Session 12. Cellular and Molecular Neurobiology — new ideas and new technologies

Lectures

L12.1

Generation of transgenic mice with neuron-type specific gene knock-down

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Generation of transgenic mice by pronuclear injection of recombined DNA has a wide array of applications and is particularly useful for studying gene function. Compared to homologous recombination, introduction of a transgene is simpler and not restricted to selected mouse strains. Moreover, breeding of transgenic mice is simpler and requires fewer animals than in case of animals carrying homologously introduced modifications. For these reasons, several attempts were made to generate transgenic mice expressing short interfering RNAs targeting a specific gene as a replacement for gene-deletion approaches. However, these attempts were usually only partially successful and often suffered from low knock-down efficiency, poor cell-type specificity or transgene toxicity causing cell-death. Here I describe the generation of mouse transgenic lines with cell-type specific knock-downs of glutamate and opioid metabotropic receptors in specific neuronal populations. The transgenes were based on bacterial artificial chromosomes (BACs) harboring promoters of genes, which expression is restricted to dopaminoceptive or dopaminergic cells in the brain. Both microRNA and shRNA strategies were tested, in the latter case the transgene was constructed to require Cre-mediated recombination for activation. The *in vivo* knock-down efficiency was estimated at >90% with no toxicity observed. There was no change in abundance of microRNA precursors or any other indication that the transgene had an appreciable effect on transcription or RNA processing. Most importantly phenotypes of strains with the same gene targeted using different strategies were generally consistent and in agreement with reported effects of gene deletion. Therefore, we conclude that the presented methodology could be a viable alternative to targeted gene deletion.

L12.2

Translational control underlying long-term synaptic plasticity

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Translational control provides a means for regulating the time, place, and amount of cellular protein synthesis. Synaptic plasticity is thought to require changes in both general and mRNA-specific translation. This suggests coordinated regulation of multiple forms of translation, but the logic of the process has not been described for any specific form of synaptic plasticity. Such information is essential for uncovering translational programs for synaptic plasticity and memory formation in specific neural circuits of the mammalian brain. The talk will start with a snapshot presentation of translational control mechanisms underlying protein synthesis-dependent forms of synaptic plasticity. I will then present new findings from our lab on translational control in long-term potentiation (LTP) in the dentate gyrus. We find that brain-derived neurotrophic signaling to MAP kinase interacting kinase (MNK) controls LTP maintenance through sequential activation of distinct modes of protein translation, suggesting the existence of a translational program.

L12.3

Synaptic translation of matrix metalloproteinase 9 and its role in Fragile X Syndrom

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Matrix metalloproteinase 9 (MMP-9), gelatinase B that regulates pericellular environment through the cleavage of protein components of the extracellular matrix, plays a role in structural plasticity of dendritic spines. Recently we have discovered that MMP-9 is locally translated and secreted at the synapse in response to synaptic stimulation. MMP-9 is involved in the activity-dependent reorganization of spine architecture and was shown to regulate spine morphology in Fragile X syndrome (FXS) which is caused by the loss of mental retardation protein (FMRP). Application of MMP-9 on neurons in culture induces formation of filopodia-like immature dendritic spines that resembles these in FXS. Furthermore, inhibiting MMP-9 activity by application of minocycline, the tetracycline analogue, to Fmr1 KO mice can rescue the abnormal spine phenotype both in vivo and in cultured neurons. Deregulation of local protein synthesis at the synapse contributes to spine dysmorphogenesis and synaptic dysfunction in patients with the Fragile X syndrome. We have shown that MMP-9 mRNA is a specific target of FMRP and that FMRP regulates its transport and translation at the synapse. In the absence of FMRP MMP-9 mRNA translation is increased and this causes an excess of matrix metalloproteinase 9 at synapses. Our data support a model in which synaptic MMP-9 is translationally regulated by FMRP. In the case of FXS the lack of FMRP leads to excessive MMP-9 protein synthesis at the synapse. We propose that such a local effect can contribute to the aberrant spine morphology observed in patients with FXS.

Oral presentations

O12.1

New humanized ataxin-3 knock-in mouse model combines genetic features, pathogenesis of neurons and glia and late disease onset of SCA3/MJD

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Spinocerebellar ataxia type 3 (SCA3/MJD) is a genetic neurodegenerative disease triggered by the expansion of CAG repeats in *ATXN3* gene. SCA3/MJD transgenic mouse models exist, however, the generation of an *Atxn3* knock-in model would represent a closer approximation of SCA3 disease

We report the generation of the first humanized knock-in ataxin-3 mouse model (Ki91) which provides insights into neuronal and glial pathology of SCA3/MJD. Mutant ataxin-3 accumulated in cell nuclei across the brain, showing diffused immunostaining and forming intranuclear inclusions. Ki91 animals showed expansion in paternal transmissions and contraction of the CAG mutation in maternal transmissions. Moreover, the CAG mutation also exhibited age-dependent tissue-specific expansion. The Serpina3n protein, a molecular sign of neurodegeneration and brain damage, was strongly increased in the brain and cultured astrocytes of Ki91 mice. The brains of the Ki91 showed astrogliosis in the cerebellar white matter and in the substantia nigra in the midbrain. Additionally, the Purkinje cells (PCs) in the cerebellum of the Ki91 mice showed a pronounced decrease of Calbindin D-28k immunoreactivity (IR) that was not dependent on protein expression. The neurodegenerative changes in the PCs occurred with different intensities and depended on the location of the PCs in the transverse zones in the cerebellum. Molecular and cellular neuropathology is accompanied by the late (90 weeks) rotarod deficit in heterozygous Ki91 animals. In summary, we created the ataxin-3 knock-in mouse model that combines molecular and behavioral disease phenotypes with genetic features of SCA3. The model can be very useful to study the pathogenesis and therapy of SCA3/MJD and other polyQ disorders.

