

Volume 9 Suppl 2, 2009

Meeting abstracts

15th Scientific Symposium of the Austrian Pharmacological Society (APHAR)

Graz, Austria

19-21 November 2009

Published: 12 November 2009

These abstracts are available online at <http://www.biomedcentral.com/1471-2210/9?issue=S2>

MEETING ABSTRACTS

A1

The targeting of US28 is dependent on GASP-I

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BMC Pharmacology 2009, **9(Suppl 2):A1**

Background: The human cytomegalovirus (HCMV) encodes the seven transmembrane (7 TM)/G protein-coupled receptor (GPCR) US28. US28 is able to endocytose and signal in a constitutive - i.e. ligand-independent - manner and is located predominantly in the membranes of intracellular organelles, especially late endosomes/lysosomes and multivesicular bodies (MVBs). It was suggested that the virions of HCMV may be budding into the membranes of these MVBs, where the viral receptors are then incorporated into the viral membranes during the final stages of virus assembly.

Methods: Protein-protein interaction between US28 and GASP-I (G protein-coupled receptor-associated sorting protein-1) was investigated by GST-fusion protein-binding assay, MBP protein competition binding assay and co-immunoprecipitation. In HEK293 cells endogenously expressing GASP-I the interaction between US28 and GASP-I was disrupted by i) overexpression of dominant negative cGASP-I or by ii) shRNA knock-down of endogenous GASP-I. The role of GASP-I in the post-endocytic trafficking of US28 was analyzed by means of immunocytochemistry and biotin protection degradation assay.

Results: Here we show that GASP-I, which sorts many GPCRs to the lysosomes, also plays an important role in the post-endocytic targeting of US28. We find that disruption of the GASP-I/US28 interaction by either i) overexpression of dominant negative cGASP-I or by ii) shRNA knock-down of endogenous GASP-I is sufficient to alter the lysosomal targeting of US28 and also its post-endocytic degradation.

Conclusion: Our data suggest that the interaction of US28 and GASP-I has important implications for the post-endocytic fate of US28.

A2

Inhibitory effects of prostaglandin EP₄ receptors on human eosinophils

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BMC Pharmacology 2009, **9(Suppl 2):A2**

Background: The accumulation of eosinophils in lung tissue is a hallmark of asthma, and it is believed that eosinophils play a crucial pathogenic role in allergic inflammation. Prostaglandin (PG) E₂ exerts anti-inflammatory and broncho-protective mechanisms in asthma, but the underlying mechanisms have remained unclear. We have shown previously that PGE₂ and the EP₂ receptor agonist butaprost inhibit eosinophil trafficking *in vitro* and *in vivo*.

Methods: Human eosinophils were purified by negative magnetic selection from peripheral blood. Cell migration was determined in microBoyden chemotaxis chambers. Ca²⁺ flux and expression of cell surface markers was recorded by flow cytometry. EP₄ receptor expression was demonstrated by immunostaining.

Results: The chemotaxis of eosinophils towards eotaxin and C5a was attenuated by the EP₄ agonist ONO-AE1-329, and the EP₄ antagonists ONO-AE3-208 and GW627368x partially reversed the inhibitory effect of PGE₂ on eosinophil migration. ONO-AE1-329, and also PGE₂, but not butaprost, inhibited the Ca²⁺ flux and the production of reactive oxygen species in eosinophils. ONO-AE1-329 also inhibited eosinophil degranulation and the up-regulation of the adhesion molecule CD11b. Selective kinase inhibitors revealed that the inhibitory effect of EP₄ stimulation on eosinophil migration depended upon activation of phosphatidylinositol 3-kinase and protein kinase C, but not cAMP. Immunostaining showed that human eosinophils express EP₄ receptors and that EP₄ receptor expression in the murine lungs is prominent in airway epithelium, and after allergen challenge, in peribronchial infiltrating leukocytes.

Conclusion: These data show that EP₄ receptor agonists potentially inhibit eosinophil trafficking and activation, and might hence be a useful therapeutic option in eosinophilic diseases.

A3**GPR55: signaling pathways and functions**

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BMC Pharmacology 2009, 9(Suppl 2):A3

Background: We have recently shown that the G protein-coupled receptor 55 (GPR55) mediates intracellular effects of cannabinoids and other, non-cannabinoid ligands in addition to the classical cannabinoid 1 (CB₁) and 2 (CB₂) receptors. Here we show different signaling pathways triggered by GPR55 in response to a panel of its agonists. In addition the cytoskeleton rearrangement mediated by GPR55 is investigated.

Methods: HEK-293 cells stably expressing the GPR55 receptor were characterized in terms of signaling properties. To this end, FLEX calcium release, reporter gene, dynamic mass redistribution (DMR) and phalloidin actin staining assays have been performed.

Results: Here we show that GPR55 is activated by lysophosphatidylinositol (LPI), AM251, SRI141716A (rimonabant) and AM281. GPR55 activation induces intracellular calcium release, NF-κB, NFAT and CREB activation. Stimulation of GPR55 induces F-actin formation under the control of Gα13, RhoA and ROCK. We also show the suitability of Corning[®] Epic[®] DMR assay for GPR55 ligand screening.

Conclusion: GPR55 as the novel cannabinoid receptor triggers distinct signaling pathways in response to LPI and some classical CB₁ receptor antagonists. Stress fiber formation mediated by GPR55 might show the function of this receptor *in vivo*.

A4**The sorting protein GASP-I regulates the constitutive signaling capacity of the virally encoded chemokine receptor US28**

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BMC Pharmacology 2009, 9(Suppl 2):A4

Background: Human cytomegalovirus (HCMV) is a widespread pathogen that has been shown to be present in various malignancies and it is also thought to be linked to vascular

diseases. HCMV encodes the seven transmembrane (7 TM)/G protein-coupled receptor (GPCR) US28, which constitutively activates the Gα_q/phospholipase C (PLC) pathway and downstream transcription factors such as the nuclear factor-κB (NF-κB) or the cyclic AMP responsive element binding protein (CREB). In this study we set out to elucidate the role of the GPCR-associated sorting protein-1 (GASP-1) in the regulation of the constitutive signaling capacity of US28.

Methods: To elucidate the role of GASP-1 in the regulation of the constitutive signaling capacity of US28 we disrupted the US28/GASP-1 interaction by either overexpression of dominant negative cGASP-1 or shRNA knock-down of endogenous GASP-1. To monitor the US28-mediated signaling we conducted inositol phosphate (IP) accumulation assays as well as luciferase reporter gene assays to check the activation of the transcription factors NF-κB and CREB.

Results: We find that GASP-1 is indeed able to modulate the signaling activity of US28. Disruption of the GASP-1/US28 interaction by either i) overexpression of dominant negative cGASP-1 or by ii) shRNA knock-down of endogenous GASP-1 alters the US28-mediated Gα_q/PLC/IP accumulation as well as the activation of the transcription factors NF-κB and CREB.

Conclusion: By identifying the sorting protein GASP-1 as a key regulator of the constitutive signaling activity of US28, we may be one step closer to gaining a better understanding of this viral receptor and its significance in the pathogenesis implicated by HCMV.

A5**Interactions of the G protein-coupled receptor-associated sorting proteins (GASP) 1 and 2 with the novel cannabinoid receptor GPR55**

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BMC Pharmacology 2009, 9(Suppl 2):A5

Background: GPR55 was recently found to be a novel cannabinoid receptor containing a seven transmembrane-spanning domain and being a member of the G protein-coupled receptor (GPCR) subfamily A. GPR55 is activated by different cannabinoid ligands as well as by the lipid ligand lysophosphatidylinositol (LPI). Generally, the activity of GPCRs is coordinated by receptor signaling, receptor desensitization and receptor resensitization. The latter two mechanisms are typically associated with the sorting of the GPCRs between degradation or recycling pathways and are highly regulated. Several sorting proteins have recently been identified, for example the G protein-coupled receptor-associated sorting protein-1 (GASPI). GASPI was originally found to target δ opioid receptors (DORs) to lysosomes, hence a degradative pathway. Moreover, it was shown that the interface of the DOR-GASPI protein-protein interaction is predominantly located in the C-terminal portion of the receptor protein.

Results: Here we show that the novel cannabinoid receptor GPR55 can internalize after ligand activation and is subsequently targeted to intracellular vesicles of the lysosomal compartments.

This result and the close similarity of GPR55 to the cannabinoid receptor CB₁ - which is targeted to lysosomes via the GASPI protein - suggested that GASP may be involved in targeting GPR55 to lysosomes. In fact, the C-terminus of GPR55 binds GASPI, cGASPI (the C-terminal part of GASPI) and GASP2 (the closest homologue to GASPI) *in vitro*.

Conclusion: This work provides the first evidence that the novel cannabinoid receptor GPR55 is targeted to lysosomes after prolonged agonist stimulation and that this mechanism is likely regulated by members of the newly discovered G protein-coupled receptor-associated sorting proteins, i.e GASPI and GASP2.

A6

The effect of carbamylation on the functionality of high-density lipoprotein

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BMC Pharmacology 2009, 9(Suppl 2):A6

Background: Increasing interest has focused on the relative functionality of high-density lipoprotein (HDL), highlighted by observations that cardiovascular events can occur even in the presence of high levels of HDL cholesterol. Myeloperoxidase (MPO), a heme protein abundant in leucocytes, colocalizes with HDL in the human artery wall and has emerged as a potential participant in multiple phases of the atherosclerotic process. Recently, the MPO/H₂O₂/SCN⁻ system has been demonstrated as a dominant pathway to promote protein carbamylation within atherosclerotic plaques. Therefore, we determined whether HDL is carbamylated in the human artery wall.

