim of our work was to compare the classical method of picking up mice by the tail, with new alternative methods using a handling tunnel or cupping mice on the open hand. The latter two methods have recently been shown in the scientific literature to lead to voluntary approach, lower anxiety and stress. The three handling methods were tested in three lines of laboratory mice: outbreed Hsd:ICR(CD-1), inbred "fat" line (FLI), 18 animals (9 male, 9 female) and inbred "lean" line (FHI), 12 animals (6 male, 6 female). Mice were housed in IVC cages, 3 animals per line and sex. Handling was done by three handlers with a different level of experience in working with experimental animals for nine days. The same protocol was used, the only difference was in the method of handling: tail, tunnel or open hand. Each mouse was lifted above the cage, held for 30 seconds and transfer into another cage. In addition to handling, the first, fifth and ninth day of the experiment, interaction with hand was assessed: the hand was put in the front of the cage, behavior of mice was observed and interaction quantified for 60 seconds before and after handling. Mice handled with tunnel or open hand spent most of the time closer to the hand, moving on the hand, smelling, putting paw on hand and climbing around the hand. On the other hand, mice picked up by the tail spent less time interacting with the hand, independently of sex and line. We can conclude that the use of new alternative methods of handling: tunnel and open hand can reduce the anxiety, stress and discomfort at laboratory mice.

Keywords: laboratory mice, handling, handling tunnel, open hand, anxiety

A new role of an old player: IFNγ-mediated crosstalk between the immune and endocrine systems in viral infections

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Pro-inflammatory cytokines of a Th1-signature, normally associated with viral infection, are known to promote insulin resistance (IR) in obesity, but the physiological role of this mechanism is unclear. Surprisingly, it is also unknown whether and how infection induces loss of glycemic control in subjects at risk for developing diabetes mellitus type 2. We find in mice and humans that viral infection causes short-term systemic IR. Virally-induced IFNγ directly targets the skeletal muscle to downregulate the insulin receptor but does not cause loss of glycemic control because of a compensatory increase of insulin production. Hyperinsulinemia enhances antiviral immunity through direct stimulation of CD8 effector T cell function. In pre-diabetic mice with hepatic IR caused by diet-induced obesity, infection resulted in long-term loss of glycemic control and aggravation of microvascular complications of DM2. Thus, upon pathogen encounter, the immune system transiently reduces insulin sensitivity of the skeletal muscle tissue to induce hyperinsulinemia and promote antiviral immunity, which derails to glucose intolerance in pre-diabetic obese subjects.

Keywords: insulin resistance, interferon gamma, obesity, diabetes mellitus 2, insulin, CD8, lymphocytes T, MCMV

In vivo and *in vitro* models in diabetes research

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Diabetes is one of the major health problems worldwide with rapidly growing incidence. It is progressive disease characterized by the inability to produce or/and properly use insulin. Regardless of medical treatment, diabetic patients as well as animals have recurring episodes of hyperglycemia that permit the development of diabetic complications. Episodes of hyperglycemia after eating are particularly harmful. It was known that glucose, resulting from digestive process, goes across intestinal membrane and is absorbed in the blood stream. Partial reduction of glucose uptake in small intestine of diabetic organism might prevent hyperglycemia-induced stress and the development of diabetic complications. The use of *in vivo* and *in vitro* models in studying disease is fundamental to the advancement of understanding of mechanisms, disease dysfunctions and development and testing of therapeutics. The presentation will be focused on mouse models, which we have used in research with the special accent on a new method for analysis of diabetic/control urine. In addition, we will present our preliminary results observed by using Caco-2 cell line. Namely, human intestinal cells have been extensively used to study absorption and intestinal physiology. Thereby, our attention will be focused on the investigation of reducing glucose uptake after cell treatment with Ca Zeolite A.

Keywords: diabetes, mouse, hyperglycemia, Caco-2 cell

3Rs – Animal Cell Technology – Achievements and Challenges

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Animal cells adapted to conditions *in vitro*, are the powerful tool that has given us enormous scientific advancement in understanding molecular mechanisms of life. Currently there are three cell-based models, established as either 2D or 3D cell culture, widely applied in research and technology: primary cells, immortalised cell lines and stem cells. The endpoint of using these relatively simple research models is to create tissue-mimicking environment that can yield relevant and reproducible data used eventually for diagnostics and treating diseases, while considerably reducing the number of experimental animals required for that same purpose. A similar approach we find in highly controlled biotech processes developed for large-scale production of vaccines and biological compounds, in which immortalized animal cells had been adapted to robust industrial conditions, thus replacing expensive, laborious and often unsafe and unpredictable treatment of animals as biocatalysts or bioreactors. Although cell based technologies have entirely substituted animals in biotech production, gaining properly validated facts about novel substances and therapies may still demand for animal models in order to guarantee human safety, but at the same time to push scientists to direct their research strategies in a new way.