O12.2

TIMP-1 loaded nanoparticles: a therapeutic strategy for neuroprotection

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Introduction: There is a marked, deleterious increase in expression of Matrix Metalloproteinase-9 (MMP-9) during numerous pathologic conditions such as ischemic stroke, epilepsy and various excitotoxic/neuroinflammatory processes. Therefore, inhibition of MMP-9 is considered as a potential therapeutic target for neuroprotection. Currently available chemical inhibitors of MMP-9 are poorly specific and have many off-targets leading to unanticipated side effects. As development of specific inhibitors is always a challenging task therefore, we planned to evaluate neuroprotective effects of an endogenous inhibitor of MMP-9, Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1), which is a 28 KDa protein. However, the major obstacles of using TIMP-1 as a neuroprotective agent are its *in vivo* short half-life and low brain permeability. Hence, we planned to explore a nanotechnological approach for delivery of TIMP-1, by using poly lactic-co-glycolic acid (PLGA) based Nanoparticles (NPs), so in the future it can be developed as a neuroprotective agent.
Results: Here, we have developed TIMP-1 loaded PLGA NPs which can deliver TIMP-1 in a sustained release manner and can cross the blood brain barrier (BBB). These NPs were coated with polysorbate 80 (Ps80) to improve their BBB penetration. These NPs were characterized by SEM, DLS, PDI, Zeta potential, protein loading and drug release. We evaluated these NPs for their in vitro and in vivo BBB penetration by using primary rat brain endothelial cell model and by tail vein injection in mice respectively. The in vitro and in vivo results have shown that NPs are non-toxic to endothelial cells and they have BBB penetration. Finally, we evaluated their neuroprotective effects on organotypic hippocampal slice culture using propidium iodide staining and LDH assay which have shown that TIMP-1 and TIMP-1 loaded have neuroprotective effects against Kainic Acid (KA) induced excitotoxicity. Moreover, we have shown through gelatinase assay that these effects are mediated through MMP-9 inhibition. Currently, we are exploring in vivo neuroprotective effects of TIMP-1 NPs.

O12.3

Cell type-specific transcriptional responses of neurons and astrocytes *in vivo*

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The mechanism of glucocorticoid action in the brain involves glucocorticoid receptor (GR) binding and its translocation into nucleus, where it acts as a transcription factor and regulates gene expression. However, GR-dependent transcriptional regulation in different types of neural cells is not fully understood.
Our previous data obtained from *in vitro* studies conducted on primary cultures of murine striatal and hippocampal astrocytes and neurons demonstrated that stimulation of astrocytes with a specific GR agonist dexamethasone (Dex) (100 nM for 4 h) caused a significant increase in expression of GR-dependent genes such as *Fkbp5*, *Sgk1 and Tsc22d3*. Strikingly, we did not observe transcriptional changes in primary neurons, which suggested that astrocytes could be the primary cellular target of glucocorticoids in the central nervous system.
Here we have acutely isolated primary astrocytes and neurons from mice treated with Dex (4 mg/kg for 4 h) or morphine (20 mg/kg for 4h), as we previously demonstrated that morphine stimulation results in an increase of GR-dependent genes in mouse brain *via* activation of hypothalamic-pituitary-adrenal axis. Neurons and Glast-positive astrocytes were isolated from striatum and hippocampus of 21 days-old C57BL/6J mice utilizing magnetic cell sorting technology. Transcriptional profiles of selected genes were evaluated with qPCR.
We show that magnetic sorting of neurons (*Npas4*) and astrocytes (*Gjb6*, *Plin4*) from mice gives high purity populations of cells. We corroborate the results from our previous *in vitro* studies demonstrating that GR-dependent genes such as *Fkbp5*, *Sgk1 and Tsc22d3* are strongly upregulated in astrocytes but not in neurons *in vivo*. This study suggests that astrocytes constitute a primary transcriptional target of glucocorticoid action in the brain.

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O12.4

Novel protocol for differentiation of induced Pluripotent Stem Cells (iPS) into dopamine and melanin producing cells

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Induced Pluripotent Stem cells (iPS) are unlimited source of different cell types. Thanks to their traits they can found application not only in clinic but also as *in vitro* models of diseases.
One important type of cells which can be generated from iPS cells are dopamine producing cells. They are particularly vulnerable to degeneration in many neuronal disorders such as Parkinson's disease (PD). IPS-derived dopamine producing cells *in vitro* comprise perfect model not only for drug testing but also for elucidating pathogenesis of PD.
Here we present distinct protocol for differentiation of iPS cells in a multi-step procedure. Firstly, iPS cells cultured on feeder layer were transferred to suspension culture. In next step progenitor cells were selected and expanded in serum-free medium. In final step cells were terminally differentiated into dopamine-producing cells. On each step cells were characterized for expression of specific markers (neuronal — Nestin, Tuj-1, TH, DAT, Tyrosinase and embryonic) on the level of mRNA (by RT-PCR) and protein (by immunocytochemistry). Dopamine production was proved by HPLC with MS and appearing black pigment was identified as melanin by EPR and Fontana-Masson staining.
We show protocol for iPS cells differentiation into dopamine and melanin producing cells. Our model is useful for pharmacological tests both *in vitro* and *in vivo* but moreover it is valuable in deciphering pathogenesis of neurodegenerative diseases.