Methods and results: Immunohistochemical studies confirmed colocalization of carbamylated epitopes with apoA-I and macrophages in human atherosclerotic lesions. We performed shotgun proteomic analysis of *in vitro* carbamylated HDL to identify specific carbamylation sites of apoA-I. We could identify apoA-I-associated lysine residues in the α -helical lipid binding domains that are specifically carbamylated, indicating that carbamylation of apoA-I affects the functional integrity of HDL. In line with this observation, we observed that carbamylation of HDL (i) leads to "non-productive" binding to the HDL receptor (SR-BI), (ii) decreased SR-BI-mediated cholesterol efflux, and (iii) reduced HDL mediated anti-inflammatory activity.

Conclusion: Taken together, our data provide strong evidence that carbamylation renders HDL dysfunctional and proinflammatory.

A7

Advanced oxidation protein products are antagonists of the HDL receptor SR-BI

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BMC Pharmacology 2009, 9(Suppl 2):A7

Background: Advanced oxidation protein products (AOPPs) are carried by oxidized plasma proteins, especially albumin, and are important risk factors for cardiovascular events in patients with renal disease. Renal patients have a high prevalence of coronary and carotid arteriopathy and face an excessive cardiovascular mortality. Therefore the role(s) of AOPPs in the development of cardiovascular disease might be of great importance.

Results: Herein, we demonstrate that albumin isolated from hemodialysis (HD) patients and *in vitro*-generated AOPP-albumin binds with high affinity to the high-density lipoprotein (HDL) receptor scavenger receptor class B type-I (SR-BI). AOPP-albumin blocked HDL association to SR-BI and effectively inhibited SR-BI-mediated cholesterol ester (CE) uptake, dependent on the AOPP content of albumin. Furthermore, we demonstrate that AOPP-albumin effectively reduces SR-BI-mediated lipid tracer uptake in mice. AOPP-albumin administration increased the plasma half-life of [³H]CE-HDL in control mice 1.6-fold (p = 0.01) and 8-fold (p = 0.0003) in mice infected with adenoviral vectors encoding human SR-BI.

Conclusion: The observed inhibitory activity of albumin isolated from HD patients is of clear physiological relevance. Our data indicate that a physiological molar excess of HD-albumin over HDL may block up to 50% of HDL-CE delivery to SR-BI. Summing up, we provide strong *in vivo* and *in vitro* evidence that AOPPs are proinflammatory mediators that directly impair HDL metabolism and might therefore be potential key players in the development of cardiovascular disease.

A8

Prostaglandin E₂ acts via the EP₄ receptor to inhibit platelet aggregation

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BMC Pharmacology 2009, 9(Suppl 2):A8

Background: Platelets play a central role in haemostasis. Blood vessel injury leads to platelet aggregation and also invokes an inflammatory response leading to the formation of prostanoids like prostaglandin E₂ (PGE₂) and prostacyclin (PGI₂). It is known that low concentrations of PGE₂ enhance and high concentrations inhibit platelet aggregation. PGE₂ mediates its effect through four receptors: EP₁ (G α_q signalling), EP₃ (three isoforms present; signals via G_i, G_s or G_q based on cell type), EP₂ and EP₄ (G_s signalling). PGI₂ is known to inhibit platelet aggregation through its IP receptor (G_s signalling). The role of EP₃ in exacerbating platelet aggregation has been well described. However, the role of EP₄ which acts via the same G protein

coupling like IP has not been explored in detail. The aim of this study was to investigate the role of EP₄ in platelet aggregation.

Methods: Platelet aggregation assays were performed *ex vivo* using a platelet aggregation analyser (Aggregometer II). Blood from healthy human donors was used to obtain platelet-rich plasma. Aggregation was induced using ADP or collagen. Different agonists and antagonists were added to investigate their effects on platelet aggregation. Ca²⁺ flux changes caused by addition of agonists were also examined using a fluorescent Ca²⁺ dye (Fluo-3 AM) by flow cytometry.

Results: As expected, PGE₂ (up to 300 nM) and an EP₃ agonist (sulprostone) enhanced platelet aggregation, whereas an EP₂-selective agonist (butaprost) seemed to have no effect on platelet aggregation. On the contrary, an EP₄ agonist (ONO AEI-329) inhibited platelet aggregation in a concentration-dependent manner, and this effect could be reversed by using EP₄ antagonists (ONO AE3-208 and GW627368x) but not an IP or a DP antagonist. Inhibition of protein kinase C prevented the inhibitory effect of the EP₄ agonist, while inhibition of adenylate cyclase had no effect. The EP₄ agonist ONO AEI-329 also attenuated Ca²⁺ flux in platelets that had been stimulated with ADP.

Conclusion: These results are suggestive of an exclusive EP₄ effect on inhibition of platelet aggregation and EP₄ could be a potential target of antithrombotic therapy.

A9

Involvement of dynorphin in anxiogenic effects of estrogen

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BMC Pharmacology 2009, 9(Suppl 2):A9

Background: Since several years dynorphin, a member of the opioid peptide family, was suggested to play a regulatory role in numerous functional pathways of the brain, including anxiogenic effects in male mice [1]. However, emotional control and stress response depend on the hormonal state and differ between sexes, therefore we now investigated female prodynorphin-deficient (Dyn KO) mice.

Methods: Dyn KO mice were generated by replacement of the entire coding region of the prodynorphin gene [2] and backcrossed onto C57Bl/6N. Age and testing experience-matched female intact and ovariectomized (OVX) Dyn KO and wildtype (WT) mice at 3-8 months age were tested in all experiments. Anxiety (open field test, OF; elevated plus maze test, EPM; light dark test, LDT) and stress-related behaviour (forced swim test, FST; tail suspension test, TST) was investigated in correlation to the estrous cycle in intact female WT and Dyn KO mice and in OVX WT and Dyn KO mice treated with the general estrogen receptor (ER) agonist 17β-estradiol (E₂), and specific agonists for ERα (PPT), ERβ (DPN) or GPER (G1) two hours before testing.

Results: In the EPM, Dyn KO mice showed a significant anxiolytic phenotype with about double time spent, distance travelled and entries in the open arm at all estrous stages compared to WT mice, while differences in the OF and LDT

were less prominent than in male mice. Strikingly, the drop in ambulation observed in the OF, LDT and EPM during the proestrus phase in WT was absent in Dyn KO animals. In addition, the influence of the estrous stage on the behaviour in stress tests was abolished by the prodynorphin deficiency. Significant differences between OVX WT and Dyn KO mice were observed after DPN and G1 treatment, which both elicited anxiogenic effects in WT, but not in Dyn KO mice. In contrast, no differences were observed regarding the anxiolytic effects of PPT.

Conclusion: Our data suggest that the anxiogenic effects mediated by activation of ERβ and/or GPER may depend on the activation of κ opioid receptors. Pharmacological experiments aiming to solve this question are presently conducted.

Acknowledgements

This project was supported by the Austrian Science Fund (P 20107) and the Tiroler Wissenschaftsfonds.

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A10

Endothelial prostaglandin I₂ restrains eosinophil trafficking

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BMC Pharmacology 2009, 9(Suppl 2):A10

Background: Enhanced eosinophil extravasation into the tissue is a characteristic feature of bronchial asthma and other allergic diseases. The barrier-forming vascular endothelial cells release prostaglandin I₂ (PGI₂, prostacyclin) as the major prostanoid, and it has been previously observed that PGI₂ receptor (IP)-deficient mice show enhanced eosinophilic inflammation in response to allergens. Our aim was to define the role of PGI₂ in endothelial function and in eosinophil trafficking across endothelial monolayers.

Methods: Eosinophils were freshly isolated from human blood. Eosinophil chemotaxis through cell-free filters and adhesion to fibronectin were studied. Cell interaction assays, like adhesion and transmigration of eosinophils, were performed on confluent monolayers of human lung microvascular endothelial cells. The endothelial barrier properties were analyzed by measurements of transendothelial electrical resistance (TEER). Morphological studies were performed with immunofluorescence microscopy.

Results: Exogenous PGI₂ markedly attenuated the chemotaxis of isolated eosinophils through cell-free filters. This effect was prevented by the IP receptor antagonist CAY10441 and the adenylyl cyclase inhibitor SQ29548. Expression of IP receptors on eosinophils was shown by indirect flow cytometry and

Western blot. PGI₂ reduced eosinophil adhesion to fibronectin, inhibited the activation and up-regulation of CD11b/CD18 adhesion molecule, and blocked podosome formation in response to eotaxin. PGI₂ production of endothelial cells was abolished by diclofenac, a non-selective COX inhibitor, which resulted in enhanced eosinophil adhesion to endothelial monolayers. Similarly, the IP receptor antagonist CAY10441 enhanced the adhesion of eosinophils to endothelial cells. Transendothelial migration of eosinophils was likewise augmented by diclofenac. The diclofenac treatment itself decreased the electrical resistance of endothelial monolayers and disrupted the intercellular junctions as visualized by VE-cadherin and F-actin staining.

Conclusion: Based on these observations, endothelium-derived PGI₂ might be an important protective factor in keeping inappropriate eosinophil infiltration under control and might modulate allergic responses by inhibiting eosinophil responsiveness to chemoattractants in terms of adhesion and migration, and by strengthening the barrier function of the endothelium against infiltrating leukocytes. Therefore, IP agonists might be a useful therapeutic option for otherwise inadequately controlled inflammation in eosinophilic diseases, by blunting their extravasation into tissue.

