Lectures

L4.1

Cholinergic-mediated microRNA control of hnRNPs A/B regulates cortical alternative splicing and neural functioning

Amit Berson1, Galit Shaltiel1, Shahar Barbash1, Yael Goll1, Geula Hanin1, Keren Ofek1, Maya Ketzef1, Yehudit Gnatek1, Alon Friedman1, Hermona Soreq1

1The Edmond and Lily Safra Center of Neuroscience and Institute of Life Sciences, Department of Biological Chemistry, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel; 2Zlotowski Center for Neuroscience, Department of Physiology, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

Genetic studies link neurodegeneration to inherited errors in RNA metabolism. Here, we report cholinergic-mediated alternative splicing errors in sporadic Alzheimer’s disease (AD). AD entorhinal cortices presented impaired exon exclusions, selectively reduced hnRNP A/B splicing regulators and increased levels of the hnRNP A/B-targeted miRNA-211. Supporting functional relevance, hnRNP A/B knockdown in mice induced memory and electro-corticographic impairments, dendrite and synapse loss and splicing abnormalities. In-vivo neurotoxin-mediated destruction of cholinergic neurons, but not APP or Tau mutagenesis caused cortical AD-like decrease in hnRNP A/Bs and reduced exon exclusions. Inversely, cholinergic excitation increased hnRNP A/B levels, suppressed miR-211 and elevated the cholinergic enhancer miRNA-132 which was drastically reduced in the AD cortex. Furthermore, suppressing miR-132 elevated miR-211 in primary neurons whereas miR-211 did not change miR-132. Our findings uncover hierarchical cholinergic and miRNA-mediated hnRNP A/B loss and consequent RNA metabolism impairments as novel targets for interference with sporadic neurodegenerative processes.

L4.2

RNA in pathogenesis and experimental therapy of polyglutamine diseases

A. Mykowska, A. Fiszer, M. Wojciechowska, W. J. Krzyzosiak

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

e-mail: Wlodzimierz Krzyzosiak <wlodkrzy@ibch.poznan.pl>

Mutant transcripts containing expanded untranslated CUG repeats are well documented pathogenic agents in myotonic dystrophy type 1 (DM1). The mutant RNA sequesters the MBNL1 splicing factor and causes misregulation of the alternative splicing of multiple genes that are linked to clinical symptoms of the disease. Here we show that alternative splicing defects are also caused by translated CAG repeats present in transcripts from mutant genes implicated in spinocerebellar ataxia type 3 (SCA3) and Huntington’s disease (HD) as well as by untranslated CAG repeats. We propose that alternative splicing deregulation by mutant CAG repeats may contribute to the pathological features of polyglutamine disorders. We also demonstrate selective targeting of mutant HD transcripts, in the presence of their normal alleles and other human mRNAs containing CAG repeats, by RNA interference reagents. This selectivity may lead to promising therapeutic modalities for HD and other polyglutamine disorders.
**L4.3**

**Intersectins and neurodegenerative diseases**

Alla Rynditch, Liudmyla Tsyba, Mykola Dergai, Dmytro Morderer, Inessa Skrypkina, Oleksii Nikolaenko, Oleksandr Dergai, Sergii Kropyvko

Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Department of Functional Genomics, Kyiv, Ukraine  
e-mail: Alla Rynditch <rynditch@imbg.org.ua>

The rich binding capability of the multidomain, adaptor and scaffolding proteins of intersectin (ITSN) family has linked them to multiple functions such as clathrin-mediated endocytosis, mitogenic signalling, actin cytoskeleton rearrangements and apoptosis. One of these family members, ITSN1, is characterized by high level of expression in neurons. ITSN1 gene is mapped to chromosome 21 in the Down syndrome (DS) critical region. Abnormalities of ITSN1 expression were associated with the endocytic anomalies reported in Down syndrome brains, early stages of Alzheimer's disease as well as with neurodegeneration in Huntington's disease. DS neurons are characterized by enlarged early endosomes as well as enlarged early endosomes are the earliest neuropathological alterations identified in sporadic Alzheimer's disease.

In order to highlight the role of ITSN1 gene in neurons, we studied its expression in normal and pathological brains as well as analyzed the composition of ITSN1-containing protein complexes in neurons. We have identified neuron-specific alternative splicing of microexon 20 that affects the structure and binding abilities of the SH3A domain of ITSN1. Structure modelling and mutational analysis revealed that the insertion encoded by the microexon shifts negatively charged amino acid residues towards the interaction interface within the n-Src loop of the SH3A domain. This event leads to dramatic change of the SH3A domain binding properties, in particular to increasing the binding affinity to endocytic proteins dynamin and synaptojanin. In contrary, binding affinity of neuron-specific SH3A domain for to Ras guanine nucleotide exchange factor Sos1 and E3 ubiquitin-ligase Cbl has decreased.

Analysis of ITSN1-containing protein complexes revealed novel neuron-specific ITSN1 protein partners: stable tubule-only polypeptide/MAP6 microtubule-associated protein 6 (STOP/MAP6) and synapsin. STOP/MAP6 regulates microtubule stability and is involved in generation of synaptic plasticity, which is thought to be a molecular background of learning and memory. Moreover, STOP knockout mice were proposed to be used as an animal model of schizophrenia. Since both, ITSN1 and STOP, are implicated in functioning of synapses and associated with mental disorders, further investigations of their interactions are of particular interest.