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Posters

P12.1

Podosome organizing protein ArhGef5 interacts with the dystrophin glycoprotein complex and localizes to the sites of neuromuscular junction remodeling

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Neuromuscular junctions (NMJs) are specialized synapses that allow for the transmission of signals from motor neurons to muscle fibers. At birth, NMJs have a simple plaque-like shape but in the early postnatal period they grow in size and become perforated with scattered openings. Subsequently, these perforations become more numerous and form indentations between ACh-rich branches, transforming the "plaque" into a complex pretzel-like structure. It had been widely assumed that the nerve terminal, which apposes and mirrors the postsynaptic machinery, dictates the remodeling of the NMJ. Recent studies, however, suggest that cultured muscle cells have an intrinsic program for AChR remodeling. We have previously reported an unexpected discovery that cultured muscle cells utilize podosomes, actin-rich adhesive organelles capable of the remodeling of the extracellular matrix (ECM), for developmental reorganization of the postsynaptic machinery. To get insight into the molecular mechanism of the AChR cluster remodeling we focused our analysis on α-dystrobrevin-1 (αDB1), a component of the dystrophin-associated glycoprotein complex (DGC), which was previously proposed to play a role in synaptic remodeling. We designed a peptide-based biochemical screen that allowed us to identify proteins that interact with αDB1 specifically upon its phosphorylation. These experiments led to the identification of Arhgef5 – a GEF for the Rho family GTPases, phosphoinositide-3 kinase (PI3K), and an adaptor protein GRB2. Interestingly, these proteins form a complex that is known to initiate the formation of podosomes in fibroblasts. Our experiments demonstrated that Arhgef5 binds directly to phosphorylated αDB1 and that this interaction is specific to phosphorylated tyrosine Y713 but not other phosphorylated tyrosines of the αDB1 protein. Immunohistochemical analysis of muscle tissue revealed that Arhgef5 is localized to the postsynaptic machinery of the NMJ, where it concentrates in the perforations and indentations between AChR-rich branches, areas where we expected podosomes to form. Thus, our results support our hypothesis that podosomes form in vivo and are involved in the remodeling of the NMJ postsynaptic machinery.

P12.2

Stromelysin-1, gelatinase B and TIMP-1 in serum during thrombolytic treatment of acute ischaemic stroke

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Introduction: The recommended treatment of acute phase of ischaemic stroke (IS) in humans is limited to recombinant tissue plasminogen activator (rtPA). Previous studies have indicated that intracranial bleeding during rtPA administration can be caused by high activity of matrix metalloproteinase(MMP)-9 (gelatinase B). MMP-9 is capable to digest type IV collagen, the component of basal membranes. During thrombolysis the enzymatic cascade that follows through plasmin and MMP-3 (stromelysin-1) is triggered by rtPA. Finally, MMP-3 activates MMP-9, theoretically leading to the destruction of basal membranes of brain vessels and intracranial bleeding thereafter. Our objective was to find the relationship between rtPA treatment *vs.* MMP-9 activity, MMP-3, and TIMP-1 (tissue inhibitor of MMP) serum levels related to patients' neurological status during acute ischaemic stroke.
Material and Methods: Thirty five ischaemic stroke patients were enrolled. Fourteen of them underwent thrombolytic therapy with rtPA (rtPA group). The serum samples were obtained at 3 time-points in rtPA group (time-point 0: 1^{st}–4^{th} hour of stroke; time-point 1: immediately after rtPA administration; time-point 3: on day 5–7 from stroke onset). In remaining patients venous blood was obtained at two time-points: time-point 1: 5^{th}–10^{th} hour of stroke and time-point 2: on day 5–7 of stroke. MMP-9 was analyzed with gelatin zymography, MMP-3 and TIMP-1 serum levels were analyzed using an ELISA method. Neurological status was assessed by National Institutes of Health Stroke Scale (NIHSS) performed on the admission and discharge of patients. NIHSS improvement ratio (IR) was calculated as a difference between NIHSS score at the admission and discharge of patient.
Results: The active form of MMP-9 (86 kDa) was not observed in any analyzed samples. MMP-9 activity was estimated as the sum of activities of each detected form 92 kDa, 130 kDa and 200 kDa (corresponding to pro-MMP-9, heterodimer MMP-9/lipokalin and homodimer MMP-9/MMP-9, respectively). MMP-9 was significantly elevated and MMP-3 was decreased in serum at time-point 1 in rtPA group in comparison to non-rtPA group. MMP-3 was negatively correlated with IR values for all stroke patients but the results did not reach full statistical significance ($p=0.06$).
Conclusion: Thrombolysis applied for the treatment of ischaemic stroke increases MMP-9 activity in serum, however rtPA seems not to facilitate the conversion of pro-MMP-9 into the active form. In addition, our results can suggest the involvement of MMP-3 to the biochemical processes occurring during acute phase of IS.

P12.3

Chronic lithium administration in mice increases nuclear β-catenin in the thalamus but not in the cortex and hippocampus

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Nowadays lithium salt is one of the most effective drugs used to treat bipolar disorder. Unfortunately, there is a thin line between its therapeutic properties and severe toxicity. In order to find targets for future alternative drugs, the molecular mechanism of lithium action must be ascertained. We are looking for molecular targets of lithium ions in the brain, focusing on the canonical Wnt signaling pathway. The effector of the pathway is β-catenin, an activator of the LEF1/TCF transcription factors. When the Wnt signal is not present, β-catenin is phosphorylated by GSK3 and subsequently degraded via the proteasome pathway. In response to Wnt signaling, GSK-3β is inhibited and β-catenin is stabilized. It is well known that GSK3 is inhibited by lithium ions. It is still controversial, however, whether this phenomena occurs in vivo upon therapeutic lithium treatment, and if yes, whether it can affect β-catenin in the brain.
C57BL/6 mice were given 600 mg/l lithium chloride in drinking water during one week, which served as a model of lithium therapy. Then serum and brain samples were collected. The lithium levels in serum were measured by inductively coupled plasma mass spectroscopy to verify whether its concentration was within the therapeutic range. The cortex, hippocampus and thalamus were isolated, and the samples were fractionated into the cytoplasm, nucleoplasm and membranes by differential centrifugation, which was optimized for the brain tissue. Then the levels and localization of β-catenin were examined by quantitative Western blot. A twofold increase in nuclear β-catenin was observed specifically in the thalamus, whereas in the hippocampus and cortex the level of β-catenin either did not change or increased in the cytoplasm. No changes were seen in membranous fractions of all the structures. Concluding, *in vivo* lithium treatment can activate b-catenin signaling pathways, but only in the thalamus.