A11

Store-operated calcium entry into rat basophil leukaemia cells: contribution of TRPC3 and Orai1
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BMC Pharmacology 2009, 9(Suppl 2):A11

Background: Before the discovery of STIM and Orai proteins, mammalian TRPC channels, including TRPC3, were considered as candidates for mediating store-operated Ca²⁺ entry (SOCE). This calcium entry pathway governs diverse cellular processes from exocytosis, cellular remodelling to gene transcription. Although a prominent role of Orai1 is now well established for immune cells, the role of TRPC proteins and the possible crosstalk between these two ion channels families to the overall store operated calcium entry into cells of the immune system is still elusive.

Results: Calcium imaging experiments with Fura2-AM-loaded rat basophil leukaemia (RBL) cells overexpressing either TRPC3, Orai1 or dominant negative mutants of these channel proteins sustain evidence for a complex interaction network between TRPC and Orai pathways. Overexpression of either wild-type protein (TRPC3 or Orai1) resulted in promotion of calcium entry as compared to controls. PYR3, a novel inhibitor of NFAT activation, which has recently been demonstrated as a selective inhibitor of TRPC3 channels [1], also suppressed thapsigargin-induced calcium entry into RBL cells, being most efficient in TRPC3-overexpressing cells. Expression of dominant negative mutations of either channels reduced SOCE to levels significant below controls, revealing a PYR3-resistant calcium entry component that was significantly larger in dominant negative Orai1-knock-out cells.

Conclusion: These results demonstrate a combined involvement of TRPC3 and Orai1 in SOCE of RBL cells. It remains to be clarified if a crosstalk between these channels exists. Investigation of down-stream signalling events such as NFAT activation and degranulation confirmed the activity of PYR3 as an inhibitor of mast cell function. In aggregate, our results support evidence for a complex interaction network of TRPC and Orai channels in immune cells.

Acknowledgements

Supported by the FWF projects PI8475 and PI9820.

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A12

Mutations in the amino-terminus impair amphetamine-induced efflux by inducing inward-facing conformations of the serotonin transporter
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BMC Pharmacology 2009, 9(Suppl 2):A12

Background: The serotonin transporter (SERT) is responsible for the rapid termination of neurotransmission by removing serotonin from the synaptic cleft. We have explored the functional significance of a highly conserved threonine residue, at position 81, located within the amino-terminus of SERT.

Methods and results: Our findings indicate that, although the mutated transporters are normally targeted to the plasma membrane, they exhibit marked functional defects, such as: (i) a dramatic decrease in amphetamine-induced efflux (despite retaining normal amphetamine-induced currents), (ii) a 3-fold reduction in transporter turnover numbers (indicating impaired substrate translocation) and (iii) a 4-fold decrease in inhibitor affinity (due to a declined on-rate and an enhanced off-rate). The latter suggests that the mutated SERTs have a preference for inward-facing transporter conformations, as further supported by our molecular dynamics simulation experiments. By studying several H-bond and hydrophobic interactions of the wild-type T81, compared to its mutations to alanine or aspartate, structural changes were detected in the juxtamembrane N-terminus region of SERT. The computer models demonstrate a degradation of N-terminus interactions with IL2 and IL3 (which are likely involved in the transition between inward- and outward-facing SERT conformations) and a shift of the C-terminus away from the N-terminus upon mutation. Moreover, truncation of the first 64 residues of the amino-terminus results in functional defects comparable to the sole mutation of T81.

Conclusion: Hence, alterations in the amino-terminus region of SERT induce inward-facing transporter states, causing hindrance to conformational changes required for amphetamine-stimulated release, without simultaneously obstructing the transporter's ability to operate in its channel or uptake mode of action.

A13

The gut-mood axis: a novel role of the gut hormone peptide YY on emotional-affective behaviour in mice

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BMC Pharmacology 2009, 9(Suppl 2):A13

Background: Peptide YY (PYY) and neuropeptide Y (NPY) are members of the PP-fold peptide family. PYY is expressed by endocrine cells of the gut, whereas NPY occurs in enteric, sensory, cerebral and autonomic neurons. While PYY is involved in the regulation of gut function and satiety, NPY is known to contribute to the regulation of emotional-affective behaviour, cognition, seizure activity, pain and satiety. Since we have previously found that the NPY system plays a role in the gut-brain axis, we explored in which way knockout of PYY and PYY plus NPY alters the emotional-affective behaviour in mice and how this phenotype is altered by experimental colitis.

Methods: Male wildtype (WT), PYY (PYY^{-/-}) and PYY plus NPY (PYY+NPY^{-/-}) knockout mice, all with a mixed C57BL/6:129/SvJ (1:1) background, were used. Mild colitis was induced by adding dextran sulphate sodium (DSS, 2%) to the drinking water for 7 days. Inflammation was assessed by the colonic myeloperoxidase content, anxiety-related behaviour was evaluated with the elevated plus maze (EPM) and open field (OF) tests, and depression-like behaviour was estimated with the forced swim test (FST).

Results: When the animals were phenotyped in the absence of colitis, anxiety-like behaviour in the EPM (reduction of open arm time) was increased in both PYY^{-/-} and PYY+NPY^{-/-} mice, whereas locomotion remained unaltered. The results obtained in the OF test were largely similar. The depression-like behaviour (immobility) in the FST was markedly enhanced in both PYY^{-/-} and PYY+NPY^{-/-} mice. DSS-induced colitis was associated with an increase in the colonic myeloperoxidase content. On the behavioural level, colitis had genotype-dependent effects on emotional-affective behaviour. Most conspicuous was that colitis increased anxiety primarily in WT mice so that in the EPM test WT mice spent less time on the open arms than the knockout animals. Similar changes were observed in the OF test. In contrast, colitis did not alter depression-like behaviour in WT and PYY+NPY^{-/-} mice but reduced immobility in PYY^{-/-} mice.

Conclusion: These data show for the first time that the gut hormone PYY has a significant impact on emotional-affective behaviour, because its deletion enhances anxiety- and depression-related behaviour. Colitis enforces anxiety-like behaviour only in the presence of PYY, whereas in the absence of PYY depression is less pronounced in animals with colitis. These data attest to an important role of PYY in the gut-mood axis.

A14

Characterization of receptors involved in serotonin contractions of isolated human umbilical artery in uncomplicated pregnancy

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BMC Pharmacology 2009, 9(Suppl 2):A14

Background: Serotonin (5-HT), is a vasoactive substance involved in physiological functioning of fetoplacental blood flow and continuous modulation of smooth muscle tone in umbilical vein and arteries. Since umbilical blood vessels have been shown to be deficient in autonomic innervation, the action of local autocrine vasoactive substances (including serotonin) is of considerable importance. Although seven main groups of 5-HT receptors (5-HT₁₋₇) have been characterized, it has been established that only several 5-HT receptor subtypes may be involved in vascular effects of this biogenic amine. Therefore, the aim of this study was to characterize receptors involved in serotonin-induced effect on isolated human umbilical artery (HUA) in uncomplicated pregnancy.

Methods: The experiments were performed on intact vascular rings of HUA isolated from umbilical cords that were obtained immediately after vaginal delivery in women with uncomplicated pregnancy. Only the remnant tissue, which would have been otherwise disposed of, has been utilized. Isometric tension of suspended artery rings was continuously recorded. Contraction induced by each concentration of 5-HT was later expressed as a percentage of the maximal contraction induced by Krebs bicarbonate solution with 60 mM KCl.

Results: 5-HT (1 nM - 30 μM) produced concentration-dependent contractions of HUA. Control contractions produced by 5-HT were notably reduced by methiothepin (a nonselective 5-HT_{1/5-HT₂} receptor antagonist; 0.01-1 μM), with typical irreversible competitive antagonism exposed. On the other hand, increasing concentrations of ketanserin (a selective 5-HT_{2A} receptor antagonist; 0.03-0.3 μM) significantly shifted 5-HT control curves to the right in a concentration-dependent manner with reversible competitive antagonism shown.

Conclusion: Schild's analysis of the effect produced by ketanserin, taken together with the results obtained with methiothepin, suggest that the transduction mechanism of HUA responses to serotonin in uncomplicated pregnancy involves activation of a mixed population of 5-HT₁ and 5-HT_{2A} receptors.

Acknowledgements

This research was supported by grant 145015B from the Ministry of Science, Serbia.

A15

Modification of actin fibers changes the electrical phenotype of cardiac myofibroblasts

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BMC Pharmacology 2009, 9(Suppl 2):A15

Background: Slow conduction and ectopic activity are major determinants of cardiac arrhythmogenesis. Both of these conditions can be elicited by myofibroblasts (MFBs) following establishment of heterocellular gap junctional coupling with cardiomyocytes. MFBs appear during structural remodeling of the heart and are characterized by the expression of α -smooth muscle actin (α -SMA) containing stress fibers. In this study, we investigated whether pharmacological interference with the actin cytoskeleton affects myofibroblast arrhythmogenicity.

Methods: Experiments were performed with patterned growth strands of neonatal rat ventricular cardiomyocytes coated with cardiac MFBs. Impulse conduction velocity (θ) and maximal upstroke velocities of propagated action potentials (dV/dt_{max}), expressed as % action potential amplitude change (%APA) per ms, were measured optically using voltage sensitive dyes. Actin was destabilized by latrunculin B (LtB) and cytochalasin D and stabilized with jasplakinolide. Data are given as mean \pm S.D. ($n = 5-22$). Single cell electrophysiology was assessed using standard patch-clamp techniques.