Keywords: animal cell culture, cell-based systems, *in vitro*, biotechnology

Classification of animal pain and distress levels

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Animal related research presents an ethical dilemma: if pursued, animals may suffer; if not, important benefits to humans, non-human animals or the environment may be lost. Severity classification is used to classify the adverse effects experienced by animals used for scientific purposes. When determining an appropriate severity classification, it is necessary to consider the following: what is being done to the animal, with what effect, how much suffering it may cause and what refinements can be included to reduce the impact. An estimate of severity (proposed or prospective) during the design of the study aids the application of optimal refinements (3Rs), monitoring tools, frequency, type of scoring and humane end points. This is done by the Institutional Ethics Committee and Competent Authority during the Project Evaluation (PE) and is based on the most severe effects likely to be experienced by an individual animal after all refinements have been applied. The actual severity of procedures can be determined after completion of the study and will be reported by Member State in the annual statistical returns as the highest severity experienced by each individual animal during the course of a procedure. There will therefore be differences in severity between prospective and actual severity for each of the animals used, coming from unexpected events. All projects classified as "severe" during PE by the Applicant must undergo Retrospective Assessment.

Keywords: severity, classification, prospective, actual, retrospective

Stem cell therapy in animal model of kidney injury-preclinical promises and challenges for translation

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Mesenchymal stem cell (MSCs) therapy is recognized as a promising innovative strategy to treat various degenerative and immune/inflammatory diseases. Numerous studies using animal models of kidney failure have shown that MSCs transplantation resulted in the improvement of kidney structure, function and animal survival. One of the strategies to improve MSCs survival following their transplantation is to reduce inflammatory overload of damaged tissue with immunosuppressive agents. We have shown that immunosuppression may improve MSCs transplantation in the acute and chronic phase of kidney failure. Despite evidence for the therapeutic potential of MSCs, underlying mechanisms remain unclear. It is known that injected exogenous MSCs can home into injured tubules, but more frequently they act through a differentiation-independent process (i.e. paracrine or endocrine process). It is believed that when the MSCs act in a paracrine manner to protect or stimulate the endogenous renal cells, then they only need to survive for a few days and immune environment may not be important. However, recent studies show that the situation is not so simple as it was suggested. Some concerns about potential side effects of MSCs therapies still exists. Namely, there is a lack of studies regarding behavior of MSCs in the late period after transplantation, especially in the context of possible tumorigenicity and immunogenicity. These and other unanswered questions raise serious concerns in clinicians. Consequentially, the stem cell therapy approaches for the kidney failure in human are still at an early stage. The speaker will critically summarize the results of MSCs transplantation in animal models of kidney injury, highlight the latest evidence from the ongoing clinical trials and expose main experience-based concerns associated with the translation of MSCs transplantation results from animal models to a clinical therapy of kidney failure.

Keywords: MSC, kidney injury, animal model, stem cell therapy, acute kidney injury

Opto- and pharmacogenetic approaches in animal models

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Optogenetics was the method of the year in 2010 according to Nature Neuroscience. Since then this method has become widespread, the use of virally delivered genetic tools has extended to other fields such as pharmacogenetics, and optogenetic techniques became frequently applied with genetically manipulated animals for *in vivo* circuit analysis and behavioural studies. However, several issues should be taken into consideration when planning such experiments. We aimed to summarise the critical points concerning opto- and pharmacogenetic manipulation of a specific brain area in mutant mice. First, the appropriate vector should be chosen to allow optimal manipulation. Adeno-associated viral vectors are the most common carriers with different available serotypes. Light-sensitive channels and modified G-protein coupled receptors (DREADDs) are available in many forms and the expression of the delivered genetic material can be influenced in many ways. Secondly, selecting the adequate stimulation/inhibition protocol is also essential. The pattern, intensity and timing could be determinative parameters for optogenetics, while the dose and timing for pharmacogenetics. Thirdly, a mutant strain might have a phenotype that influences the observed behaviour. In conclusion, detailed preliminary experiments and numerous control groups are required to choose the best vector and protocol and to ensure that the mutant animals do not have a specific phenotype, which can influence the examined behaviour.

Keywords: optogenetics, pharmacogenetics, adeno-associated virus vector, behaviour

SAŽETCI POSTERA / POSTER ABSTRACTS

Wistar-Zagreb serotonin (WZ-5HT) rats - a new animal model of obesity

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The continuously increasing prevalence of obesity worldwide urges development of animal models sharing features of human obesity, that would enable a search for more effective preventive and/or treatment strategies. Currently available rodent models of obesity are based on genetic manipulations of one or few target genes or on exposures to obesogenic environment such as high-fat diet. Serotonin (5HT), an ancient biogenic amine, plays a vital role in energy homeostasis and has been implicated in the pathogenesis of obesity. Here we will present a potentially novel animal model in obesity research, the two sublines of the Wistar-Zagreb (WZ) 5HT rats, developed by selective breeding toward physiological extremes of platelet serotonin parameters. Selective breeding procedure resulted in high-5HT and low-5HT subline of rats with constitutionally upregulated and downregulated, respectively, 5HT tone. High-5HT animals have a lifelong increased body weight and elevated adiposity as compared to low-5HT rats. On the other hand, low-5HT rats seem to be more sensitive to obesogenic effects of high-fat diet. Our animals are kept 3 per cage with free access to commercial rat chow (Mucedola, 4RF21 or MD.06415, 45% kcal from fat) and tap water, and are housed under controlled conditions (23±2°C, 55±10% humidity, 12h light/12h dark). All experiments are conducted in accordance with national legislations (NN 55/2013; 102/2017) and the EU directive on the protection of animals used in scientific purposes (2010/63/EU). 3R principle and the ARRIVE guidelines are followed as much as possible and healthy status of animals is tested four times a year (Faculty of Veterinary Medicine, University of Zagreb).