P12.4

A method for efficient transfection of differentiated and non-differentiated cells

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During cell transfection with a plasmid, DNA must be delivered to the nucleus, where the transcription of the encoded gene occurs. Nuclear transport of plasmids occurs most efficiently during the cell cycle, when the nuclear envelope becomes disassembled. Therefore, the transfection of post-mitotic cells, such as differentiated muscle cells and neurons, is often highly inefficient. Here we report a simple and cost-effective method for efficient transfection of dividing and non-dividing cells with synthetically prepared mRNA. Unlike plasmid DNA, mRNA transfection bypasses the requirement for the genetic material to be transported into the nucleus, since the protein synthesis from mRNA occurs in the cytoplasm. We demonstrate that mRNA transfection yields up to 100% of transfected differentiated muscle cells, HEK-293 or HeLa cells and up to 50% of transfected cultured hippocampal neurons. Furthermore, we provide evidence that injection of mRNA-Lipofectamine complexes is a simple method of brain tissue transfection. Finally, we generated a series of universal plasmid vectors that can be used for conventional plasmid transfection or as templates for the production of synthetic mRNAs. These plasmids can be used in a variety of experiments since they contain tags that enable the localization and purification of fusion proteins. Moreover, they have a uniquely designed multicloning site, the expression-enhancing WPRE sequence, the SV-40 origin of replication for the propagation of plasmid in T-antigen containing mammalian cells (COS-7, HEK-293T), as well as the puromycin resistance gene for the quick selection of transfected cells.

P12.5

Inhibition of Cyclin-dependent kinase 5 attenuates NF-κB – regulated gene expression in the brain during lipopolysaccharide-evoked systemic inflammation

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Cyclin-dependent kinase 5 (Cdk5) is involved in modulation of a broad range of neuronal functions; it regulates neuronal cytoskeleton, synaptic development and function, oxidative stress and apoptosis. Activity of Cdk5 is controlled by binding of regulatory subunits (p35 or p39) and by phosphorylation on Tyr15 and Ser159. Overactivation of Cdk5, which is induced by the cleavage of p35 by calpain, was implicated in neuronal dysfunction in various neurodegenerative disorders, including Alzheimer's and Parkinson's disease. Moreover, it was recently proposed that Cdk5 may be involved in regulation of inflammatory processes. The aim of this study was to analyze the role of Cdk5 in regulation of inflammation-related gene expression in the mouse hippocampus during lipopolysaccharide (LPS)-evoked systemic inflammatory response (SIR). LPS (1 mg/kg b.w.) was administered i.p. to adult male mice C57BL6, and after 3 and 12 h molecular alterations in hippocampi were analyzed. Cdk5 inhibitor Roscovitine was injected i.p. at 50 mg/kg b.w. Our data indicated a transient increase in gene expression for *Cdk5* and *Cdk5r1* (p35) in hippocampi of LPS-treated mice. Moreover, phosphorylation of Tyr15 was enhanced, suggesting activation of Cdk5 during SIR. In addition, we observed enhancement of calpain activity and p35 degradation, which is a molecular hallmark of Cdk5 activation. To investigate the significance of Cdk5 in progression of neuroinflammatory signaling, the effect of Roscovitine was analyzed. It was found that Roscovitine reduced the activity of inflammation-related transcription factor NF-κB in hippocampus, and downregulated the mRNA level of NF-κB-controlled genes, *Nos2*, *Tnfa* and *Il6*. Moreover, Roscovitine prevented the increase in TNF-α and IL-6 protein level. Our results indicate that Roscovitine significantly affects pro-inflammatory gene expression in the brain; therefore, we conclude that inhibition of Cdk5 may be a promising protective strategy in neuroinflammatory processes.

Acknowledgements

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P12.6

CacyBP/SIP and its role in dysregulation of protein ubiquitination in Huntington's disease transgenic mice model YAC128

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Mutated huntingtin was shown to affect gene expression in the brain of Huntington's disease (HD) patients [1] and in brains of HD models [2].We found that the level of several genes and proteins involved directly (*Calb2*, *Cib1*, *Cib2*, and *Carl*) or indirectly (*CacyBP/SIP* and *Hap1*) in calcium homeostasis was at least 2-fold increased in the striatum of YAC128 mice. One of these proteins, a calcyclin-binding protein (CacyBP/SIP) is involved in beta-catenin ubiquitination *via* Siah1 E3 ubiquitinating enzyme [3]. We found that overexpression of CacyBP/SIP protein was observed only in the striatum of the YAC128 model, but not in the motor cortex and cerebellum. The striatum is the brain region first and most affected in HD. The aim of the present work was to establish the role of the CacyBP/SIP in protein ubiquitination in YAC128 model. Our preliminary data indicate that the total protein ubiquitination is decreased in HD transgenic mice as compared to the wild type mice. Moreover, we found that the level of beta-catenin is higher in the striatum of HD mice than in the control ones. We are now analyzing if the increased level of CacyBP/SIP is responsible for the observed changes in beta-catenin and protein ubiquitination.