**Oral presentations**

**O4.1**

**The neurotrophic factors mRNA expression in U87 glioma cells and its regulation by hypoxia and ischemic conditions are dependent from ERN1 gene function**

O. V. Hubenya¹, D. O. Minchenko¹,²,³, A. P. Kharkova¹, M. Moenner³, O. H. Minchenko¹,³  
¹Department of Molecular Biology, Palladin Institute of Biochemistry National Academy of Sciences of Ukraine, Kyiv, Ukraine; ²Department of Pediatrics, Bohomoletz National Medical University, Kyiv, Ukraine; ³INSERM U920 Molecular Mechanisms of Angiogenesis Laboratory, University Bordeaux 1, Talence, France  
e-mail: Olena Hubenya <gubenia.al@yandex.ua>

Gliomas are characterized by diffuse infiltrative growth in the surrounding brain parenchyma, which precludes complete surgical resection and is responsible for local recurrences. Endoplasmic reticulum to nuclei-1 (IRN1) is a central mediator of the endoplasmic reticulum stress. Blockade of ERN1 transduction pathways in experimental tumor models suggests that its activity is linked to the neovascularization process.

The main goal of this work is to study the role of neurotrophic factors in malignant gliomas progression under the inhibition of endoplasmic reticulum to nuclei-1 signaling pathways as well as the effect of hypoxia and ischemic conditions on the expression of several genes encoded the neurotrophic factors. We have shown that the expression levels of BDNF (brain-derived neurotrophic factor) and PRNP (prion protein) are increased but NPDC1 (neural proliferation, differentiation and control), PSEN1 (presenilin) and SLC1A1 (neuronal epithelial high affinity glutamate transporter) – decreased in glioma cells with suppressed activity of endoplasmic reticulum to nuclei-1. It was also shown that hypoxia is induced the expression levels of BDNF, NPDC1, PNPLA6 (neuropathy target esterase) and SLC1A1 in control glioma cells, but in glioma cells with suppressed activity of endoplasmic reticulum to nuclei-1. At the same time, the expression levels of PRNP and SLC1A1 is decreased in genetically modified cells. Glutamine deprivation condition leads to increase the expression levels of PSEN1 and PRNP in both cell types, however the expression levels of PNPLA6 is increased in control glioma cells and decreased in cells with suppressed activity of endoplasmic reticulum to nuclei-1. We have also shown that glucose deprivation condition leads to increase the expression levels of PRNP, PNPLA6 and SLC1A1 in control glioma cells and to decrease the expression levels of NPDC1, BDNF, PNPLA6 and SLC1A1 in genetically modified cells. Thus, our results showed that blockade of the activity of endoplasmic reticulum to nuclei-1 signaling enzyme changes the expression levels of neurotrophic factors and modulate effect of hypoxia and ischemia on these genes expression.
Identification of novel targets of STAT3 transcription factor in glioma cells
Karolina Swiatek-Machado, Michal Dabrowski, Jakub Mieczkowski, Piotr Przanowski, Alicja Adach Kilon, Bozena Kaminska
Nencki Institute of Experimental Biology, Department of Cell Biology, Warsaw, Poland
e-mail: Karolina Swiatek-Machado <k.swiatek@nencki.gov.pl>

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor constitutively activated in diverse human tumors, including gliomas and contributing to malignant transformation, tumor progression and resistance to apoptosis. Using microarray techniques, hundreds of genes have been identified as potential STAT3 targets, however the small fraction of these genes have been proven to be direct STAT3 targets. Here we report identification of novel, direct STAT3 target genes using microarray techniques, computational methods for the genome-wide identification of transcription factor binding sites and chromatin immunoprecipitation. We determined global changes in gene expression in C6 glioma cells treated for 24 h with either DMSO (control) or 25 μM the inhibitor of JAK/STAT3 signaling pathway. The analysis revealed that inhibition of JAK/STAT3 signaling significantly modulates expression of numerous genes involved in many different biological processes such as inflammatory response, apoptosis, signal transduction, proliferation, but also RNA processing or lipid metabolism. Next, we analyzed promoter regions of differentially expressed genes. First, we searched for conserved non coding region between rat and human sequences was used to select the binding sites most likely to be functional. For further validation we selected 12 genes with at least two putative binding sites within the promoter regions. All of the tested sites were bound by STAT3 in C6 glioma cells, as assessed by chromatin immunoprecipitation. Altogether, our results demonstrate identification of a group of novel STAT3 targets, shed light on complexity of a genetic network regulated by JAK/STAT3 signaling in glioma cells and its functions.

Acknowledgements
The study was supported by grant N N405 621938 from the Polish Ministry of Science and Higher Education (BK).

Activation of transcription factor NFκB in malignant and nonmalignant glioma cell lines
Joanna Bem, Magdalena Tyburczy, Bozena Kaminska
Nencki Institute of Experimental Biology, Department of Cell Biology, Warsaw, Poland
e-mail: Joanna.Bem <bemjoanna@gmail.com>

NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) is known to be one of the most important inflammation regulators. It plays a key role in autoimmune diseases and cancer development. NF-κB regulates transcription of a variety of genes including cytokines, chemokines, adhesion molecules and many others. Correlation between increased Akt phosphorylation and high levels of NFκB activity in malignant gliomas has been reported. We recently reported that inhibition of NFκB transcriptional activity by inhibitors of PI3-K/Akt signaling or the NFκB inhibitor - BAY11-7082, affects MMP-2 expression and impairs glioma invasion (Kwiatkowska et al., 2011). Elucidating mechanisms of NFκB activation and its role in different glioma types would increase our understanding of brain tumor invasion. Inactive NF-κB is bound to IκB (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) and retained in cytoplasm. A few pathways of NF-κB activation have been characterized. The classical pathway involves phosphorylation of IκB by IκB kinases (IKK-β, IKK-γ) that leads to its degradation by a proteasome complex. NF-κB is released and translocates to the nucleus, where it can regulate expression of target genes. Our aim was to elucidate if there are differences in NF-κB regulation in malignant glioma and nonmalignant cell lines. First, the relative expression of (IKK-β, IKK-γ) was determined in various established and primary glioma cell lines by quantitativePCR. The activity of NF-κB was further determined in transfected cells using NF-κB binding site-driven reporter luciferase gene using a dual light reporter luciferassay. We found that the expression of IKK-β and IKK-γ varies between malignant glioma versus nonmalignant cells. Those differences were corroborated by various levels of NF-κB transcriptional activity in malignant gliomas and nonmalignant cells. Our data show that in malignant gliomas NF-κB activity is higher than in non-malignant tumors (SEGA — subependymal giant cell astrocytoma) and in normal human astrocytes. It corresponds to the levels of phospho-IκB detected by immunoblotting. The classical activation pathway is likely involved in this process. Inmalignant and nonmalignant cell lines, NF-κB activity corresponds to the expression of IKK-β and IKK-γ kinases.
Alzheimer’s disease (AD) is the most common age-related dementia characterized by progressive neuronal loss. About 95% of AD cases occur sporadically and have unknown etiology (SAD), whereas the remaining familial AD cases (FAD) are mostly caused by autosomal dominant mutations in the presenilin 1 (PS1) gene. PS1 is the main enzymatic component of gamma-secretase complex responsible for release of toxic beta-amyloid from its amyloid precursor protein (APP). Growing evidence suggests that in AD neurons re-enter the G1 cell cycle phase, which leads to cell death. Since some alterations in AD are seen also in peripheral cells, we checked if cell cycle changes occur in AD lymphocytes. The study involved immortalized lymphocytes derived from a group of Polish patients: 8 FAD patients with distinct PS1 mutations, 18 SAD patients, and 34 age-matched individuals. Our PCR arrays experiments showed that 43% of the 90 investigated cell cycle genes were down-regulated in SAD, whereas 4% were up-regulated comparing to controls. Interestingly, 19% of the cell cycle genes were differentially expressed in the SAD versus FAD. Most significant changes in the expression referred to the genes engaged in the G1/S control. Therefore we assessed the levels of key proteins involved in the G1/S transition with immunoblotting. The most striking difference we found regarded p21 protein, which was significantly elevated in SAD comparing to control and FAD lymphocytes. Furthermore, our FACS analysis demonstrated that alterations in the cell cycle genes expression and p21 increase observed for SAD lymphocytes were accompanied by increased % of cells in G1 phase and decreased % of cells in S phase. Using FACS cell cycle analysis after nocodazole treatment and pulse chase BrdU labeling we showed that observed G1 arrest in SAD cells was a consequence of G1 phase prolongation and S phase shortening. Moreover, we found that the observed cell cycle changes were independent on gamma-secretase activity.

Our data demonstrate that cell cycle abnormalities are common for SAD, but not for FAD lymphocytes. Thus, this study brings to light differences in the mechanisms of FAD and SAD pathogenesis. Furthermore, these results indicate that human lymphocytes sustain a useful model for further analyzes of AD pathogenesis and for the development of new diagnostic methodologies targeting cell cycle proteins, such as p21.

Introduction: Enhanced expression and activities of matrix metalloprotease (MMPs) have been observed under numerous pathologic conditions. Therefore inhibition of MMPs is considered as a potential therapeutic target. After cerebral ischemia it has been shown that there is a marked increase of MMP-9 expression which has been implicated in breakdown of Blood brain barrier (BBB). Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1) is a 28 KDa endogenous inhibitor of MMP-9.

Objectives: In this study we plan to deliver TIMP-1 using PLGA Nanoparticles across BBB and investigate whether this can be an effective strategy to prevent BBB disruption after ischemia and ameliorate ischemic cell death.

Methods: In order to get TIMP-1, the protein was cloned in pCMV plasmid with a Histag at C terminal. The plasmid was expressed in HEK 293T Cell lines and isolated using Cobalt based affinity column followed by dialysis. TIMP-1 loaded PLGA nanoparticles were synthesized by multiple emulsion and solvent evaporation method and coated with Tween 80 for BBB delivery.

Results: TIMP-1 was characterized using western blot, reverse zymography, gelatinase assay and protein sequencing. PLGA NPs were analysed by using SEM, DLS, PDI, Zeta potential, protein loading and drug release. The mean size of NPs as measured using SEM was found to be 90.2 ± 5 nm. The mean hydrodynamic diameter of NPs as measured by DLS was 330 nm. The zeta potential of TIMP-1-NPs was 7.78 ± 2.5 mV. The encapsulation efficiency of TIMP-1-NPs was 71 ± 5.1% (n=3); i.e., 71% of the added protein was entrapped into NPs. The release of TIMP-1 from NPs was sustained, with 17.1 ± 1.4% of release in 24 h, 65.1 ± 1.1% in 1 wk. At present the formulation is being tested for BBB penetration, MMP-9 inhibition and neuroprotection in vivo.
Cyclosporine A-induced endoplasmic reticulum stress triggers autophagy of malignant glioma cells

Iwona Ciechomska, Bozena Kaminska
Nencki Institute of Experimental Biology, Department of Cell Biology, Warsaw, Poland
e-mail: Iwona Ciechomska <jcjech@nencki.gov.pl>