Keywords: Wistar-Zagreb 5HT rat, serotonin, high-fat diet, obesity, animal model

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Rat model of sex specific chronic stress response

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Scientific findings are often biased because mostly young males are used in experimental studies. Chronic stress is associated with development of Alzheimer´s disease (AD) which disproportionately affects more women than men. We developed a unique protocol for chronic stress and aging in male and female rats. Male and female rats were divided in young (6.5 months) and old (14.5 months) experimental groups. Two stressors were applied per day during three 10-day stress sessions. Protocol included stressors such as exposure to cold, forced swimming, restraint, disturbance of the night cycle. Neuroplastin (Np) and glucocorticoid receptor (GR) were analyzed in hippocampus (HIP) by Western blotting. Amyloid precursor protein (APP) was analyzed in HIP by fluorescent immunohistochemistry. Passive avoidance test was used to determine memory impairment. Experiments were approved under class 602-04/14-08/06 and registration number 2158-61-07-14-118. Np decreased in young males ($p=0.049$) while APP was increased in old females upon chronic stress. GR expression was increased in all animal groups upon chronic stress. Passive avoidance test determined cognitive decline in old females compared to young females ($p=0.0004$). Chronic stress protocol is validated by increased GR in all animal groups. Cognitive tests and expressional changes of Np in young males and APP in old females imply gender and age differences in molecular mechanisms of chronic stress response.

Keywords: chronic stress, gender difference, neurodegeneration

Acknowledgements: This study has been funded by Croatian Science Foundation project IP-09-2014-2324

Lizard as a potential behavioral model: comparison of exploratory behavior and brain catecholamine levels between Italian wall lizard and Wistar rat

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Besides representing an important model for studies in the fields of conservation biology, ecophysiology and evolution, lizards may be interesting as a model in translational research for elucidation of the underlying mechanisms of behavior and its adaptive value. The Italian wall lizard (*Podarcis sicula*) is a highly adaptable species that occupies a variety of habitats (the main area of distribution is Italy and the eastern Adriatic coast with surrounding islands) and undergoes population specific adaptations in morphology, physiology and behavior. In this study, we explored the potential of *P. sicula* as a model for exploratory behavior by comparing its behavioral and neurochemical parameters to those of Wistar rat, the long-used behavioral model well established in our Laboratory. Exploratory behavior of 28 lizards was analyzed in open field as time spent in movement, total distance travelled, percent of time spent in the central area, and number of rearings. The levels of dopamine (DA) and noradrenaline (NA) were determined in brain tissue homogenates of 20 lizards using the standard ELISA kit for human catecholamines. *P. sicula* displayed whole spectrum of behavior in open field with comparable average number of rearings and % time in center to those of rats. They spent more time in movement, with lower speed, travelling therefore less total distance than rats in a 10 min experiment. We were able to reliably measure catecholamine levels in 50x diluted whole-brain homogenates, and they amounted to 417 ± 116 pg/mg DA and 917 ± 170 pg/mg NA, which is about one order of magnitude higher than in rat brain cortex. Our results speak in favor of the use of *P. sicula* as a potential model for studying neurochemical basis of behavioral adaptation in laboratory conditions.

Keywords: lizard, behavior, dopamine, noradrenaline

Transplantation of neural stem cells influenced the programmed cell death as a consequence of after ischemic stroke

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Stroke is nowadays one of the most common cause of severe disability and has already reached epidemic proportions. Cell-based treatment is the fastest developing field in the history of biomedicine. The aim of the study was to analyse how transplantation of neural stem cells in the brain affected by stroke, influences expression of genes involved in the programmed cell death. After inducing stroke by occlusion of the middle cerebral artery in mouse, animals were, 24 hours after induction of ischemia, transplanted either by neural stem cells or medium enriched by growth factors. Neural stem cells were isolated from the telencephalic wall of 14.5 days old mouse embryos, obtained from the B6.Cg-Tg(Thy1-YFP)16Jrs/J transgenic mice strain. Fourteen days after transplantation, 4 groups of animals were analysed: those affected by stroke, affected by stroke and treated by medium, affected by stroke and treated by stem cells, and sham-operated animals. Brain affected by stroke increased expression of genes with protective effects (*Iduna*), while damage-supportive genes (*Casp8 and Aif*) were downregulated. In addition, we distinguished between effects obtained by stem cells (*Iduna, Aif,, Ripk1, Ripk3, Mlkl*) and those ones as well obtained by supporting tissue with enriched medium (*Casp8*). We also report a significant downregulation of *Ripk1*, the major trigger of necroptotic cell death, but upregulation of *Ripk3 and Mlkl,* other elements of this pathway, which suggests a complex role of necroptosis in both tissue damage and recovery. The transplantation of neural stem cells in the mouse brain affected by stroke significantly increased expression of genes with protective effects on hypoxic damage, while their antagonists, damage-supportive genes were significantly downregulated, which suggests a complex role of necroptosis in both tissue damage and recovery.