Results: As revealed by immunocytochemistry, exposure of MFBs to LtB (0.01-10 μ mol/L) profoundly disrupted stress fibers which led to drastic changes in cell morphology with MFBs assuming an astrocyte-like shape. In control cardiomyocyte strands (no MFB coat), LtB had negligible effects on θ and dV/dt_{max} . In contrast, LtB applied to MFB-coated strands increased θ dose-dependently from 197 ± 35 mm/s to 344 ± 26 mm/s and dV/dt_{max} from 38 ± 5 to $78 \pm 3\%$ APA/ms, i.e., to values virtually identical to those of cardiomyocyte control strands (339 ± 24 mm/s; $77 \pm 3\%$ APA/ms). Highly similar results were obtained when exposing the preparations to cytochalasin D. In contrast, stabilization of actin with increasing concentrations of jasplakinolide exerted no significant effects on impulse conduction characteristics in MFB-coated strands. Whole-cell patch-clamp experiments showed that LtB hyperpolarized MFBs from -25 mV to -50 mV, thus limiting their depolarizing effect on cardiomyocytes which was shown before to cause arrhythmogenic slow conduction and ectopic activity.

Conclusion: Pharmacological interference with the actin cytoskeleton of cardiac MFBs affects their electrophysiological phenotype to such an extent that they lose their detrimental effects on cardiomyocyte electrophysiology. This result might form a basis for the development of therapeutic strategies aimed at limiting the arrhythmogenic potential of MFBs.

A16

In vitro and in vivo profiling of P-glycoprotein in human neuroblastoma and rhabdomyosarcoma cells under simvastatin exposure

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BMC Pharmacology 2009, 9(Suppl 2):A16

Background: Drug efflux via ATP-binding cassette (ABC) transporters is one of the main causes for chemoresistance of

tumours. P-glycoprotein (ABCB1) is the main representative of these efflux pumps, which needs full glycosylation for proper function. We could previously show that statins have a double impact on P-glycoprotein [1]. Firstly, statins inhibit P-glycoprotein which results in higher intracellular accumulation of chemotherapeutics like doxorubicin. This could be shown in human neuroblastoma and rhabdomyosarcoma cells. Secondly, statins reduce the glycosylation level of P-glycoprotein and thereby also lead to reduced efflux of anthracyclines, which results again in enhanced apoptosis via the mitochondrial pathway. The question arises if these effects are also seen *in vivo* and in other tumour cells.

Methods and results: In a mouse xenograft model, the liver and rhabdomyosarcoma were analysed for P-glycoprotein levels in the absence and presence of simvastatin. On protein level P-glycoprotein was significantly down-regulated in the presence of pharmacological doses of simvastatin. Preliminary data confirm compensatory elevation of mRNA for P-glycoprotein by real time PCR. On cellular level the compensation of mRNA induction of P-glycoprotein is seen only after long-time simvastatin exposure of more than 24 hours. Other ABC-transporters are present in neuroblastoma and rhabdomyosarcoma cells and are currently under investigation, most importantly MRPI (ABCC1), which allows also doxorubicin export.

Conclusion: Taken together, these data show that pharmacological doses of simvastatin are sufficient to down regulate P-glycoprotein in normal and tumour tissue *in vivo*. Thus, inhibition and down-regulation of an ABC transporter by simvastatin represents a novel mechanism of action which clearly has clinical implications for cancer therapy.

Reference

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A17

Nucleotides excite sensory neurons via two P2Y receptors and a dual signaling cascade

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BMC Pharmacology 2009, 9(Suppl 2):A17

Background: Sensory neurons innervating the skin provide information about physical contact between organisms and the environment including stimuli that lead to pain sensation. Metabotropic P2Y receptors have been suggested to be important in the signaling of sensory neurons, but their effects and signaling mechanism remained controversial.

Methods: Patch-clamp recordings were performed in primary cultures of dorsal root ganglion (DRG) neurons from neonatal rats, P2Y receptor ligands and signaling interceptors were applied.

Results: ADP (EC_{50} : 7.5 μ M), ATP (EC_{50} : 0.5 μ M), UTP (EC_{50} : 0.8 μ M), and thio-UTP (EC_{50} : 0.4 μ M) increased the number of action potentials fired in response to current injection; UDP failed to affect action potential firing. The effect of ADP was attenuated by a P2Y₁ antagonist. This enhancement of excitability

was abolished by flupirtine (30 μM), a $\text{K}_{\text{V}7}$ channel opener, and slightly, but insignificantly attenuated by iodoresiniferatoxin (0.3 μM). Under voltage clamp, the same nucleotides inhibited currents through $\text{K}_{\text{V}7}$ channels in a concentration-dependent manner with similar EC_{50} values. The P2Y_1 -specific agonist MRS2365 also caused an inhibition of $\text{K}_{\text{V}7}$ channels (EC_{50} value of 8.68 nM), and the P2Y_1 antagonist MRS2179 attenuated the inhibition by ADP. Treatment of sensory neurons with the phospholipase C inhibitor U73122, with the Ca^{2+} -ATPase inhibitor thapsigargin, or the Ca^{2+} chelator BAPTA-AM abolished the inhibition of $\text{K}_{\text{V}7}$ channels by ADP. Moreover, ADP and ATP increased amplitudes of currents through TRPV1 receptors evoked by capsaicin.

Conclusion: Activation of P2Y_1 and P2Y_2 receptors increases the excitability of sensory neurons via a dual mechanism: an inhibition of $\text{K}_{\text{V}7}$ channels via phospholipase C and increases in intracellular Ca^{2+} , and a sensitization of TRPV1 receptors, with the former mechanism being the decisive one.

Acknowledgements

Supported by FWF.

A18

P2Y_1 receptors are linked to $\text{K}_{\text{Ca}2}$ channels in PC12 cells

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BMC Pharmacology 2009, 9(Suppl 2):A18

Background: P2Y_1 receptors are widely expressed in the brain, but their signalling mechanisms in neurons remained largely unknown. In sympathetic neurons, recombinant P2Y_1 receptors inhibit voltage-gated Ca^{2+} currents (I_{Ca}) and M-type K^+ currents.

Methods: Patch-clamp recordings were performed in PC12 cell cultures, P2Y receptor ligands and signaling interceptors were applied.

Results: In PC12 cells stably expressing rat P2Y_1 receptors (PC12- P2Y_1), but not in wild type PC12 cells (PC12-wt), ADP induced rises in intracellular Ca^{2+} with half-maximal effects at $15 \pm 1.3 \mu\text{M}$. In whole-cell patch-clamp recordings, ADP inhibited I_{Ca} of PC12- P2Y_1 cells (EC_{50} : $6.3 \pm 1.7 \mu\text{M}$) and of PC12-wt (EC_{50} : $3.8 \pm 1.3 \mu\text{M}$); this effect was not altered by the P2Y_1 antagonist MRS 2216 (1 μM), but abolished by P2Y_{12} antagonists. In perforated-patch recordings, ADP inhibited I_{M} relaxation amplitudes of PC12- P2Y_1 cells with half-maximal effects at $2.0 \pm 1.8 \mu\text{M}$, but in PC12-wt no such effect was observed. In PC12- P2Y_1 , but not in PC12-wt cells, ADP (1-100 μM) caused transient increases in outward currents determined at -30 mV in the perforated-patch, but not the whole-cell mode. ADP-induced currents had reversal potentials between -80 and -90 mV which was close to the calculated K^+ equilibrium potential (-89 mV). Replacement of 100 mM extracellular Na^+ by K^+ shifted the reversal potential of ADP-induced currents to about -10 mV which was again close to the K^+ equilibrium potential (-17 mV). ADP-induced currents were prevented by thapsigargin (1 μM) and by the phospholipase C inhibitor U73122 (3 μM), but not by an inactive analogue. Finally, the ADP-induced currents were significantly reduced by 100 nM apamin.

Conclusion: These results reveal channels of the $\text{K}_{\text{Ca}2}$ family as novel targets for P2Y_1 receptor signalling.

Acknowledgements

Supported by FWF.

A19

Adult neurogenesis in a psychopathological mouse model of trait anxiety and comorbid depression-like behavior: effect of antidepressants

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BMC Pharmacology 2009, 9(Suppl 2):A19

Background: Evidence has been provided linking neurogenesis to mood disorders. Notably, it has been shown that chronic experimental stress resulting in enhanced depression-like behavior decreases neurogenesis in the dentate gyrus (DG), while antidepressants reverse these stress-induced effects. However, in most studies "normal" animals reflecting physiology rather than pathophysiology are used. Therefore, we aimed to investigate neurogenesis in the DG of a mouse model of high-trait anxiety and comorbid depression (HAB), which mimics important features of human psychopathology, and their normal anxiety/depression (NAB) controls.

Methods: BrdU (5-bromo-2'-deoxyuridine) was administered to female HABs and NABs. Mice were sacrificed at 15 and 42 days post BrdU to study cell proliferation and survival respectively. At 42 d the mice were subjected to a forced swim test. Double labelling of BrdU and c-Fos (a marker for neuronal activation) was performed to observe if newborn cells functionally integrated into the DG network. Furthermore, effects of the selective serotonin reuptake inhibitor (SSRI) fluoxetine on depression-like behavior and cell survival were assessed. Finally, gene array studies were conducted in the DG.

Results: Compared to NABs, HAB mice displayed enhanced depression-like behavior in the forced swim test and reduced newborn cell proliferation and survival in the DG. Double-labelling of BrdU and c-Fos revealed that some of the newborn cells in the survival paradigm functionally integrated in NABs, while no such evidence was found in HABs. Gene array studies revealed lower abundance of cyclin-dependent kinase 5 (Cdk5) and brain-derived neurotrophic factor (BDNF) in HABs which might contribute to reduced neurogenesis. Finally, although chronic treatment with fluoxetine reduced the depression-like behavior exclusively in female HABs, it did not alter cell survival or functional integration of newborn neurons in the DG.