Autophagy is a self-digestion process allowing cell survival during starvation but functions also as an alternative death mechanism under certain conditions. Autophagy is accompanied by the progressive formation of vesicle structures from autophagosomes to autophagolysosomes, and involves both autophagy effectors (Atg proteins) and regulators (i.e. mTOR - mammalian target of rapamycin is a negative regulator). Malignant gliomas are highly resistant to available therapies which induce apoptosis, thus induction of the alternative cell death is an attractive strategy. We demonstrate that cyclosporine A (CsA, an immunophilins/calcineurin inhibitor) induces cell death with some apoptotic features but also accompanied by the appearance of numerous cytoplasmic vacuoles, immunostained for endoplasmic reticulum (ER) stress and autophagy markers. The induction of ER stress in glioma cells by CsA was evidenced by detection of unfolded protein response activation (phosphorylation of PERK) and accumulation of ER stress associated proteins (BIP and CHOP). Upon CsA treatment several autophagy features were observed: formation of the acidic vesicular organelles, increase in punctuate GFP-LC3 (microtubule-associated protein light-chain 3) and LC3-II accumulation. Decrease of phosphorylation 4E-BP1, p70-S6K1, and its downstream target molecule S6 ribosomal protein suggests that CsA affects mTOR signaling. Salubrin, which protect cells from ER stress, partially blocked CsA-induced decrease of p70-S6K1 and 4E-BP1 phosphorylation, and accumulation of LC3-II. It suggests that ER stress was primary to CsA-induced autophagy. Surprisingly, selective silencing of Atg1, Atg5 or Atg7 increased the level of active caspases 3, 7 and PARP degradation in CsA-treated cells. Our results demonstrate that CsA induces both apoptosis and autophagy in malignant glioma cells via induction of ER stress and inhibition of mTOR/p70-S6K1 pathway, however autophagy is cytoprotective in this context.

Acknowledgements
Studies supported by grant N N301 092036 from The Polish Ministry of Science and Higher Education.

A novel approach of gene delivery in cortex and hippocampus of mouse brain to study Mmp-9 gene regulation in depolarized neurons

Krishnendu Ganguly, Emilia Rejmak, Michał Stawarski, Leszek Kaczmarek
Nencki Institute of Experimental Biology, Department of Cellular and Molecular Neurobiology, Warsaw, Poland
e-mail: Krishnendu Ganguly <L.Kaczmarek@nencki.gov.pl>

Understanding of the molecular underpinnings of gene regulation during depolarization-driven synaptic plasticity is a major challenge of modern neuroscience. MMP-9 (matrix metalloproteinase), is a crucial enzyme that helps in transient alteration of neuronal circuits by affecting extracellular matrix (ECM) components. The molecular mechanism behind Mmp-9 gene transcription during depolarization of brain neurons in vivo is poorly understood. Notably, presently available in vitro reporter assay-based systems for gene regulation do not simulate adequately the gene regulation that occurs in the brain. Therefore, this study was directed to develop a strategy to study the transcriptional regulation of Mmp-9 gene in mouse brain neurons in response to pentylenetetrazole (PTZ)-induced seizure. The transcriptional response of Mmp-9 gene was determined by real time based quantitative PCR and in situ hybridization. Furthermore, wild type and mutated versions of Mmp-9 reporter gene in both plasmid and BAC (Bacterial Artificial Chromosome) vectors were introduced into the brain by means of electroporation. Both the reporter gene transcripts and proteins levels were increased in PTZ-induced brain neurons. In conclusion, this is the first effort by which we studied the in vivo transcriptional regulation of Mmp-9 gene by reporter based assay system, during synaptic plasticity driven by depolarization of mammalian brain neurons.
**P4.6**

**The effects of antiepileptic agents on fusion of synaptic vesicles in cell-free model of exocytosis and the role of cholesterol in this process**

Vitaliy Gumenyuk¹, Tamara Kuchmerovska², Irene Trikash¹

Palladin Institute of Biochemistry of National Academy of Sciences of Ukraine; ¹Department of Neurochemistry and ²Department of Biochemistry of Vitamines and Coenzymes, Ukraine

e-mail: Vitaliy Gumenyuk <vitakli@yandex.ru>

The neurochemical mechanisms underlying seizure-associated dysfunctions of central nervous system are multiple and poorly understood. The antiepileptic drugs which directly or non-directly attenuate brain dysfunction can use to restore the balance between the excitation and inhibition of neurons. To elucidate the effects of ethosuximide, sodium valproate, and gabapentin on the exocytosis process the calcium-dependent fusion of synaptic vesicles (SVs) in cell-free model system was investigated. All studies were carried out on SVs isolated from rat brains. Membrane fusion was assayed with the fluorescence probe R18. To estimate the role of membrane cholesterol in synaptic vesicle fusion the cholesterol content in membrane was modulated by methyl-β-cyclodextrin used as a cholesterol binding agent. It has been found that all studied antiepileptic agents increase the Ca²⁺- and Mg²⁺/ATP-dependent SVs fusion. The 20% reduction of membrane cholesterol leads to decrease of the rate of calcium-triggered fusion of homotypic membranes of SVs that indicates the disturbances in function of proteins driving this process. At the same time, the reduction of cholesterol level in SVs membrane did not change the ability of antiepileptic drugs to enforce the fusion process. These findings suggest that antiepileptic drugs are capable to bind to SVs proteins which are not located in cholesterol rich lipid microdomains. Thus, it was shown that SVs proteins can be the functional targets for the action of ethosuximide, sodium valproate, and gabapentin in regulation of exocytosis in the nerve terminals. This may be the basis for further studies of new antiepileptic drugs action at the level of SVs proteins and SVs fusogenic activity.