Keywords: neural stem cells, stroke

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Combining synchrotron-radiation based CT, MRI, and micro-CT for Mouse Ischemic Brain Lesion Evaluation

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Preclinical trials of new stroke therapies are in constant need for novel ischemic brain imaging modalities. Volumetric analysis helps establishing new therapeutical approaches for revealing every neuroprotective aspect of the tested drug. Multimodal imaging approaches would help overcome the loss of information obtained when only one imaging modality is used. Multimodality offers the possibility to reduce the number of animals used, simultaneously allowing macroscopic and microscopic 3D evaluation of the ischemic lesion evolution. C57BL/6 mice underwent 60-minute cerebral ischemia induced by filament occlusion of the middle cerebral artery. The first two groups of animals underwent different fixation protocols: the Evaporation-of-Organic-Solvent (EOS) dehydration method for synchrotron imaging and a hydrated preparation method for MRI imaging. The other two groups underwent different contrast agent staining procedures using phosphotungstic acid or non-ionic iohexol monomer staining. Different fixation protocols influenced MRI quality and contrast of the visualized lesion, which was easily delineated by SRμCT imaging when the EOS method was applied. Volumetric analysis of the ischemic lesion and brain edema showed a clear difference in the measured values depending on the preparation method used. Tissue preparation procedures differently affect the injured area and the healthy tissue producing a huge variability in the volumetric analysis. MRI enables good gross morphology visualization of the brain tissue, while micro-CT and SRμCT provided high-resolution neuroarhitectonic depiction.

Keywords: ischemic lesion, magnetic resonance imaging, micro-CT, synchrotron

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Apoptosis and proliferation in teratocarcinoma of the testis and the experimental mouse teratocarcinoma model

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Teratocarcinoma is a mixed testicular germ cell tumor composed of teratoma and embryonal carcinoma. Established experimental mouse teratocarcinoma model accepted for studying human teratocarcinoma was defined only in the frame of histological and histochemical features. The molecular signature was not comprehensively studied, and leaves the question of translational value open in the age of molecular-medicine. The aim of this study was to compare the molecular signatures and the validity of the model, starting with the rate of apoptotic and proliferative activity in the experimental mouse model/human teratocarcinomas. Formalin-fixed paraffin-embedded tissue from testicular teratocarcinoma and animal model tumors was used for immunohistochemical detection of Caspase-3 and PCNA expression. Slides were analyzed semi-quantitatively by pathologist, graded from 0-3, depending on the percentage of reactive cells. Data was analyzed in GraphPad Prism using the Mann-Whitney test. The results have shown difference in the rate of apoptosis between the human-teratocarcinomas and the mouse-model, with the mouse model showing a higher rate (>25% of positive cells) in 64% of tumors compared to 30% of human tumors with highest reaction. PCNA quantification has shown comparable levels of PCNA expression in the EC regions of the experimental mouse model and human teratocarcinomas, while in the teratoma regions the mouse model has a higher proliferative activity. Some of the difference could be attributed this being a pilot study with a relatively small sample pool, the intragroup and intergroup difference in biological development. Western Blot analysis is needed to verify the results and a larger cohort should be studied.

Keywords: TGCT, teratocarcinoma, mouse model, apoptosis, proliferation

Examples of the use of confocal microscopy and bioluminiscence resonance energy transfer (BRET^2)

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Confocal microscopy is primarily used at the Institute of Preclinical Sciences at Veterinary Faculty, University of Ljubljana to determine i) toxins induced changes in the intracellular calcium activity ([Ca^{2+}]i) in various mammalian cell lines, ii) intracellular localization of the members of G protein-coupled receptors (GPCR), their internalization and colocalization with actin cytoskeleton, and iii) morphological changes associated with the cytoskeleton rearrangement. Confocal microscopy was important for monitoring the influence of various biologically active substances on calcium level changes in the time and concentration correlation and their effect on the morphology of NG105-15 and A10 cells, which represent an *in vitro* model of respiratory neurons and vascular smooth muscle cells. Using fluorescence markers for cytoskeletal labeling, adverse effects of xenobiotics microcystin-LR on morphology and blastomer cytoskeleton were studied in various stages of early embryonic development. To study the localization of various GPCR receptors (long form of the D2 dopamine receptor), colocalization studies with different intracellular organelles (e.g. calnexin) were performed. The role of $G\alpha_{12}$ and $G\alpha_{13}$ proteins in alternative signaling pathways of ghrelin receptor was observed through the redistribution of the cytoskeleton in HEK293 cells and showed the formation of stress filaments and changes in the polymerization of microtubules in various grelin receptor mutants. For these purposes Leica Multispectral Laser Confocal Microscope (Leica TCS NT, Heidelberg, Germany) was used.