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P12.7

SH3BP2, liprin-alpha1 and alpha-catulin are involved in the organization of the neuromuscular junction postsynaptic machinery

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Neuromuscular junctions (NMJs) are synapses formed between motor neurons and muscle fibers. Abnormalities in NMJ development lead to various neuromuscular disorders, such as myasthenia gravis. However, the mechanisms that orchestrate NMJ development are still poorly understood. The dystrophin-associated glycoprotein complex (DGC) is a major laminin receptor in the muscle, which anchors and stabilizes components of the postsynaptic machinery, including receptors for the neurotransmitter acetylcholine (AChRs), by linking them to the extracellular matrix (ECM) and the cytoskeleton. Alpha-dystrobrevin-1 (aDB1) is a cytoplasmic component of the DGC that was shown to play a role in the stabilization of AChR and the development of AChR clusters. To understand the molecular mechanism underlying the assembly of AChR clusters, we performed a biochemical screen for aDB1-interacting proteins. Our experiments identified several novel targets including three scaffold proteins: liprin-alpha1, SH3BP2, and alpha-catulin. We show that all three are concentrated at the neuromuscular synapse, where they localize to AChR-rich branches of the postsynaptic membrane. We also demonstrate that myotubes depleted in alpha-catulin or SH3BP2 fail to cluster AChRs. Currently we are testing our hypothesis that alpha-catulin regulates AChR assembly through the interaction with the NFkappaB pathway, which controls expression of rapsyn, a scaffold protein that directly clusters AChRs at the postsynaptic membrane.

P12.8

Serum response factor (SRF) ablation in forebrain neurons of adult mice leads to increased vulnerability to seizures and aberrant expression of activity regulated genes

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Aberrant synaptic plasticity is known to play a pivotal role in epilepsy, yet the molecular mechanism underlying this pathology remains still unknown. SRF is a transcription factor that plays a prominent role in various programs of gene expression in the brain, including neuronal plasticity. Moreover, SRF protein accumulation and phosphorylation as well as increased binding of SRF to DNA was observed after pilocarpine and kainic acid (KA)-induced seizures, what suggests that SRF activation can be engaged in plasticity changes associated with the development of epilepsy. To study the role of SRF in the context of epilepsy, we used conditional, inducible, forebrain specific SRF knockout mice. Epileptogenesis was induced by intra-hippocampal injection of KA and animals were monitored with continuous video-EEG recording. We showed, that SRF knockout animals developed more spontaneous seizures and seizures were more severe. To identify target genes regulated by SRF we used microarray profiling analysis. Animals from both genotypes were injected intraperitoneally with KA or saline. 6 hours after KA- induced seizures/saline injection dentate gyrus of the mouse hippocampus was dissected and RNA was isolated and analyzed by microarrays. Statistical analysis of microarray results revealed that in the basic conditions (saline treated animals) SRF KOs do not display any significantly downregulated transcripts, except the SRF itself (Tukey $p<0.05$, fold change 0.66). As an outcome of our experiments, 431 genes altered after seizures and significantly changed in SRF KOs were identified (ANOVA interaction genotype: treatment p 0.0005, correction FDR 1.5 or 0.66). More than 260 genes showed increased expression after seizures in WT animals and were significantly down-regulated in SRF KOs. Among those genes we distinguished and verified some genes, including: Arc, BDNF, Lcn2, Acan. Decreased levels of these genes can lead to hyperactivity. Our results suggest, that SRF-dependent genes may function as "molecular brakes" giving a negative feedback to decrease the hyperexcitation in response to strong neuronal stimulation.

P12.9

Blood platelets for peripheral modeling of presynaptic events: Glutamate forward and reversed transport assessment

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Introduction: Platelets express neuronal and glial glutamate transporters EAAT1-3 in the plasma membrane and vesicular glutamate transporters VGLUT1-2 in secretory granules. The aim of this study is to assess whether platelets are adequate model of both presynaptic glutamate uptake and release.
Methods: spectrofluorimetry, flow cytometry, glutamate dehydrogenase assay, laser scanning confocal microscopy, radiolabelled assay.
Results: The absence of unstimulated release of endogenous glutamate from platelets was shown even after the inhibition of glutamine synthetase and glutamate uptake by methionine sulfoximine or DL-threo-β-benzyloxyaspartate, respectively. The depolarization of plasma membrane by high K^+ did not evoke glutamate release from platelets shown with glutamate dehydrogenase and L-[^{14}C]glutamate. The glutamate efflux also was not registered with transportable inhibitor of glutamate transporters DL-threo-β-hydroxyaspartate (DL-THA) by means of heteroexchange. The protonophore cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP) and inhibitor of H^+-ATPase V-type bafilomycin A1 did not stimulate glutamate release from platelets. However, high K^+, DL-THA, FCCP and bafilomycin A1 did not prevent the exocytotic release of glutamate from secretory granules in response to thrombin stimulation.
Discussion: The depolarization of the plasma membrane in isolated nerve terminals (synaptosomes) at Ca^{2+}-free conditions caused transporter-mediated release of endogenous glutamate from the cytosol into the extracellular space (glutamate transporter reversal). In contrast to nerve terminals, the heteroexchange, transporter-mediated and unstimulated release of glutamate are not inherent to platelets. The reverse function of vesicular glutamate transporters of platelets also seems unlikely.
Conclusion: Glutamate is released from platelets by means of exocytosis only. Hence, the platelets are the most appropriate experimental and diagnostic model for the forward glutamate transport, exclusively.
Key words: glutamate transporters, synaptosomes, platelets, exocytosis