Conclusion: Taken together, the enhanced depression-like behavior in a psychopathological animal model is accompanied by decreased hippocampal neurogenesis as well as BDNF and Cdk5 expression possibly contributing to the phenotype. Since the antidepressant effect of fluoxetine is discerned from neurogenesis, it is suggested that mechanisms other than adult

neurogenesis underlie the therapeutic action of SSRIs in the HAB model, which, however, has to be confirmed by using additional SSRIs. Thus, the present data do not support the idea that neurogenesis is a prerequisite for therapeutic actions common to all antidepressants. Moreover, these findings highlight the importance of psychopathological animal models in order to investigate molecular mechanisms of effective antidepressants.

Acknowledgements

Supported by FWF DK SPIN WI206-B05.

A20

L-type voltage-gated calcium channels in hippocampal neurons and their potential as anti-epilept(ogen)ic drug targets

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BMC Pharmacology 2009, 9(Suppl 2):A20

Background: Neuronal L-type voltage-gated calcium channels (LTCCs) were shown to be involved in the control of neuronal excitability, synaptic plasticity and gene expression. These mechanisms are altered in epileptic tissue and are thought to contribute to epileptogenesis. Hence, LTCCs are interesting targets for epileptic and anti-epileptic therapy. However, their role in epilepsy, whether LTCCs enhance or reduce epileptiform/epileptogenic activity, remained unclear. The aim of this study was to identify in which manner LTCCs contribute and/or modulate electrical excitation.

Methods: Current-clamp experiments were performed on hippocampal neurons in culture using the perforated patch-clamp method to record membrane voltage. The neurons were continuously superfused and LTCC activity was modulated by application of the dihydropyridines BayK 8644 (LTCC agonist) and isradipine (LTCC antagonist), all in the presence of TTX. Electrical excitation was evoked by incremental current injections, whereby the neurons were depolarised experimentally beyond the LTCC activation threshold.

Results: With 8 s long depolarisations LTCC-mediated effects appeared as bumps, oscillatory activity or hyperpolarising sags. Using ion channel blockers and ion-exchange experiments we provide evidence that LTCCs couple to both SK(K_{Ca}2.x) and CAN (probably TRPM) channels, that these couplings underlie the various LTCC-mediated effects and show up as after-depolarisations (ADPs) or after-hyperpolarisations (AHPs) following the current pulse. These coupling modes operate in parallel, because blocking one type of afterpotential uncovered the other. Varying pulse length and current strength we obtained evidence that ADPs are activated at a lower LTCC activity than AHPs. Most notably, irrespective of the predominant coupling mode leading to a depolarising or hyperpolarising modulation of the voltage responses, the initial effect of LTCC activation (e.g. the one occurring within the first second) was, in all cells, an enhancement of the depolarisations.

Conclusion: Varied predominance of LTCC coupling may explain the controversy surrounding LTCC blockers as anti-epileptic drug targets. However, short excitatory signals were always subject to LTCC-mediated augmentation. Brief (≤ 1 s),

excessive depolarisations (so-called paroxysmal depolarisation shifts or PDS) were recently implied as important elements in epileptogenesis, appearing prior to actual seizures, probably altering neuronal circuits by causing repetitive, synchronised pulsative cytosolic Ca²⁺ rises. In the framework of this hypothesis our data point to a potential use of LTCC inhibitors to counteract PDS, and hence epileptogenesis.

Acknowledgements

Supported by FWF grant P19710.

A21

Ca_v1.3 L-type calcium channels modulate depression-like behavior in mice independent of deaf phenotype

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BMC Pharmacology 2009, 9(Suppl 2):A21

Background: Mounting evidence suggests that neuronal voltage-gated Ca_v1.2 and Ca_v1.3 L-type calcium channels (LTCCs) can modulate mood and anxiety behaviors. In Ca_v1.2 dihydropyridine (DHP)-insensitive mice (Ca_v1.2DHP^{-/-} mice), systemic application of the DHP channel activator BAYK 8644 induced pro-depression-like behavior providing evidence for a possible role of Ca_v1.3 channels in mood behavior. We therefore explored the role of Ca_v1.3 LTCCs in depression- and anxiety-like behaviors using Ca_v1.3-deficient mice (Ca_v1.3^{-/-}). However, Ca_v1.3^{-/-} mice are congenitally deaf and it is so far unclear how deafness affects emotional behavior in mice. We therefore used another mouse model suffering from congenital deafness, claudin 14-deficient mice (Cldn14^{-/-}) as a control to address this question. As Ca_v1.3 channels are expressed in the retina we also investigated Ca_v1.3^{-/-} mice for possible disturbances in retinal morphology and visual function that could interfere with behavioral analysis.

Methods: Depression-like behavior was assessed using forced swim and tail suspension tests (FST and TST) whereas elevated plus maze (EPM) and stress-induced hyperthermia (SIH) were performed to test anxiety-like behavior. Morris water maze, electroretinography and immunofluorescence stainings were performed to evaluate the consequence on visual acuity and retinal morphology of Ca_v1.3 deletion in Ca_v1.3^{-/-} mice.

Results: We showed that Ca_v1.3^{-/-} mice displayed less immobility in the FST as well as in the TST, indicating an antidepressant-like phenotype. In the EPM, Ca_v1.3^{-/-} mice

entered the open arms more frequently and spent more time there indicating an anxiolytic-like phenotype which was, however not supported in the SIH test. By performing parallel experiments in *Cldn14^{-/-}* mice, an influence of deafness on the antidepressant-like phenotype could be ruled out. On the other hand, a similar EPM behavior indicative of an anxiolytic phenotype was also found in the *Cldn14^{-/-}* animals. Using electroretinography and visual behavioral tasks we demonstrated that in mice, $Ca_v1.3$ channels do not significantly contribute to visual function. However, distinct morphological changes were revealed in synaptic ribbons in the outer plexiform layer of *Ca_v1.3^{-/-}* retinas by immunohistochemistry. Although these changes have no major effects on visual function, they indicate a possible role of this channel type in structural plasticity at the ribbon synapse.

Conclusion: $Ca_v1.3$ LTCCs modulate depression-like behavior but are not essential for visual function. The findings raise the possibility that selective modulation of $Ca_v1.3$ channels could be a promising new therapeutic concept for the treatment of mood disorders.

Acknowledgements

Supported by the Austrian Science Fund (P-20670 and W11).

A22

3,5-Di-*t*-butyl catechol (DTCAT) as an activator of the human skeletal muscle ryanodine receptor Ca^{2+} channel and its evaluation as a test substance for the assessment of susceptibility to malignant hyperthermia

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BMC Pharmacology 2009, 9(Suppl 2):A22

Introduction: 3,5-Di-*t*-butyl catechol (DTCAT) has been shown to release Ca^{2+} from rat skeletal muscle sarcoplasmic reticulum (SR) vesicles, which makes it a possible candidate for use as a substitute for halothane or caffeine in the *in vitro* contracture test (IVCT) for the assessment of susceptibility to malignant hyperthermia (MHS).

Methods: To characterize the effect of DTCAT at the cellular level, Ca^{2+} release experiments were performed on cultured, human skeletal muscle cells using the fluorescent Ca^{2+} indicator fura2-AM. DTCAT was also used for the first time in the IVCT to induce contractures in human skeletal muscle bundles obtained from individuals diagnosed susceptible (MHS), normal (MHN) or equivocal (MHE); these effects were compared to those elicited by the standard test substances caffeine and halothane.

Results: In single cultured skeletal muscle cells, DTCAT released Ca^{2+} from intracellular stores with a higher potency when compared to caffeine. This effect, however, was unspecific, since the release of Ca^{2+} from stores other than the SR was evident, as well as a Ca^{2+} influx, possibly triggered by depletion of intracellular Ca^{2+} stores. DTCAT induced contractures in

skeletal muscle bundles in a concentration-dependent manner with an EC_{50} value of $160 \pm 91 \mu M$. However, the reaction to DTCAT in muscles from MHS individuals was similar to reactions to DTCAT in MHE or MHN muscles.

Conclusion: Due to its low specificity in inducing the release of Ca^{2+} from SR stores and the additional activation of Ca^{2+} influx, DTCAT is not an appropriate test substance for the diagnosis of MH.

A23

Benzodiazepines modulate GABA_A receptors by reducing a gamma-subunit-mediated inhibition of GABA sensitivity

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BMC Pharmacology 2009, 9(Suppl 2):A23

Background: Heterologous expression of α_1 , β_2 and $\gamma_{2S}(\gamma_1)$ subunits produces a mixed population of GABA_A receptors containing $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_{2S}(\gamma_1)$ subunits. GABA sensitivity (lower in receptors containing γ_1 or γ_{2S} subunits) and the potentiation of GABA-activated chloride currents (I_{GABA}) by benzodiazepines (BZDs) are dependent on $\gamma_{2S}(\gamma_1)$ incorporation [1]. A variable γ subunit incorporation may affect the estimation of I_{GABA} potentiation by BZDs. We propose an approach for estimation of BZD efficiency that accounts for a mixed population of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}(\gamma_1)$ receptors.

Methods: We investigated the relation between GABA sensitivity (EC_{50}) and BZD modulation by analyzing triazolam-, clonazepam- and midazolam-induced potentiation of I_{GABA} in *Xenopus* oocytes under two-microelectrode voltage clamp.