BRET technology is used to study protein-protein interactions and conformational changes in proteins in living cells, in which energy transfer from a bioluminescent donor to a fluorescence acceptor with resonant energy transfer occurs. We use the TriStar LB 942 panel reader (Berthold Technologies, Bad Wildbad, Germany). The BRET^2 technique was used to monitor the conformational changes caused by the binding of ligand to GPCRs. We studied the influence of the activation of wild type and mutants of D2L-R with the agonist on the kinetics of internalization and conformational changes of β-arrestin2. To this purpose, we used double-labeled mutant of β-arrestin2 (RLuc/β-arr2R393E, R395E/GFP2), with the luminescence marker (RLuc) linked to the N-terminal part and the GFP2 label bound to the C-terminus.

Binding of the agonist resulted in conformational changes in β-arrestin[2], which resulted in a further proximity of the described labels and an increase in the basal signal BRET[2].

Keywords: bioluminiscent resonance energy transfer; BRET technology; confocal microscopy

Comparison of the lethal toxicity test of snake venom from Croatia on mice and crickets

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Snake venoms are complex mixture of enzymes and non-enzymatic proteins used for both the immobilization and digestion of prey. It is known that determination of 50% lethal doses (LD_{50}) of snake venoms on mice appears to be an important step to assess (and compare) venom toxic activity. There are three species of venomous snakes in Croatia: long nosed viper (*Vipera ammodytes –Va*), European adder (*Vipera berus - Vb*) and meadow (karst) viper (*Vipera ursinii - Vu*). Eating habits of venomous snakes in Croatia are different. *Va* and *Vb* primarily eat small mammals. The karst viper in Croatia (*VuCro*) favours high-mountain dry grasslands so its choice of food is limited on insects. For proteomic investigation and biological (toxinological) profile of the venom of *Vu*, we need a method to prove insecticide component in venom. Therefore, we decided to compare their venoms using lethal toxicity test on both mice and crickets, because venom toxicity test only on mice is not suitable to evaluate *VuCro* venom potency. The *VuCro* venom is less lethally toxic in mice than the *Va* or *Vb* venom however; the pattern of mice dying indicates the presence of a strong neurotoxic component. Crickets (*Gryllus assimilis*) were used as a model, which mimics the natural insect prey. The obtained results that *VuCro* venom is as twice as more toxic than *Va* and *Vb* venom on crickets. Taken all together, *VuCro* venom might be a good starting material for the discovery of a novel neurotoxic component in Vipera venoms with potentially insecticidal activity. Therefore, this test helps us to discover a component, which makes those venoms different. The lethal venom potency test on crickets is accordant with 3R principles.

Keywords: viper, venom, lethal toxicity test, LD_{50}, mice, crickets, Croatia

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An insight into the toxic effects of irinotecan and delta 9-tetrahydrocannabinol through measurements of cholinesterases activities and markers of oxidative stress in rat plasma

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Irinotecan (IRI) is one of the most important antineoplastic drugs primarily intended for use in chemotherapy of metastatic colorectal cancer. Patients treated with IRI often manifest acute cholinergic syndrome, which is the reason behind the growing use of legally prescribed preparations that contain cannabinoids as well as illicit ones that sometimes contain very high delta 9-tetrahydrocannabinol (THC) levels. The objective of this pilot study was to evaluate the toxic effects of a concomitant intake of IRI and THC in male Wistar rats. IRI was administered once intraperitoneally (at 100 mg/kg b.w.) and THC per os repeatedly for 1, 3, and 7 days (at 7 mg/kg b.w.). The rats were sacrificed 24 h after the last treatment. The plasma samples were collected and stored for biochemical analyses. IRI either alone or in combination with THC inhibited acetylcholinesterase activity by 30 and 20 % according to control after 1 and 3 days. Repeated application of THC for 3 and 7 days resulted with inhibition of butyrylcholinesterase activity. Although IRI and THC treatments generally did not affect biomarkers of oxidative stress (lipid peroxidation, superoxide dismutase and catalase), an increase of total antioxidant capacity in the plasma of rats treated with THC was noticed after 1 and 7 days. The biomarkers used in this study provide a static assessment of the adverse effects of IRI and THC in rats so future research should focus on evaluating their interactions by molecular methods.