P12.10

Control of the medial prefrontal cortex (mPFC) pyramidal neuron membrane potential by muscarinic receptors in young rats

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Introduction: Abnormalities in the muscarinic control of prefrontal cortical neurons occur in neuropsychiatric disorders such as schizophrenia and senile dementia. The purpose of this study was to clarify the mechanisms responsible for the muscarinic-dependent depolarization of pyramidal neurons of the medial prefrontal cortex (mPFC).
Material and methods: Experiments were conducted on slices of 18- to 22-day-old rats. The membrane potential was recorded under current-clamp conditions using the perforated patch method. Ca++ ions were absent from the extracellular solution, and voltage- and TTX-dependent Na+ currents were blocked by TTX. Recordings were performed in the presence of glutamatergic and GABAergic transmission blockers.
Results: It was determined that muscarinic receptor stimulation by carbamoylcholine chloride (CCh; 100 μM) evoked a depolarization (10.0±1.3 mV, n=33) that was eliminated by the M1 receptor antagonist pirenzepine (2 μM) or by the removal of Na+ ions from the extracellular solution. The application of the of G-protein βγ subunit-dependent signaling inhibitor gallein (20 μM) reduced the amplitude of CCh-dependent depolarizations to 1.7±0.42 mV (n=12). This CCh-induced depolarization was not affected by the application of the following blockers to the extracellular solution: Kir channels (Ba^{2+} ions, 200 μM), GIRK channels (tertiapin Q, 300 nM), Ih channels (ZD 7288, 50 μM), non-selective cationic channels (flufenamic acid, 200 μM; 2-APB; 200 μM and SKF 96365, 50 μM), the Na+/Ca++ exchanger (benzamil, 20 μM), intracellular transduction systems coupled to kinase C (U73122, 10 μM and chelerythrine, 5 μM), or kinase A (SQ22536, 100 μM and H-89, 10 μM). We also demonstrate that mPFC pyramidal neurons express type Nav1.9 channels.
Conclusion: We conclude that the muscarinic M1 receptor-dependent depolarization of layer-V pyramidal neurons depends on the activation of signal transduction involving G protein βγ subunits. The prime candidate for the membrane effector responsible for CCh-dependent depolarization is the TTX-resistant, low threshold, Na+ type Nav1.9 channel current.

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P12.11

Levels of biogenic amines in the midbrain of rats with acute alcohol intoxication and its correction by tauzinc

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It is known, that zinc ions are included in the active centers of many enzymes regulating of alcohol metabolism, reducing its negative impact (Truong-Tran AQ *et al*., 2001, *BioMetals* **14**: 315–330). While zinc deficiency and alcohol abuse violate the body's ability to absorb taurine, which protects brain cells from the many negative impacts. Considering that taurine has membrane-protective and antioxidant effects in a variety of systems (Shan-Shan Yu, 2007, *BMC Develop Biol* **7**: 51), and that zinc increases the activity of alcohol dehydrogenase and accelerates the excretion of metabolic products of ethanol (Murillo-Fuentes ML *et al*., 2010, *J Trace Elem Med Biol* **3**: 200–206), the aim of the work became research the modulatory effect of drug based on taurine and zinc (tauzinc) on the levels of biogenic amines in the midbrain of rats in case of acute ethanol administration.
The rats of the experimental group once per day intragastrically was injected ethanol at a dose of 4.5 g/kg for 10 days. Animals second group together with ethanol received a preparation based on taurine and zinc aspartate — "Tauzinc" at a dose of 100 mg/kg of body weight. Animals were decapitated 24 h after the last injection of the investigated compounds. For the study took midbrain, in which by HPLC were determined levels of biogenic amines.
After a ten-day ethanol administration were observed a marked increase of the concentration of the amino acid precursor of catecholamines - tyrosine with decreased levels of the individual catecholamines: dopamine (DA), dihydroxyphenylalanine (DOPA), epinephrine, as well as their metabolite — 3-methoxy-4-hydroxyphenylglycol. A similar situation was observed with indolamines: there was a decrease in serotonin levels (5-HT) and its metabolite — 5-hydroxyindoleacetic acid (5-HIAA), also was reduced ratio 5-HT/5-HIAA pointing to weakening of the serotonin turnover. Level of the indolamines precursor — tryptophan unlike tyrosine was decreased in animals receiving ethanol.
In case of the joint admission of ethanol and Tauzinc in the midbrain of animals levels of DA, DOPA, Trp, 5-HT and ratio 5-HT/5-HIAA returned to control values, also was observed an increase in the level of DA metabolite - homovanillic acid (HVA) and ratio HVA/DA which can indicate on the activation of DA turnover. Thus, the administration of Tauzinc prevents the changes in dopamin- and serotoninergic system in the midbrain of rats during acute alcohol intoxication.