**P4.7**

**Changes in homocysteine thiolactone disposition and neurotoxicity in bleomycin hydrolase (Blmh) and paraoxonase 1 (Pon1) knock-out mice**

Kamila Borowczyk¹, Joanna Tisonczyk¹, Hieronim Jakubowski¹,²

¹UMDNJ-New Jersey Medical School, Department of Microbiology and Molecular Genetics, Newark, NJ, USA; ²Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

e-mail: Hieronim Jakubowski <jakubows@umdnj.edu>

Genetic or nutritional disorders in methionine metabolism increase homocysteine (Hcy) editing by methionyl-tRNA synthetase, which generates Hcy-thiolactone and is linked to cardiovascular and neurological diseases. Hcy-thiolactone has the ability to form isopeptide bonds with protein lysine residues, which generates toxic N-Hcy-proteins with autoimmunogenic, prothrombotic, and amyloidogenic properties. Bleomycin hydrolase (Blmh) and paraoxonase 1 (Pon1) have the ability to hydrolyze Hcy-thiolactone in vitro. To determine physiological roles of Blmh and Pon1, we studied Hcy-thiolactone disposition and neurotoxicity in mice with inactivated Blmh and Pon1 genes. We found that endogenous Hcy-thiolactone and N-Hcy-protein levels were elevated in Blmh⁻/⁻ and Pon1⁻/⁻ mice compared with wild type animals. Exogenous Hcy-thiolactone, injected intraperitoneally, was hydrolyzed less effectively by Blmh⁻/⁻ than by Pon1⁻/⁻ and wild type mice. We also found that Blmh⁻/⁻ and Pon1⁻/⁻ mice were more sensitive to Hcy-thiolactone neurotoxicity than wild type animals. Taken together, these findings indicate that Blmh and Pon1, by hydrolyzing Hcy-thiolactone and minimizing protein N-homocysteinylated, protect against Hcy-thiolactone neurotoxicity in mice.
P4.8

Co-regulation of expression of NFκB-dependent genes by the HSF1 transcription factor

Patryk Janus1, Magdalena Kalinowska-Herok1, Małgorzata Pakuła-Cis2, Wojciech Pignowski1, Katarzyna Szöltysek1, Adam Makuchowski3, Marek Kimmel3, Piotr Widłak1

1Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland; 2University of Aarhus, Aarhus, Denmark; 3Silesian University of Technology, Gliwice, Poland

e-mail: Patryk Janus <patrykjanus@gmail.com>

NFκB- and HSF1-dependent pathways are essential components of cellular responses to stress. They play the major role in pathogenesis of serious human diseases, including cancer and response to therapeutic treatments. Both of these transcription factors regulate several genes involved in cell proliferation, apoptosis, immune and inflammatory responses. Here we aimed to identify NFκB-dependent genes which expression is affected by the active HSF1.

Activation of the NFκB pathway and expression of NFκB-dependent genes was analyzed in U2-OS human osteosarcoma cells stimulated with TNFα cytokine. Cells were either preconditioned with hyperthermia to activate endogenous HSF1 or engineered to express a constitutively active form of HSF1 in the absence of heat shock. The expression of NFκB-dependent genes was analyzed by quantitative RT-PCR, using both NFκB-pathway-oriented PCR-Array and gene-specific reactions. Binding of HSF1 to promoters of NFκB-dependent genes was analyzed by chromatin immunoprecipitation assay (ChIP) with anti-HSF1 Ab (genes with hypothetical sites of HSF1 binding were pre-selected based bioinformatics analysis).

We found that hyperthermia resulted in a general blockade of activation of the NFκB signaling and expression of NFκB-dependent genes. In marked contrast, the presence of constitutively active HSF1 did not block TNFα-induced activation of the NFκB pathway and general expression of the NFκB-dependent genes in the absence of the heat shock. However, the presence of HSF1 affected expression of several specific NFκB-dependent genes in the absence of the heat shock. Four of these genes, namely TNFA, IL-6, FASLG, AGT, contained functional binding sites for HSF1 in their promoter regions.

We concluded that expression of several NFκB-dependent genes is modulated by HSF1-dependent mechanisms. Some of these genes could be regulated by HSF1 in the direct mechanism due to the binding of HSF1 transcription factor to their promoter regions.

Acknowledgements
This work was supported by the Ministry of Science and Higher Education, grants N514 41936, PBZ-MNiI 2/1/2005 and by the European Community from the European Social Fund within the RFSD-2 project.

P4.9

Identification of signal transduction pathways re-programming macrophages into tumor supportive cells — on the missing link between inflammation and cancer

Bożena Kaminska, Konrad R. Gabrusiewicz, Paweł Wisniewski, Aleksandra Ellert-Miklaszewska, Maciej Lipko, Michał Dworowski, Magdalena Kijewska, Beata Kaza, Małgorzata Porycka

Nencki Institute of Experimental Biology, Laboratory of Transcription Regulation, Warsaw, Poland

e-mail: Bożena Kaminska <bozenakk@nencki.gov.pl>

Malignant tumors benefit from support from the surrounding tumor microenvironment composed of tumor-associated fibroblasts, leukocytes, bone marrow-derived cells, blood and lymphatic vascular endothelial cells. The microenvironment provides essential cues to the maintenance of cancer stem cells/cancer initiating cells, rare cells which contribute to tumor propagation and the immunosuppressive tumor milieu. Tumor-infiltrating macrophages and immunomodulatory mediators present in the tumor microenvironment polarize host immune response toward specific phenotypes impacting tumor progression. Aggressive human brain tumors (glioblastomas) contain numerous glioma-infiltrating macrophages, which abundance correlates with malignancy and poor patient prognosis. Using immunofluorescence and flow cytometry, we demonstrated an early accumulation of activated microglia followed by accumulation of blood derived macrophages in experimental murine EGFP-GL261 gliomas. Those cells acquire the alternative phenotype, as evidenced by evaluation of the production of ten pro/anti-inflammatory cytokines and expression profiling of 28 genes in magnetically-sorted CD11b+ cells from tumor tissues. In vitro studies revealed that glioma cells secrete soluble factors which convert primary microglial cultures into amoeboid cells supporting glioma invasiveness, while attenuating inflammatory responses. Global gene expression profiling of microglial cultures, stimulated either by glioma-conditioned medium or by a classical inflammation inducer — lipopolysaccharide (LPS), revealed different patterns of gene induction, and activation of distinct signaling and metabolic pathways. Proteomic analysis of glioma-conditioned medium using HPLC fractionation followed by a tandem mass-spectrometry identified two activating proteins which are small integrin-binding ligand N-linked glycoprotein family members. Interference with ligand binding to integrins using a blocking RDG peptide or gene silencing in glioma cells, abolished microglial activation induced by glioma-conditioned medium and their influence on glioma invasion. These data provide strong evidence on the identity of signals which direct tumor-related inflammation and tumor progression.