Keywords: irinotecan, delta 9-tetrahydrocannabinol, rats, cholinesterases, oxidative stress

Procedure of hemolymph sampling in *Octopus vulgaris*

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Cephalopods (i.e. octopus, squid, cuttlefish and nautilus) are class of exclusively marine animals belonging to the phylum Mollusca, containing about 700 known living species. Unlike other molluscs, cephalopods have a functionally closed circulation system, sophisticated nervous system with well-developed sense organs and large, highly differentiated brain, and are thought to be the most intelligent invertebrates. Live cephalopods have been used in studies of neurobiology, physiology, immunology and environmental toxicology and consequently have been involved in scientific procedures where there was need to immobilize, anesthetize and euthanize them. However, only just recently, cephalopods became the only invertebrate animals included in the Directive 2010/63/EU on the protection of animals used for scientific purposes. In cephalopod research community this regulation opened a lot of questions about objective criteria for identification and assessment of pain, suffering, distress and lasting harm in cephalopods and revealed the lack of protocols for cephalopod surgery, anaesthesia, analgesia and humane euthanasia. In this work, we present a procedure of *Octopus vulgaris* anesthesia, using combination of magnesium chloride and ethanol solution, and procedure of hemolymph sampling. Hemocytes, cephalopod blood cells, could be used for *in vitro* investigations, such as cytotoxicity assays, gene transcription expression patterns under conditions of thermal stress, stress-induced neuroendocrine and immune changes, thus gaining valuable information without invasive experiments on animals.

Keywords: cephalopods, anesthesia, hemocytes, welfare impact

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Wistar rats as an experimental model for sterigmatocystin toxicity testing

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Mycotoxins are secondary metabolites produced by many mould species. More than 300 mycotoxins are known and most of their toxicity mechanisms are unknown. Humans can be exposed to mycotoxins found in food, through skin or inhalation. Sterigmatocystin (STC) is a mycotoxin produced by many *Aspergillus* mould species, which can contaminate wet buildings. STC is a precursor and has similar structure as a carcinogenic mycotoxin aflatoxin B1. The International Agency for Research on Cancer classified OTA as a Group 2B (possible human carcinogen) carcinogen. So far, STC exposure was connected to liver and kidneys damage and DNA damage of certain cell lines. The aim of the study was to determine the role of oxidative stress in mechanism of STC toxicity. Experiments were performed on male adult Wistar rats because of their sensitivity to STC exposure. Animals were treated with single oral STC doses of 1/4, 1/8, and 1/16 of LD_{50}. After 24 hours, rats were sacrificed and blood, liver, kidney, brain and lung samples were collected. The experiment was approved by the Ethic Committee of the Institute for Medical Research and Occupational Health and the Ministry of Agriculture of Republic of Croatia in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU). Parameters of oxidative damage of lipids, proteins and DNA were measured in different tissues. Levels of different oxidative stress parameters were not dose dependent and the kidney were more affected with STC treatment than other organs.

Keywords: kidneys, liver, oxidative stress, sterigmatocystin, Wistar rats

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DHEA(S) restored memory impairment and modulated apoptotic signalling following ischemic brain injury in male Wistar rats

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Ischemic brain injury, one of the major causes of death and serious long-term disability associated with cognitive and behavioral dysfunction, represents global health problem. Preclinical studies suggest that neurosteroids dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), together referred as DHEA(S), might protect neuronal cells against different neurotoxic insults and reduce cognitive decline, thus showing therapeutic potential. This study aims at better understanding of potential neuroprotective actions of DHEA(S) in preventing neuronal cell death induced by ischemic brain injury and promoting survival of remaining neurons. For that purpose, adult male Wistar rats underwent two-vessel occlusion of common carotid arteries (2VO) for 10 minutes, after which circulation was restored. Sham operated animal group went through the same procedure, except final artery occlusion. 24 hours after surgical procedure, animals received 20 mg/kg DHEA, 20 mg/kg DHEAS or vehicle. Object location test (OLT) and object recognition test (ORT) were performed 24 hours prior to sacrifice to assess spatial and non-spatial memory, respectively. On 7th post-surgical day, animals were sacrificed and prefrontal cortex and hippocampus were isolated and frozen. Cytosolic and mitochondrial extracts were prepared for Western blot analysis to determine the levels of pro- and anti-apoptotic proteins, Bax and Bcl-2. Behavioral tests revealed that DHEA(S) treatment restored memory impairment provoked by 2VO. Moreover, preliminary results of Bax and Bcl-2 protein expression ratio in cytosolic and mitochondrial brain fractions suggested that observed neuroprotective effects of DHEA(S) following 2VO might be due to the modulation of apoptotic signalling.

Keywords: ischemic brain injury, neurosteroid DHEA(S), spatial and non-spatial memory, apoptotic markers Bax and Bcl-2