Results: Plotting EC_{50} versus BZD-induced shifts of GABA concentration-response curves ($\Delta EC_{50}(BZD)$) of oocytes injected with different amounts of α_1 , β_2 and $\gamma_{2S}(\gamma_1)$ cRNA (1:1:1-1:1:10) revealed a linear regression between $\gamma_{2S}(\gamma_1)$ -mediated reduction of GABA sensitivity (EC_{50}) and $\Delta EC_{50}(BZD)$. The slope factors of the regression were always higher for oocytes expressing $\alpha_1\beta_2\gamma_1$ subunit receptors (triazolam: 1.8 ± 0.1 ; clonazepam: 1.6 ± 0.1 ; midazolam: 2.3 ± 0.2) than for oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors (triazolam: 1.4 ± 0.1 ; clonazepam: 1.4 ± 0.1 ; midazolam: 1.3 ± 0.1). Mutant GABA_A receptors ($\alpha_1\beta_2$ -R207C γ_{2S}) with lower GABA sensitivity showed higher drug efficiencies (slope factors: triazolam: 1.1 ± 0.1 ; clonazepam: 1.1 ± 0.1 ; midazolam: 1.2 ± 0.1) whereas higher GABA sensitivity of α_1 -L263S $\beta_2\gamma_{2S}$ mutant receptors was associated with lower efficiency (slope factor: clonazepam: 1.7 ± 0.1).

Conclusion: Regression analysis enabled the estimation of BZD efficiency when variable mixtures of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}(\gamma_1)$ receptors are expressed and provided new insights into the $\gamma_{2S}(\gamma_1)$ dependency of BZD action. The method for determining the slope of the regression line also allowed the determination of the percentage of $\alpha_1\beta_2\gamma_{2S}$ receptors. Assuming a twofold difference in the single channel conductance of $\alpha_1\beta_2$ and

$\alpha_1\beta_2\gamma_{25}$ receptors, at 70% current ratio of γ_{25} -containing receptors our mathematical model predicts only about 50% of γ_{25} subunit incorporation. Our data suggest that BZDs reduce a γ -subunit-mediated inhibition of GABA sensitivity.

Acknowledgements

This work was supported by FWF grant I5914 (SH).

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A24

From frog oocytes to mammalian cells: substantial differences in modulation of Na_v1.4 channel slow kinetic behaviour by the β_1 subunit

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BMC Pharmacology 2009, 9(Suppl 2):A24

Background: Voltage gated sodium channels consist of an α subunit and several modulating β subunits. Upon depolarization, the α subunit first opens and then enters into different types of inactivated states. When expressed in mammalian cells, the β_1 subunit has been shown to modulate the kinetics of fast inactivation. Here, we tested whether a very stable inactivated state, which we refer to as ultra-slow inactivation (lus), is subject to modulation by the β_1 subunit of the sodium channel. Previously, we showed that Na_v1.4 channels, containing the mutation K1237E in the selectivity filter, had enhanced entry into lus when expressed in *Xenopus* oocytes. Coexpression of the β_1 subunit in this system had no effect on lus. However, the kinetic behaviour of Na_v1.4 may vary between the *Xenopus* oocyte system and mammalian expression systems. As both systems are widely used in ion channel research, it appeared of interest to evaluate the kinetic effect of coexpression of β_1 in a mammalian expression system. Therefore, we tested whether lus could be reproduced in TSA201 mammalian cells and whether it is subject to modulation by the β_1 subunit in this system.

Results: The time course of recovery from lus was assessed by depolarizing the cells to -30 mV for 600 seconds, followed by repetitive 25 ms test pulses from -120 mV to -20 mV, at 5 s intervals. Fitting of a double-exponential function to the time course of recovery at -120 mV revealed that 45% of K1237E channels recovered with a time constant of ~140 s, characteristic for recovery from lus. Coexpression of the construct with β_1 substantially reduced the fraction of channels recovering from lus to 28%.

Conclusion: These results suggest that lus can be reproduced in mammalian cells. However, unlike in *Xenopus laevis* oocytes, in a mammalian expression system this kinetic state can be modulated by the β_1 subunit.

Acknowledgements

Funding support: Austrian Science Fund P210006-B11.

A25

Double mutant gating perturbation analysis predicts a high conformational stability of the domain IV S6 segment of the voltage-gated Na⁺ channel

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BMC Pharmacology 2009, 9(Suppl 2):A25

Background: The S6 segment of domain IV (DIV-S6) of voltage-gated Na⁺ channels is considered to be a key player in gating and local anesthetic drug block. Thus, mutations at several sites of DIV-S6 are known to substantially alter the channel's inactivation properties.

Methods: For a comprehensive analysis of the kinetic role of DIV-S6 in fast inactivation we performed a cysteine scanning analysis of sites 1575-1591 in the DIV-S6 of the rNa_v1.4 channel. These mutations were engineered into the wild-type channel and into rNa_v1.4 carrying the mutation K1237E. K1237E is located in the P-loop of domain III and mutations at this site have dramatic effects both on permeation and gating properties. Hence, K1237E most likely causes a complex conformational change of the channel. We sought to explore whether K1237E changes the pattern of gating perturbations produced by the serial cysteine replacements in DIV-S6. The constructs were expressed in *Xenopus laevis* oocytes and studied by means of two electrode voltage-clamp.

Results: The half-point of availability following a 50 ms conditioning prepulse (V05) was -44 ± 1 mV and -51 ± 1 mV in wild-type and K1237E, respectively ($p < 0.001$). Most serial amino acid replacements by cysteines in DIV-S6 produced shifts in V05, both in the background of wild-type and in the background of K1237E, ranging from $+17 \pm 1$ mV to -9 ± 2 mV. A plot of the shifts in V05 by single DIV-S6 mutants relative to wild-type vs. the shifts in V05 by double mutants relative to K1237E showed a significant positive correlation ($r = 0.92$, $p = 0.002$).

Conclusion: This indicates that the general pattern of gating perturbations in DIV-S6 is not affected by K1237E, suggesting a high conformational stability of the DIV-S6 segment during the fast inactivated state.

Acknowledgements

Funding support: Austrian Science Fund P210006-B11.

A26

Attenuation by a novel synthetic analogue of ACTH₄₋₇ of the learning and memory deficits in juvenile rats treated with amphetamine *in utero*: role of nitric oxide

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BMC Pharmacology 2009, 9(Suppl 2):A26

Background: Drug abuse among pregnant women continues at alarming frequency. Exposed children often show selective impairments of attention and other disturbances which might develop to major cognitive disorders. This work seeks to examine the impact of prenatal stress (PS) induced by the psychostimulant drug amphetamine (AMPH) on memory functions in male offspring of rats and to study the possible neuroprotective action of the novel Russian peptide Semax (a synthetic analogue of ACTH₄₋₇). In addition, the role of the neuronal messenger nitric oxide (NO) as well as the intensity of lipid peroxidation (LPO) in mechanisms of PS was examined.

Methods: Pregnant Wistar rats received a daily intraperitoneal injection of 10 mg/kg AMPH (IUAMPH) or saline for control dams (IUV) between E17 and E20. Nitric oxide generation was measured by electron paramagnetic resonance technique.

Results: Juvenile IUAMPH rats at 25 days of age showed delayed alternation deficits and impairments of acquisition of a fixed platform position in the water maze demonstrating impaired working memory. Both NO and LPO levels were elevated in the hippocampus of IUAMPH rats as compared with control animals. Pretreatment with Semax reversed the PS-induced learning deficits in offspring rats and prevented the increase of NO generation.

Conclusion: Thus, AMPH-elicited PS induces delayed memory deficits and significant learning impairments in juvenile offspring of rats. Therefore, *in utero* AMPH exposure resulted in a significant oxidative stress, which may be related to impaired learning ability. Modulation of the activity of NO and LPO might lead to a significant recovery of the memory functions in PS rats that open new approaches for neuroprotection and cognitive rehabilitation of prenatal brain damage.

A27

Importance of the carboxyl terminus for folding and trafficking of the serotonin transporter

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BMC Pharmacology 2009, 9(Suppl 2):A27

Background: The human serotonin transporter is the plasma membrane Na⁺/Cl⁻-dependent transporter responsible for uptake of serotonin from the synaptic cleft.

Methods: We studied the importance of the carboxyl terminus in folding and trafficking of the serotonin transporter by generation of SERT mutants by site-directed mutagenesis (alanine scanning mutagenesis), transient expression in HEK293 cells, localization by epifluorescence microscopy and confocal laser scanning microscopy, biochemical characterization (binding studies, uptake studies) and test for possible pharmacochaperoning effect of SERT ligands.

Results: Our data show that the mutation in P601G602-AA, R607I608-AA and RII-AAA (Sec24 binding site) causes intracellular retention and abolishes uptake and binding. We could

rescue the mutant (RI-AA and RII-AAA) by ibogaine (100 mM) and DMSO (2%) but not with 5-HT (100 mM), imipramine (10 mM) or low temperature (31°C). However, the mutant PG-AA could not be rescued by any of these compounds. We studied the effect of the mutations on mis-folding by expression of our mutants in a bacterial system [1] but we could not use this protocol for SERT, so we turned to co-immunoprecipitation of SERT (wild-type and mutant) with calnexin antibody as has been described by Duvernay *et al.* [2], and we could immunoprecipitate calnexin (preliminary data).

Acknowledgements

This work was supported by SFB35-10 and a stipend from Egyptian Ministry of Higher Education and State for Scientific Research.

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A28

Cytokine signalling in human melanoma cells determines susceptibility to statin-induced apoptosis

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BMC Pharmacology 2009, 9(Suppl 2):A28

Background: Melanoma is one of the most aggressive and chemoresistant cancer types in humans. Especially in late stages, effective therapeutic approaches are not available. Statins have been investigated for their anti-proliferative and pro-apoptotic effects in many tumor cells including melanoma [1]. Beside paracrine signalling, melanoma cells rely on a wide range of autocrine cytokine loops.