Acknowledgements
Studies supported by the Ministry of Science and Higher Education grant N N301 7862 (BK).
A role of osteopontin — a small integrin-binding ligand in glioma pathology

M. Kijewska, M. Sielska, A. Ellert-Miklaszewska, K. Gabrusiewicz, B. Kaminska
Laboratory of Transcription Regulation, Nencki Institute of Experimental Biology, Warsaw, Poland
e-mail: Magdalena Kijewska <bozenakk@nencki.gov.pl>

Tumor-associated macrophages represent the major component of the stroma of many tumors, including gliomas, and their high content correlates with malignancy and poor patient prognosis. We have demonstrated that glioma cells release soluble factors which induce accumulation and a non-inflammatory activation of brain macrophages associated with pro-invasive function of these cells. Proteomic analysis of glioma-conditioned medium (G-CM) revealed that one of these factors is Osteopontin (OPN, known as secreted phosphoprotein 1 — SPP1), a metastasis-associated small integrin-binding ligand and a N-linked glycoprotein family member. OPN was highly overexpressed in rat and some human glioma cells when compared to non-transformed astrocytes. To evaluate the role of OPN in glioma biology, we constructed plasmids encoding shRNA against OPN (shOPN) or negative control shRNA (shNeg). We obtained C6 glioma cells stable transfected with indicated plasmids. The silencing of OPN in shOPN clones was confirmed on RNA and protein level. Silencing of OPN had no impact on cell proliferation and survival. To evaluate the role of OPN in glioma invasiveness we performed Matrigel invasiveness assay. We demonstrate that silencing of OPN diminished invasiveness of glioma cells and abolished the pro-invasive effects of microglia co-cultured with glioma cells. To follow the role of OPN in glioma pathology in vivo, we injected shOPN and shNeg C6 cell clones into the striatum of Wistar rats. Tumor volumes and microglia and lymphocytes infiltration into the tumor were evaluated. Our studies indicate that tumor-derived OPN may be a crucial factor mediating interactions between glioma and tumor-associated brain macrophages and is involved in glioma pathogenesis.

Acknowledgements

The study was supported by grant GP2908 from the Ministry of Science and Higher Education.

Expression of HIF mediates endogenous neuroprotection of hippocampal neurons during the ischemic and anoxic precondition

Anastasiia M. Maistrenko, Irina V. Lushnikova, Maxim M. Orlovsky, Victor E. Dosenko, Galina G. Skibo
Bogomoletz Institute of Physiology, Department of Cytology, Ukraine
e-mail: Anastasiia Maistrenko <Sayra@yandex.ru>

Brief anoxia or episodic hypoxic preconditioning can enhance a brain resistance to subsequent prolong ischemia. However, a little is known about events occurring in the time frame between anoxic preconditioning (APC) and subsequent formation of ischemic resistance. Existing reports in this area are predominantly focused on APC morphological features, in particular, the inhibition of neuronal apoptosis, neurogenesis or survival of the neurons. Although it has been shown that hypoxia may induce extensive injury to susceptible brain neurons, it does not necessarily follow that such injury results in neuronal death, but rather a marked impairment of brain functioning, such as behaviours, stress response, learning and memory. Thus while APC could protect brain from hypoxic/ischemic injury, the mechanisms of this action require further investigations.

Recent investigations indicate that mechanisms of such stimulated endogenous neuroprotection are related to the family of hypoxia-inducible factors (HIF), however there are still little data concerning the role of HIF family members in hippocampus — a brain structure, highly sensitive to oxygen deficiency. We have used the model of cultured hippocampal slices and single-cell quantitative RT-PCR to study HIF-1α and HIF-3α mRNA expression following triple 5-min mild anoxia, 30-min oxygen-glucose deprivation and their combination. The viability was evaluated with PI-staining. We also tested the effects of HIF prolyl-hydroxylase inhibition with 2,4-pyridinedicarboxylic acid diethyl ester pre-treatment followed by 30-min oxygen-glucose deprivation. It was found that neuronal damage induced by oxygen-glucose deprivation was accompanied by a significant decrease in both HIF-1α and HIF-3α mRNA levels in CA1 but not CA3 neurons. Anoxia preconditioning did not affect cell viability and HIF mRNA levels but applied before oxygen-glucose deprivation prevented neuronal damage and suppression of HIF-1α and HIF-3α mRNA expression. It was also found that effects of the prolyl-hydroxylase inhibitor were similar to anoxia preconditioning. These results suggest that anoxia preconditioning increases anti-ischemic neuronal resistance which to a certain extent correlates with the changes of HIF-1α and HIF-3α expression.
β-catenin is constitutively present in the nuclei of postmitotic thalamic neurons due to WNT-independent mechanism

Katarzyna Misztal¹, Marta B. Wisniewska¹, Mateusz Ambrozkiewicz¹, Jacek Kuznicki¹,2

¹International Institute of Molecular and Cell Biology, Warsaw, Poland; ²Nencki Institute of Experimental Biology, Warsaw, Poland

Wnt activation promotes β-catenin accumulation upon inhibition of β-catenin degradation. Stabilized β-catenin translocates to the nucleus where it triggers transcription of the Lef1/Tcf target genes. Wnt/β-catenin signaling is essential for nervous system development as well as division and maturation of neuronal progenitors in adult brain.