A non-invasive rat model of perinatal hypoxic brain lesion

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The Rice-Vannuci hypoxic-ischemic-brain lesion in the rat, and its various modifications, used as a standard model for hypoxic brain lesions, are invasive surgical treatments classified as severe procedure (EU Directive 63/10, Article 15). We aim to introduce a non-invasive hypoxic brain lesion rat model of mild to moderate severity, to serve for the research targeting the fetal hypoxic brain lesions occurring during midgestation (23-32 weeks post-conception) in humans. In the present study, Wistar rats (9 females and 10 males) were randomly divided into hypoxic and control group on postnatal day 1 (P1) when hypoxia was induced in a warm (≈ 25°C) hypobaric chamber (Atm 350 mmHg, $pO_2$73 mmHg) during 2 hours, while controls were kept in normal housing conditions. Possible behavioral deficits were examined in a battery of tests: open field, hole board, the T-maze and social choice at P30 and P70. Samples of brain tissue from adult animals (P105-120) were used for histochemical examination of cytoarchitectonics (Nissl staining), interneurons (parvalbumin immunohistochemistry) and perineuronal nets (*Wisteria floribunda agglutinin* histochemistry). Compared to controls, hypoxic animals had intact exploratory, anxiety-like and social behavior, but displayed significantly impaired learning. There were no disturbances in the brain macro-morphology or any other pathoanatomical consequence of the treatment, and the cytoarchitecture, as well as the laminar and structural organization of the telencephalon, were preserved. However, changes in morphology, number, and distribution of the parvalbumin-immunoreactive neurons and perineuronal nets, distinct in different regions of the telencephalon were observed. In conclusion, the proposed rat model of non-invasive hypoxic brain injury has indicated consistent disturbances in brain connectivity related to cognitive processes, that mimic perinatal mild post-hypoxia condition in humans. Further characterization and evaluation of the model, on molecular, cytological and connectivity levels, is needed to disclose developmental disturbances that are not compensated after the provoked hypoxia and therefore lead to cognitive deficits.

Keywords: Rice-Vannuci model, hypoxia, parvalbumin, brain development, perineuronal nets, learning

Acknowledgements: This study was co-financed by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, grant agreement No. KK.01.1.1.01.0007, CoRE - Neuro.

Novel transgenic mouse line with bioluminescent and fluorescent neurons was developed for *in vivo* imaging in preclinical studies of brain diseases

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Genetically modified animals are essential models for studying human diseases at the whole-organism level. The aim of this study was to generate a transgenic mouse line with bioluminescent and fluorescent neurons, which could be a useful model for studies of brain repair after injury. A vector with a transgenic cassette composed of NFH (neurofilament heavy) promoter and a reporter with fused luc2 (bioluminescent) and FP635 (fluorescent properties) genes, TurboLuc, was constructed. The Golden Gate method based on the use of restriction enzymes type IIs was applied to clone the DNA plasmid with the desired construct. The construct functionality was validated *in vitro* in differentiated neural stem cells. Subsequently, the construct was microinjected in the pronuclei of the mouse zygotes to generate founders of the transgenic mouse line. Founders were selected among new-born pups and subsequently functionally validated using *in vivo* optical imaging. After successful pronuclear injections, three founder animals were selected. Bioluminescent signal was shown in these transgenic animals using *in vivo* optical imaging. The chosen promotor sequence was expected to provide expression of reporter, TurboLuc, in mature neurons. Expression of reporter in transfected differentiated neural stem cells *in vitro*, and bioluminescent signal in founders *in vivo* was located in the areas where mature neurons can be found. The novel transgenic mouse lines could be appropriate for the research of differentiation of neural stem cells, both *in vitro* and *in vivo*.

Keywords: transgenic animal model, bioluminescent and fluorescent neurons, TurboLuc reporter

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Analysis of apoptotic and proliferative activity in experimental mouse teratocarcinoma model after epigenetic modulator treatments

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Testicular Germ Cell Tumors (TGCT) are the most frequent malignancies in young men and believed to be initiated by epimutations. Teratoma is the most differentiated TGCT type encompassing three germ layer-derived tissues. Mouse teratoma is a well-established *in vitro* model harvested by cultivating mouse embryos. It represents a practical system for examining the effect of most prominent epigenetic drugs and agents. After isolation, embryos were treated for two hours with 5-azacytidine, Trichostatin A, Valproate, esiNanog, esiOct3/4 and esiTrrap. The embryos/teratomas were measured during 7 days of culturing. For proliferative and apoptotic activity analysis, immunohistochemistry and Western blot were performed. Signal intensity was measured by morphometric analysis. For gene expression analysis, specific gene-related RNA was analyzed by both qPCR and ddPCR. Epigenetic modulators significantly reduced embryo/teratoma growth. Most noticeably, the 5-azacytidine and esiOct3/4. 5-azacytidine treatment almost completely disrupted tissue architecture and cellularity and induced an increase in apoptotic activity. Increase in apoptotic activity was also induced by Trichostatin-A and Valproate. Proliferative activity was not decreased by any epigenetic modulator. The esiOct3/4, esiTrrap and surprisingly 5-azacytidine, in fact, showed a slight increase in proliferation. Differentiation increase was induced by esiNanog and esiTrrap. Expression of analyzed stemness and differentiation genes panel was significantly disrupted by 5-azacytidine, Valproate and esiOct3/4. This research shows a strong adverse influence of epigenetic modulators on experimental germ cell tumor development. It seems that this effect is a consequence of an induced change in stemness and expression of differentiation-related genes.