Methods and results: We have therefore screened the serum-free supernatant of simvastatin-treated 518A2 melanoma cells for cytokines. While INF- γ , TNF- α , IL-1 α , IL-1 β , IL-10 and IL-12 were not regulated by simvastatin, most strikingly, IL-6 levels were significantly decreased. IL-6 is an important prognostic marker in late stage melanoma. Due to this crucial role in the autocrine regulation of the tumour growth this cytokine was investigated in greater detail. A375 and 518A2 melanoma cells were transfected with a fluorescent Stat-3 fusion protein and showed IL-6-mediated translocation of Stat-3-YFP into the nucleus. This was followed by a transient phosphorylation of Stat-3. Conversely, the "IL-6-insensitive" melanoma cell lines, WM278 and WM793B, showed constitutively active Stat-3 phosphorylation and virtually no regulation upon IL-6 addition. Interestingly, the latter cells were approximately 10-fold less susceptible toward statin-induced caspase 3 activation compared to A375 and 518A2 melanoma cells. Moreover, addition of IL-6 to simvastatin-treated A375 and 518A2 melanoma cells abrogated the pro-apoptotic effect of statins.

Conclusion: Taken together, these data may open a possible new therapeutic window for statins in late-stage melanoma therapy which is based on IL-6 suppression by simvastatin in the metastatic melanoma cell lines A375 and 518A2, while early-stage melanoma cell lines, WM278 and WM793B were virtually insensitive to statin treatment.

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A29

Unexpected role of STAT1 serine727 for NK cell function

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BMC Pharmacology 2009, 9(Suppl 2):A29

Background: Natural killer (NK) cells are important key players of the innate immune system and provide immediate defense against viral infection and tumor transformation. Therefore, their potential in cancer immunotherapy has grown prominently in the last years [1]. As is known from the literature, the Signal Transducer and Activator of Transcription I (STAT1) plays an important role for NK cell function, since STAT1-deficient mice display impaired basal NK cytolytic activity *in vitro* and are unable to reject transplanted tumors *in vivo* [2]. STAT1 mediates signals downstream of interferons and gets activated by phosphorylation of several tyrosine and serine residues. In particular phosphorylation of S727 is considered a prerequisite for the full-fledged activation of STAT1 [3]. The aim of this project is to investigate the role of STAT1-S727 in NK cell-mediated cytotoxicity and tumor surveillance.

Methods: The analysis of STAT1^{-/-}, STAT1-S727A and wild-type mice includes the preparation of primary splenic NK cells and their investigation regarding proliferation (³H]thymidine-incorporation), cytotoxicity (standard [⁵¹Cr]-release) and cytokine production (ELISA, multi-plex bead arrays). *In vivo* tumor models are employed using NK-sensitive tumor cell lines (v-abl⁺ leukemia, B16 melanoma, 4T1 breast cancer).

Results: Wild-type NK cells from untreated healthy mice display basal phosphorylation on STAT1-S727 *in vivo*. Surprisingly, disruption of this phosphorylation site by mutating serine727 to alanine (STAT1-S727A) significantly enhances NK cell cytotoxicity towards various target cells *in vitro* compared to wild-type. Moreover, we demonstrate that STAT1-S727A mice do not only display delayed leukemia onset but also lower susceptibility to intravenously administered B16 (melanoma) and 4T1 (metastasizing breast cancer) cells compared to STAT1^{-/-} and wild-type mice.

Conclusion: Obviating the phosphorylation of STAT1-S727 seems to increase NK cell cytotoxicity *in vitro* and *in vivo*. As an ultimate goal we aim to find the underlying mechanism(s) and upstream regulator(s), since loosening the brake on NK cell functions could represent a potential novel strategy in cancer therapy.

Acknowledgements

We thank the Austrian Academy of Science for supporting Eva Maria Putz with a DOC-fORTE fellowship.

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A30

Regulation of CFTR expression and function by NOS isoforms

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BMC Pharmacology 2009, 9(Suppl 2):A30

Background: The cystic fibrosis transmembrane conductance regulator (CFTR) is the gene of interest in the eponymous disease, cystic fibrosis (CF). CFTR is expressed in epithelial cells and functions as a chloride channel. Several hundred mutations are known to occur in CF patients, but the most prevalent mutation is the deletion of phenylalanine at position 508 (Δ F508). Although the mutated protein is retained in the endoplasmic reticulum, the channel has been shown to be at least in part functional. A possible strategy for the treatment of CF therefore aims at facilitating surface expression by e.g. increasing total protein expression or by chaperoning the protein on its route from the endoplasmic reticulum to the cell surface. Polymorphisms in nitric oxide synthase (NOS) have been shown to influence disease severity in CF patients. Insights into the molecular mechanisms underlying the disease modifying effect of NOS will not only help understanding variation in the clinical course of cystic fibrosis but it may also provide information that can be exploited to improve and individualize treatment strategies for CF patients.

Results and conclusions: Using cell culture systems, we have investigated the effect of NOS on CFTR expression levels. Preliminary results show an up-regulation of wild-type and mutant CFTR by two NOS isoforms. This CFTR up-regulation is independent of the enzymatic activity of NOS. Deletion of a C-terminal PDZ domain interaction motif (TRL) does not block NOS-mediated increase in CFTR expression. In future experiments we will investigate if the observed up-regulation of CFTR is mediated via a direct or indirect interaction and if this enhanced expression translates into a higher level of functional chloride channels formed by CFTR Δ F508 at the cell surface.

A31**Ion channel impairments in dystrophic cardiomyocytes**

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BMC Pharmacology 2009, 9(Suppl 2):A31

Background: Muscular dystrophies comprise a heterogeneous group of inherited diseases that are characterized by progressive muscle weakness and degeneration. Severe forms, e.g. Duchenne muscular dystrophy (DMD), which is caused by a mutation in the dystrophin gene, lead to loss of ambulation, respiratory failure, and premature death. In many types of the muscular dystrophies the cardiac muscle is also affected - cardiomyopathy and/or cardiac arrhythmias regularly represent life threatening complications. The current understanding of the pathomechanisms underlying these cardiac diseases in various muscular dystrophies is still very limited. Here we tested the hypothesis that dysfunctional ion channels may be critically involved in dystrophy-associated cardiac disease.

Methods: The functional properties of voltage-gated sodium and calcium channels in cardiomyocytes derived from normal and dystrophic neonatal mice were studied by using the whole cell patch clamp technique. Besides the most common mouse model for human DMD, the dystrophin-deficient mdx mouse, we also used mice additionally carrying a mutation in the utrophin gene. The mdx-utr double mutant mouse exhibits a more severe disease phenotype than the mdx mouse, and may represent a more suitable animal model for human DMD.

Results: We found that dystrophic cardiomyocytes show reduced sodium current density compared to wild-type cardiomyocytes. In addition, extra utrophin-deficiency altered sodium channel activation and inactivation properties, which was not observed in only dystrophin-deficient (mdx) cardiomyocytes. Preliminary experiments also suggest an impairment of calcium channel inactivation in dystrophic cardiomyocytes.

Conclusion: We found significant impairments in ion channel function in dystrophic cardiomyocytes. These may perturb electrical impulse propagation in the dystrophic heart, and thus contribute to cardiac complications associated with muscular dystrophies.

Acknowledgements

Supported by the Austrian Science Fund (FWF, P19352-B11).

A32**A_{2A} adenosine receptor interacting partners (interactome)**

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BMC Pharmacology 2009, 9(Suppl 2):A32

Background: The A_{2A} adenosine receptor is a member of the G protein-coupled receptor family. It is known to possess several unique structural and functional features that differ from established signaling paradigms. Recently, the exceptionally long (>120 amino acids) C-terminus of the receptor has raised interest for its capacity to act as a binding site for several "accessory" proteins. In search for interaction partners we identified a list of possible candidates via a yeast two-hybrid system. From the identified potential interaction partners we further investigated (i) ARNO/cytohesin-2, a guanine nucleotide exchange factor for the small G protein ARF6, and (ii) SAPI02, "synapse-associated protein of 102 kDa", and their effects on the signaling properties of the A_{2A} receptor.

Methods and results: Cell-lines (HEK-293 and PC12) with inducible expression of ARNO or its catalytic inactive mutant E156K, as well as cell-lines expressing ARF6 or the dominant negative mutant T27N were created. None of these proteins altered the receptor expression, signaling via adenylyl cyclase after activation or long-term de- and resensitization kinetics in PC12 cells. On the other hand, ARF6 and its guanine nucleotide exchange factor ARNO are recruited to the cell membrane after agonist stimulation of the receptor, where they seem to stabilize the receptor/G protein complex. The mutant proteins are recruited to the membrane in a similar way but do not stabilize the receptor/ G protein complex. SAPI02, as its eponymous name suggests, is located at synaptic sites and is known to recruit glutamate receptors during different stages of the brain development. The overexpression of SAPI02 in mouse hippocampal neurons showed no apparent effects on the receptor signaling properties. However, the role of SAPI02 as an interaction partner may lie in the alteration of receptor mobility. To visualize possible differences in A_{2A} receptor membrane diffusion we utilize a single-particle tracking approach. Epitope-tagged receptors are labeled with quantum dots and their trajectories are recorded under various conditions. To further characterize the A_{2A} receptor interactome under more physiological conditions we use a two-step proteomics approach combining the tandem affinity purification method and 2D-nano-LC-MS/MS. This requires the introduction of an epitope-tagged version of the A_{2A} receptor. After establishing the method in heterologous expression systems we want to continue with this approach in native mouse tissues. Therefore we will generate transgenic mice expressing a tagged A_{2A} receptor.