We showed recently that nuclear β-catenin is abundant in vivo in non-dividing neurons of adult thalamus, where it is involved in gene transcription of CACNA1G gene (Wisniewska et al., 2010, J Neurosci). Here we demonstrate spontaneous accumulation of β-catenin in 40% of cultured thalamic neurons and lack of such accumulation in cortical neurons. This phenomenon does not depend on soluble factors produced by glia or cortical neurons, since neither conditioned medium of cortical cells nor glial cells co-culture affect the number of β-catenin positive cells. This suggests that nuclear localization of β-catenin in thalamic neurons is not a consequence of paracrine stimulation.

We also observed that Wnt receptor inhibitor DKK1 and dnDVL3 had no major effect on the number of β-catenin positive thalamic neurons. Thus, autocrine WNT stimulation is not responsible for nuclear β-catenin accumulation in these neurons. We analyzed expression of APC, AXIN1 and GSK3β that are involved in degradation of β-catenin and detected lower level of APC and GSK3β in thalamic neurons when compared to cortex and hippocampus. Reduced levels of these proteins were also observed in cultured thalamic neurons compared with cortical cultures. Finally, pulse-chase experiments confirmed that cytoplasmic β-catenin turnover was slower in thalamic neurons than in cortical neurons. Our observations support an idea that β-catenin accumulation is an intrinsic feature of thalamic neurons, independent of cellular environment of thalamic neurons and Wnt stimulation, but associated with low levels of proteins involved in β-catenin labeling for ubiquitination and subsequent degradation.

Acknowledgements
This work is supported by "Health-Prot" Grant no 229676 and Polish MNiSW Grant no 4245/B/P01/2010/38 and 1917/B/P01/2010/39.

Loss of snap29 impairs endocytic recycling and cell motility

Debora Rapaport¹, Yevgenia Lugassy², Eli Sprecher²,³,⁴, Mia Horowitz¹

¹Tel Aviv University, ²Department of Cell Research and Immunology, ³Sackler Faculty of Medicine, ⁴Tel Aviv Sourasky Medical Center, Department of Dermatology, ⁵Technion, Center for Translational Genetics, Israel

Intracellular membrane trafficking depends on the ordered formation and consumption of transport intermediates and requires that membranes fuse with each other in a tightly regulated and highly specific manner. Intracellular trafficking of membrane receptors dictates the kinetics and magnitude of signal transduction cascades. Membrane anchored SNAREs assemble into SNARE complexes that bring membranes together to promote fusion. SNAP29 is a ubiquitous synaptosomal-associated SNARE protein localized in intracellular membranes. Loss of functional SNAP29 results in CEDNIK syndrome (Cerebral Dysgenesis, Neuropathy, Ichthyosis and Keratoderma) causing abnormalities in epidermal differentiation and in corpuscallosum accompanied by cortical dysplasia. Using fibroblast cell lines derived from CEDNIK patients, we show that SNAP29 mediates endocytic recycling of transferrin and β1-integrin. Impaired β1-integrin recycling affected cell motility, as reflected by changes in cell spreading and wound healing. Our results emphasize the importance of SNAP29 mediated membrane fusion in endocytic recycling and in cell motility.
CD150 receptor (IPO-3/SLAM) is a member of CD2 family within the immunoglobulin superfamily. It is expressed on thymocytes, activated T and B lymphocytes, dendritic cells and monocytes. However, it is still little known about CD150 expression in non-lymphoid cells, especially in tumors. The aim of our work was to study the expression of CD150 in human glioma cell lines and in primary tumors of central nervous system (CNS). Using reverse transcriptase reaction, followed by polymerase chain reaction (RT-PCR) with several pairs of specific primers to the CD150 extracellular, transmembrane and cytoplasmic domains, we showed differential expression of the gene among human glioma cell lines. The A172 cell line highly expressed mRNA for full transmembrane form of CD150, but the protein production was not detected in these cells. We found a low level of CD150 mRNA expression in the U87, U343, NCH89 and NCH92 cell lines. Expression of CD150 at the protein level was observed in fixed U87, U343, NCH89, NCH92 and TE671 cell lines using immunofluorescent staining. However, FACS analysis did not reveal CD150 on the surface of live cells. All studied glioma cell lines were not sensitive to the infection with wild type measles virus (which uses CD150 as entry receptor) and in contrast to laboratory strain of measles virus (which uses CD46 as receptor as well). These results showed that CD150 is absent on the surface of the studied glioma cells. Sequencing of the RT-PCR fragments from the U87 cell line revealed an unusual CD150 transcript containing an 83 bp insert between exons for the transmembrane and cytoplasmic regions of the full-length mRNA. Bioinformatics analysis showed that the insert was derived from a previously unrecognized exon located 511 bp downstream of the exon for the transmembrane region. The exon designated Cyt-new is flanked with canonical AG/GT splice sites. The use of the Cyt-new exon results in the reading frame shift in the exons Cyt1-Cyt3 and production of an isoform with cytoplasmic tail lacking any known signaling motifs. Specific primers for this novel CD150 isoform were designed and used for study its expression in cell lines of different origin, primary human CNS tumors and human normal brain. Taken together, we found a novel CD150 isoform that is expressed in malignant cells of central nervous system tumors.