Keywords: mouse model, epigenetic modulators, apoptosis, proliferation, differentiation

In vitro and *in situ* study of the role of Sema5A in the developing mouse telencephalon

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The Semaphorina5A (Sema5A) is a transmembrane protein that has been shown to have a role as bifunctional axon guidance molecule during axon elongation and cell migration in the development of diencephalon and its connections. It was also shown that the down-regulation of SEMA5A expression is present in patients with autism spectrum disorder and haploinsufficiency for SEMA5A in Cri-du-chat mental retardation. According to the recent data (Allan Brain Institute), the transcripts of Sema5A gene are present in proliferative zones of the telencephalic vesicle in rodents at age E13.5 E15.5 and P14. Aiming to disclose the role of Sema5A in the development of telencephalon and its neocortical connections, we firstly, conducted functional *in vitro* study. The neocortical explants from mouse embryo (E15) were cultivated 72h on Sema5a uniform or alternately presenting carpet (Sema5A transfected Human Embryonic Kidney-293 cells membranes). The indirect immunohistochemical method for Sema5A protein was performed on *post-mortem* brain samples from early postnatal mouse brains to disclose the spatial and temporal expression of Sema5A protein throughout the developing telencephalon. The *in vitro* assays showed that Sema5a influence neocortical axons by promoting their outgrowth and elongation when it is uniformly presented on the carpet. The study also shows Sema5a does not steer axons or modulate collateralization on Sema5A-alternating carpets. The expression of Sema5A protein revealed by immunohistochemical staining was more prominent at P15 than at P5, in the entire cortex with clear regional differences in the expression. The layer II/III, V and VIa of the neocortex show transmembrane expression of the Sema5A protein at the soma and the initial dendrite segment at P15. At P5 Sema 5A is expressed in layer V and layer VIb. The insular and the piriform region of the cortex show prominent expression of Sema5A protein on the neuronal soma membrane at P15. At the same time, the Sema5A protein was not expressed in the cingulate cortex. Further post-mortem in situ, *in vitro* and *in vivo* studies are needed to get a better understanding of Sema5a roles in the developmental processes of the telencephalon in health and disease.

Keywords: cortex, axon guidance, thrombospondin repeats, autism spectrum disorder

Acknowledgements: This study was co-financed by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, grant agreement No. KK.01.1.1.01.0007, CoRE - Neuro.

RADIONICA / WORKSHOP

David Smith[1] **and David Anderson**[2]

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The new Directive 2010/63/EU on the protection of animals used for scientific purposes entered into force in Member States on January 1st, 2013. As with the previous Directive 86/609/EEC, this Directive requires that experiments are designed to cause the least pain, suffering, distress or lasting harm to the animals used.

However, there is a new and additional requirement that all procedures are assigned a severity classification in advance of the procedure being performed. Furthermore, the actual severity experienced by each individual animal must be reported in the Statistical returns. This actual severity of any previous procedure is a key consideration in determining whether or not an animal can be re-used in further procedures.

The implementation of a severity classification process, both prospectively and retrospectively, is a big challenge in animal studies as it entails legal and ethical implications. On the other hand, it is an important and useful tool for properly evaluating research projects with regards to their prospective severity classification so as to implement the least constraining procedures (refinement), and keep the level of severity of procedures to the lowest possible. The reporting of actual severity will help to refine similar procedures going forward and improve communication with the public by providing a more detailed picture of what animals experience during procedures.

The animal models used in the workshop will be based on commonly used laboratory species and will address the three steps described in the document of the Commission's Expert Working Group which was endorsed by the National Competent Authorities in 2012: designing the project, monitoring it when it is performed and assessing the outcomes after it has ended.

The workshop will consist of lectures, case studies and group participation/ discussions.

The workshop is addressed to project leaders, scientists applying for project applications, veterinarians, responsible persons for animal welfare, advisors to and members of Animal Welfare Body, senior technicians, animal care staff and members of Ethical Review Boards.

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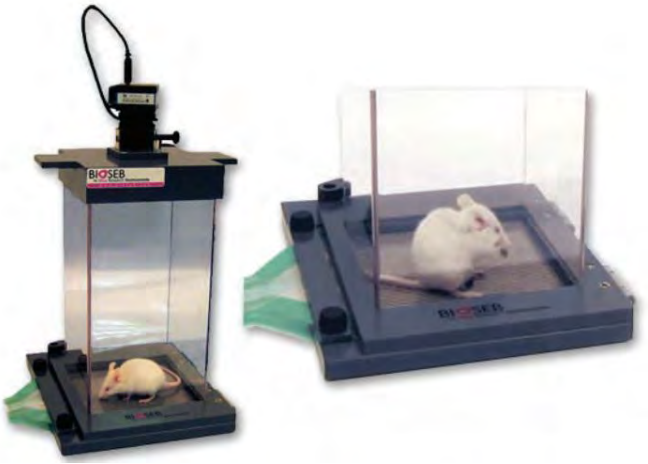

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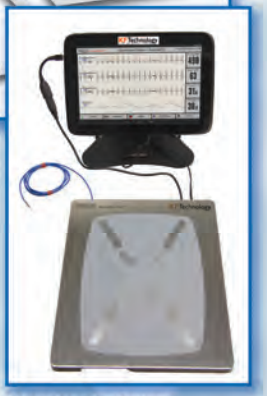

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