P12.12

Activity-dependent cleavage of Nectin-3 is mediated by metalloproteinase

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Matrix metalloproteinases are a family of proteolytic enzymes that can cleave components of the extracellular matrix and certain cell-surface proteins, making them particularly suitable to sustain neural remodeling processes. Matrix metalloproteinase-9 is secreted by neurons and plays an important role in synaptic plasticity. Several lines of evidence suggest a critical role for MMP-9 in synaptic changes associated with learning and memory. However, only one synaptic target for its enzymatic activity, beta-dystroglycan was identified to date. In this report we show that Nectin-3 is a potential substrate for MMP-9. Nectin-3 is an immunoglobulin-like cell adhesion molecule (CAM), which primarily localizes at adherens junctions. To determine whether Nectin-3 might be cleaved in response to enhanced neuronal activity, we treated hippocampal neurons in culture with either KCl or glutamate, and extracts from whole cell lysates were analyzed on Western blots. We found that glutamate treatment resulted in a significant increase in the level of cleaved 20 kDa form of Nectin-3. This cleavage was dependent on NMDA receptor function, as it was reproduced by treatment with NMDA, and it was entirely inhibited by the NMDA receptor antagonists. To verify whether MMPs could be responsible for cleavage of Nectin-3 after neuronal stimulation, the neurons were pretreated with metalloproteinase-9 inhibitor (inhibitor I) for 30 min prior to NMDA stimulation. NMDA-dependent stimulation of Nectin-3 cleavage was abolished in the presence of the MMPs inhibitor. Using EGTA, the calcium chelator, we showed that NMDA-mediated cleavage of Nectin-3 was calcium dependent. In addition, we observed Nectin-3 cleavage in the presence of calcium ionophore ionomycin and this effect was blocked by inhibitor I. To confirm that exactly MMP-9 is responsible for Nectin-3 cleavage, recombinant Nectin-3 was incubated alone or with the indicated proteinase for 16 h. The digestion of Nectin-3 with MMP-9 resulted in a digestion product of approximately 20 kDa, corresponding to the molecular mass of SPF of Nectin-3 from previous study. In contrast, proteolysis was not observed in control conditions (buffer) or in the presence of an inactive mutant of MMP-9, confirming the specificity of the assay. Moreover, shedding of Nectin-3 was not detected in the presence of MMP-9 inhibitor undoubtedly indicating that MMP-9 is responsible for Nectin-3 cleavage. Our results also suggest that ecodomain MMP-9-dependent shedding of Nectin-3 is Ca2+-regulated event.

P12.13

Expression and function of the Angiomotin family of proteins in the brain

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Proper function of synaptic connections is important for the transmission of information in the central and peripheral nervous systems (CNS and PNS). The molecular mechanisms underlying synaptic remodeling are still poorly understood. We have recently identified the scaffold protein Angiomotin-like-2 (Amotl2), as a potential regulator of neuromuscular junction (NMJ) plasticity. Interestingly, majority of the machinery that specifically regulates NMJ remodeling is also implicated in the plasticity of synapses in the brain. Therefore, we investigated the expression of angiomotin-like-2 (Amotl2) in the CNS. Our analysis also included the closely related proteins angiomotin (Amot) and angiomotin-like-1 (Amotl1), which together with Amotl2 constitute a family of proteins called angiomotins. We demonstrated that all three angiomotins are widely expressed in the brain. In cultured rat hippocampal neurons and mouse brain slices Amotl2 and Amotl1 localize to the synaptic compartment, whereas Amot was distributed in neurites with the more predominant expression in mature axons. Thus, our experiments identified a novel group of proteins that may regulate synaptogenesis both in the CNS and PNS and highlight parallels between synapses in both systems. We are currently performing knock-down experiments and generating conditional knockout mice to study the function of Angiomotins in the CNS and PNS. Results from our preliminary experiments using RNAi suggest that Amot plays a role in neurites outgrowth and Amotl2 is involved in synaptogenesis.

P12.14

Changes in the expression and properties of canonical Wnt pathway mediators during brain development in mice

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β-catenin, a mediator of Wnt signaling and activator of the lymphoid enhancer-binding 1/T-cell transcription factors (LEF1/TCF), plays a well-established role in brain development and tissue homeostasis. Mutations in components of β-catenin signaling networks have been associated with several psychiatric disorders, indicating the involvement of β-catenin and LEF1/TCF proteins in the proper functioning of the brain. Although activity of Wnt signaling during brain patterning and neurogenesis has been extensively studied, little is known about activity of its mediators both during development and in the adult brain.
In adult, we detected *Lef1* and high levels of *Tcf7l2* transcripts, as well as LEF1 and TCF7L2 proteins, in neurons of the thalamus and dorsal midbrain, i.e., subcortical regions specialized in the integration of diverse sources of sensory information. These neurons also exhibited nuclear localization of β-catenin, suggesting the involvement of β-catenin/LEF1/TCF7L2 in the regulation of gene expression in these regions. We also analyzed spatial and subcellular localization of β-catenin and LEF1/TCFs in the cortical and thalamic regions in developing mouse embryo. We found that β-catenin is accumulated in the cytoplasm, but not in the nuclei of thalamic neurons until embryonic day 18.5. Analysis of alternative splicing and promoter usage identified brain-specific TCF7L2 isoforms and revealed a developmentally coordinated transition in the composition of LEF1 and TCF7L2 isoforms. In particular, dominant negative isoforms of TCF7L2, that is isoforms without β-catenin binding domain, are predominant in the embryonic thalamus and decrease postnatally, suggesting that interactions between TCF7L2 and β-catenin are involved in changes in β-catenin cellular localization.
Concluding, TCF7L2 isoform switch in the developing thalamus might regulate the outcome of β-catenin signaling and substantially alter the response of the target genes.