The big progress was made in characterization of tumor-associated markers in central nervous system (CNS), which helps to identify cell of origin and the level of tumor cell differentiation. The efforts are focused now on new marker disclosure for routine immunohistochemical diagnostics, evaluation of invasiveness and tumor cell proliferation rate, prediction of treatment outcome and possible targets for therapy. CD150, encoded by the SLAMF1 gene, is a dual-function receptor, expressed in hematopoietic system and involved in immune regulation. Four isoforms of CD150 were identified: trans-membrane (mCD150), secreted that lacks transmembrane region, cytoplasmic, lacking the leader sequence and a variant membrane CD150 with truncated cytoplasmic tail. CD150 is one of two identified receptors for measles virus (MV) and binds hemagglutinin of both wild-type (wt) and vaccine MV strains. MV holds promise as a novel oncolytic agent in the treatment of tumors of CNS, but expression of wild type measles virus receptor CD150 was not reported on cells of the nervous system yet. Our immunohistochemical studies of primary human CNS tumors (108 cases) revealed CD150 expression in diffuse astrocytoma (66.7% of analyzed cases), anaplastic astrocytoma (81.3%), glioblastoma (88.9%), ependymoma (66.7%) and anaplastic ependymoma (75.0%). Expression of CD150 in different regions of human normal brain was not observed. Immunostaining of all samples with the antibodies to GFAP, nestin and CD68 validated the CD150 expression in the malignant cells. The majority of tested glioblastoma cell lines also expressed CD150, however we did not detect its expression on the cell surface and cells were resistant to MVwt infecton. To find which splice isoforms of CD150 were expressed by glioblastoma cells we performed RT-PCR and 3’RACE of CD150 mRNA, from the cell line U87. A variety of aberrant and normal mCD150 transcripts were found of which one contained a previously unrecognized 83 bp exon downstream of the exon for the transmembrane region. The exon insertion caused the reading frame shift and translation of a novel isoform with alternative cytoplasmic tail. Thus, we show that CD150 is frequently expressed in CNS tumors. Further studies will clarify the biological significance of CD150 isoforms expression in CNS tumors and validate the potential use of this marker in differential diagnostics and targeted therapy.
P4.16

Crosstalk between NFκB- and p53-dependent signaling pathways in HCT116 colon carcinoma cells

Katarzyna Szołtysek1, Patryk Janus1, Adam Makuchowski2, Marek Kimmel2, Piotr Widłak1

1 Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland; 2 Silesian University of Technology, Gliwice, Poland

e-mail: Katarzyna Szołtysek <kszoltysek@io.gliwice.pl>

Signaling pathways that depend on NFκB and p53 transcription factors are essential elements of cellular responses to stress. Both factors participate in regulation of a network of genes involved in control of the cell cycle, DNA repair, apoptosis, immune response and inflammation. Here we aimed to analyze the interference between these signaling pathways at the level of expression of selected NFκB- and p53-dependent genes.

Experiments were performed using human colon carcinoma HCT116 cells in two congenic lines either containing or lacking transcriptionally competent p53. Cells were incubated with TNFα cytokine to activate NFκB, and/or exposed to ultraviolet/ionizing radiation to activate p53 pathway; both factors were used in two different time combinations: stimulation with TNFα was placed either 3 hours before or 6 hours after irradiation. Activation of the NFκB and p53 pathways was monitored by Western-blot detection of selected proteins (IκBα, p53, p21, MDM2, PTEN, PARP, AKT). Expression of selected p53-dependent genes (MDM2, p21, WAF1, PTEN, NOXA) and NFκB-dependent genes (BCL3, NFKBIA, NFKB1, REL, IL1A, IL8, TNFA, TNFAIP3, JUN, LTA) was assessed by quantitative QRT-PCR; p53-dependent genes were analyzed 6, 12 and 24 hours after irradiation while NFκB-dependent genes were analyzed 1 and 6 hours after stimulation with TNFα.

We observed that radiation-induced activation of p53-dependent genes was affected in cells with activated NFκB: UV-induced expression of p53-dependent genes was further up-regulated by either mode of stimulation with TNFα. TNFα-stimulated activation of expression of NFκB-dependent genes was different in cells with different status of p53. Irradiation of p53-competent cells before stimulation with TNFα resulted in down-regulation of analyzed NFκB-dependent genes. Our data revealed interference between regulation of p53-dependent genes and activation of NFκB signaling and between regulation of NFκB-dependent genes and activation of p53 signaling.

Acknowledgements

This work was supported by the Ministry of Science and Higher Education, Grant NN 301 264536.

P4.17

Expression of pro-epileptic protease Matrix Metalloproteinases 9 is regulated by DNA methylation during epileptogenesis

Katarzyna Zybura-Broda1, Renata Amborska1, Leszek Kaczmarek1, Marcin Rylski2

1 Nencki Institute of Experimental Biology, Department of Molecular and Cellular Neurobiology, Warsaw, Poland; 2 The Medical Centre of Postgraduate Education, Department of Clinical Cytology, Warsaw, Poland

e-mail: Katarzyna Zybura-Broda <kzybura@nencki.gov.pl>

Matrix Metalloproteinase 9 (MMP-9) acts pro-epileptically, stimulating aberrant synaptic plasticity occurring during epileptogenesis. Here, we have studied epigenetic changes occurring on Mmp-9 proximal promoter during pentylene-tetrazole-induced epileptogenesis in the rat hippocampus. Rats received multiple intraperitoneal injections of pentylene-tetrazole in non-seizure inducing doses, what led to a precipitation of seizures and to a gradual progression in their strength and duration. We have shown that MMP-9 activity and expression of MMP-9 mRNA are gradually upregulated during PTZ-dependent epileptogenesis in the hippocampus. Moreover, using Methylated DNA Immunoprecipitation (MeDIP) and Methylation Specific PCR (MSP) we have demonstrated that the induction of MMP-9 gene expression is associated with a progressive demethylation of its proximal promoter. It’s probably performed by DNA demethylase Gadd45β, which increasingly binds to the proximal Mmp-9 promoter in vivo during PTZ-induced kindling. Accordingly, our results imply that important epigenetic mechanism — active DNA demethylation, play an essential role in a regulation of MMP-9 expression during development of epilepsy.