P12.15

Branch Chain Amino Acids during thrombolytic treatment of ischaemic stroke

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Introduction: Several specific to neurons or glial cells compounds are releasing through damaged blood-brain barrier into the circulation during ischaemic stroke (IS). Branch chain amino acids (BCAAs) was considered as a potential biomarker of numerous diseases including cerebrovascular diseases. Previously was noticed the decrease of BCAAs during acute phase of IS. However, there is no information about the influence of intravenous thrombolytic treatment with recombinant tissue plasminogen activator (rtPA) on BCAAs serum level. In addition, the relationship between patients' neurological status and BCAAs level during acute phase of IS is also unknown.
Material and Methods: Seventeen ischaemic stroke patients and ten healthy controls were enrolled into the study. Seven of them underwent intravenous thrombolytic therapy with rtPA (rtPA group). The serum samples were obtained at 3 time-points in rtPA group (time-point 0: 1^{st}–4^{th} hour of stroke; time-point 1: immediately after rtPA administration; time-point 3: on day 5–7 from stroke onset). Remaining stroke patients (without thrombolysis, n=10) had venous blood collection at two time-points: time-point 1: 5^{th}–10^{th} hour of stroke and time-point 2: on day 5–7 of stroke. Control serum were obtained once. Before the analysis samples were deproteinised with 6% sulphosalicylic acid in lithium–citrates buffer (pH 2.8) and centrifuged. BCAAs were determined by the automated ion-exchange chromatography with five lithium-citrate buffers using Amino Acids Analyser (AAA 400) by INGOS Corp., Praha, Czech Republic. Amino acids were separated using analytic column OSTION LG FA. The amino acids were identified in comparison to the standards provided by INGOS Corp. The original software MIKRO version 1.8.0 (INGOS) was used for BCAAs evaluation. The amino acids serum level was expressed in microM/ml.
Results: The leucine and valine serum level was gradually increased at time-point 1 and 2 in comparison to the time-point 0 at rtPA group. In addition the leucine level was significantly higher at time-point 1 of rtPA group compared to corresponding time-point at group non treated with rtPA or control patients (median value: 0.134 *vs.* 0.089 or 0.099 microM/ml in rtPA *vs.* non-rtPA or control groups respectively, $p<0.05$). Neither the significant fluctuation of isoleucine serum level nor the relationship between BCAAs serum level and the degree of neurological deficits was observed.
Conclusion: We suggest that the increase of leucine serum level in patients treated with rtPA could be the indicator of quickened recanalization resulting in the washing out of leucine from the ischaemic focus.

P12.16

Generation of transgenic mice with neuron-type specific knock-down of opioid receptors

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Opioid receptors belong to the family of G protein-coupled receptors and activate the Gi/o proteins. Their activation leads to inhibition of the neuron's activity, mainly by opening potassium channels and inhibiting calcium channels. The aim of this project is to create transgenic animals carrying miRNA selectively targeting opioid receptors in different brain regions in order to investigate their roles in the reward and aversion systems of the brain.
miRNAs targeting Oprm1, Oprd1 and Oprk1 receptors were designed with the BLOCK-iT RNAi Designer and cloned into pcDNA™6.2-GW/EmGFP-miR plasmids. For each opioid receptor we have chained two miRNAs targeting adjacent sequences in a single exon. miRNA silencing efficiency was validated in the CHO-K1 cell line co-transfected with the plasmids encoding opioid receptors and miRNA cassettes. Transfection of CHO-K1 cells resulted in at least 50% reduction in the target transcript abundance. Validated miRNAs were recombined with D1, Dbh and DAT promoter carrying bacterial artificial chromosomes (BACs) using the Red-ET recombination system that relies on homologous recombination *in vivo* in *E. coli*.
Purified transgenes were transferred by pronuclear injection to generate transgenic mice with selective inhibition of Oprm1 and Oprd1 in dopaminoceptive, Oprm1 in noradrenergic and Oprk1 in dopaminergic neurons. Presently, potential founders of the transgenic line are being genotyped using multiplex real-time PCR that additionally assays the number of transgene copies integrated. Further characterization of the line will determine if the knock-down approach has comparable efficiency to a targeted gene deletion.

P12.17

Role of Lipocalin 2 in the structural plasticity of neurons

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Lipocalin 2 is a small, 24kDa extracellular protein involved in the innate immune response. Lcn2 is mostly known as an immunomodulatory protein expressed by astrocytes and microglia, however increase of Lcn2 levels is also observed in neurons: after lipopolysaccharide (LPS) administration, after spinal cord injury and upon stress. On the cellular level Lcn2 modulates morphology of glial cells and causes time- and dose-dependent increase in the number and length of cellular processes of the astrocytes. Structural changes are also manifested by microglia undergoing cell deramification after treatment with recombinant Lcn2. Recently, it was shown that lack of Lcn2 leads to alterations in dendritic spines number under stress conditions in the hippocampus. The aim of the current study was to investigate the role of Lcn2 in structural plasticity of neurons. To study the role of Lcn2 in regulation of structural plasticity we used rat primary hippocampal cultures. In order to determine Lcn2 effects on development of dendritic tree, rat primary neuronal hippocampal cultures were incubated with recombinant protein (500 ng/ml Lcn2) at day *in vitro* 6 (DIV6) for eight days. Lcn2 treatment resulted in significant changes in the complexity of dendritic tree, characterized by increased number of secondary dendrites as well as extended total dendrite length. In order to determine the rapid effects of Lcn2 on dendritic spines remodeling, we used live imaging of the hippocampal neurons. GFP labeled neurons were incubated with or without 500 ng/ml of recombinant Lcn2 and visualized using confocal microscopy. Effects on single spines shape were analyzed at 10 min, 20 min, 30 min and 40 min after Lcn2 application. We used the length-to-width ratio parameter (length of the spine divided by its width; L/W) as a determinant of spine shape and focused on "small spines", that have the L/W value < median. Our results show that after incubation with Lcn2, spine shapes change significantly 20 minutes after application of Lcn2 and that the spines become thinner and longer. Altogether our results show that exogenous Lcn2 may exert rapid effects on the dendritic spine shape of hippocampal neurons causing elongation and thinning of the spines and long term effects increasing the complexity of dendritic tree.