A33**The role of NPY in expression and extinction of conditioned fear**

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BMC Pharmacology 2009, 9(Suppl 2):A33

Background: Neuropeptide Y is a highly conserved 36 amino acid peptide that is widely distributed in the peripheral and central nervous systems. Besides its functions in various

metabolic processes, NPY has attracted considerable attention in modulating emotional-affective behavior. NPY exerts a solid anxiolytic effect most likely mediated by Y_1 receptors, whereas stimulation of predominantly pre-synaptic Y_2 receptors results in increased anxiety. However, little is known about an involvement of NPY in processing of fear.

Methods: The current study aims to elucidate the role of NPY in Pavlovian fear conditioning, a simple form of associative learning. NPY knockout (NPY KO) mice as well as knockout mice for the different NPY receptors (Y_1 , Y_2 , Y_4 and Y_1/Y_2 double KO) were subjected to a delay fear-conditioning paradigm (5 presentations of a tone co-terminating with a mild electric foot shock, 0.7 mA). Extinction learning was performed the following day by repetitive exposure to the tone (40 presentations) in the absence of a foot shock.

Results: Compared to wild-type controls, NPY KO mice revealed faster acquisition and augmented expression of conditioned fear. Baseline freezing was increased on retention/extinction day, indicating a generalization of conditioned fear. Moreover, NPY KO mice displayed a pronounced deficit in the extinction of fear memory. Within sessions, extinction as well as extinction recall were significantly impaired in NPY KO mice. Conversely, acquisition of fear was reduced in Y_2 KO mice. Interestingly, no corresponding changes in extinction of conditioned fear were seen in Y_2 KO mice. However, Y_4 KO mice exhibited an impairment in fear extinction, similar to the one seen in NPY KO mice.

Conclusion: Our data indicate that NPY has a protective role in the acquisition of fear memories. In addition, it facilitates extinction of conditioned fear. Results from Y receptor KO mice suggest that Y_1 and Y_2 receptors are the most likely candidates for modulating the acquisition of fear, whereas for extinction a concerted action of Y_1 and Y_4 receptors seems to be conceivable.

A34

Protein kinase C- ζ is involved in the inhibition of eosinophil migration

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BMC Pharmacology 2009, 9(Suppl 2):A34

Background: The accumulation of eosinophils in lung tissue is a hallmark of asthma, and it is believed that eosinophils play a crucial pathogenic role in allergic inflammation. Therefore, eosinophils are currently considered a major therapeutic target in allergic diseases. Prostaglandin (PG) E_2 exerts anti-inflammatory and broncho-protective mechanisms in asthma, but the underlying mechanisms have remained unclear. We have shown previously that PGE_2 and the EP_2 receptor agonist butaprost inhibit eosinophil trafficking *in vitro* and *in vivo* [1].

Methods: Human eosinophils were purified by negative magnetic selection from peripheral blood. Chemotaxis was determined in 48-well microBoyden chambers and migrated eosinophils were enumerated by flow cytometry.

Results: The chemotaxis of human eosinophils towards the chemoattractant eotaxin was attenuated by PGE_2 and the selective EP_4 agonist ONO-AE1-329 in a concentration-dependent manner. Pretreatment of eosinophils with the adenylyl

cyclase inhibitor SQ-22536, the protein kinase A inhibitor H-89 or the p38 MAP kinase inhibitor SB-202190 did not prevent the inhibitory effect of PGE_2 , while the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002, tricinibine, a specific inhibitor of Akt phosphorylation, and a myristoylated pseudosubstrate of protein kinase (PK) C- ζ (mPS), partially or completely reversed the inhibitory effect of PGE_2 and ONO-AE1-329 on the migration of eosinophils towards eotaxin.

Conclusion: Protein kinase C is an increasingly diverse family of enzymes that has been implicated in a range of cellular functions within the eosinophil. The present data show that the PI3K/Akt/PKC- ζ pathway is involved in the negative regulation of the migration of human eosinophils mediated by EP_2 and EP_4 receptor activation.

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A35

Bioavailability of dodeca-2E, 4E, 8E, 10E/Z-tetraenoic acid isobutylamides after oral administration in rats and distribution in various tissues

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BMC Pharmacology 2009, 9(Suppl 2):A35

Background: Preparations from *Echinacea* are among the best-selling phytopharmaceuticals worldwide and have been widely used for the treatment of common cold and various upper respiratory infections. The most relevant active principles of *Echinacea* extracts are alkamides, caffeic acid derivatives (CADs) and glycoproteins/polysaccharides [1]. The main alkamides in *Echinacea* preparations are the isomeric dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides ("tetraenes"). Until now, a limited number of pharmacokinetic studies with alkamides have been reported, but no data exist about their distribution in tissues and transport through the blood-brain barrier (BBB). Therefore, we evaluated the pharmacokinetics of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides after a single oral dose administration of 2.5 mg/kg in plasma as well as in liver and four different brain regions (hippocampus, cerebral cortex, striatum and cerebellum).

Methods: Plasma and tissues were collected after 8, 15, 30 minutes and 1, 2, 3, and 6 hours after oral dosing and the concentrations were determined by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method with benzanilide as internal standard using the respective $[M-H]^+$ ions, m/z 248/152 for the dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides and m/z 198/105 for the internal standard.

Results: The lipophilic constituents were rapidly absorbed and well distributed to the examined tissues. The highest concentration was found in the striatum. The total tetraenoic amount in plasma was calculated as $AUC_{0-\infty}$ (794 min ng/mL), which was about 13-45% of that found in different brain parts (1764-6192 min ng/mL), and 63% of that in liver tissues (1254 min ng/g). The C_{max} in plasma was 26.4 ng/mL, while the C_{max} in the different brain regions varied between 33.8 ng/g and 46.0 ng/g.

Conclusion: The results demonstrate that the dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides are bioavailable in rats with a rapid passage across the blood-brain barrier.

Acknowledgements

Supported by the Erwin-Schrödinger scholarship (FWF) J2754-B05.

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A36

Absolute and relative bioavailabilities of dodeca-2E, 4E, 8E, 10E/Z-tetraenoic acid isobutylamides after intravenous and oral single doses in rats

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BMC Pharmacology 2009, 9(Suppl 2):A36

Background: Dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides are the main alkaloids in *Echinacea* preparations, which have been demonstrated to possess biological activities in various bio-assays, such as immune-modulating activities and effects on cannabinoid receptors [1]. Therefore, the evaluation of systemic availability of these active plant constituents is a major prerequisite for the interpretation of *in vitro* pharmacological testing. This study assessed the absolute and relative bioavailabilities of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides (tetraenes) administered as pure compounds or as an *Echinacea purpurea* root extract preparation.

Methods: Ten rats received 0.75 mg/kg dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides orally, pure and within 158.6 mg/kg *Echinacea purpurea* extract, or intravenously to compare the absorption and pharmacokinetic properties. Pharmacokinetic parameters and bioavailability data of tetraenes were obtained by non-compartmental analysis (NCA) using WinNonlin[®] 5.2 software.

Results: Mean dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamide plasma area under the concentration-time curve ($AUC_{0-\infty}$ per dose) was 3.2 ± 0.3 min ng/mL/ μ g and 1.0 ± 0.2 min ng/mL/ μ g after i.v. and oral administration, respectively, and 1.5 ± 0.2 min ng/mL/ μ g after oral administration of the *Echinacea* root extract. The absolute bioavailability of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides was 29%, which was increased to 47% (1.6 fold) by the administration of an *Echinacea* extract. The relative bioavailability was over 100%.

Conclusion: Administration of a whole *Echinacea* extract increases blood exposure with no impact on C_{max} . The high area under the curve concentration resulted in a longer elimination half-life with 123 min in comparison to 36 min after administration of the pure dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides. The approximately 2-fold higher percentage of relative bioavailability achieved with the *Echinacea* root extract resulted in a 3.4 and 3.6 times higher terminal elimination half-life and mean residence time (MRT), respectively. A rapid absorption followed by a slower elimination phase was observed.

Acknowledgements

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A37

A relationship between membrane permeability of amphetamines and serotonin efflux via serotonin transporters

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BMC Pharmacology 2009, 9(Suppl 2):A37

Background: It has been noticed by our group [1] that p-chloroamphetamine (PCA)-induced SERT current “deactivates” slower upon removal of PCA from the external solution, when compared to the deactivation of serotonin (5-HT)-induced current. This was observed in stably transfected human embryonic kidney (HEK) cells as well as in *Xenopus laevis* oocytes injected with hSERT-RNA, for PCA/5-HT concentrations between 1 and 30 μ M. Following the removal of higher concentrations of PCA (>30 μ M) from the bath solution we witnessed an intriguing, but yet paradoxical current rise.

Methods and results: Here we try to explain both findings with a model in which due to PCA leakage from the interior of the cell, SERT activity is prolonged. The increase of current following removal of high PCA concentrations is thus a consequence of the combination of “PCA leakage” and a bell-shaped dose-response relationship for the activation of the substrate-induced current. We then explored the relevancy of these findings for PCA-induced [³H]5-HT release employing superfusion experiments. Analogous to the described slow deactivation of the substrate-induced current, also a slow deactivation of 5-HT release upon washout of PCA was found.

Conclusion: This strongly supports the notion that leakage of amphetamines from the lumen of the cell is crucially implicated in amphetamine action. PCA leaking from the interior of the cell can reactivate SERT and thus increase the probability of the occurrence of outward transport. Here we propose that amphetamines can cause 5-HT efflux due to their ability to cycle (passive leak through the membrane and active reuptake through SERT), an ability that is not shared by the endogenous substrate 5-HT.

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A38

Active metabolites formed during hepatic first-pass: simulations featuring their contribution to the overall effect in altered liver clearance and drug-drug interactions

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BMC Pharmacology 2009, 9(Suppl 2):A38

Background: Phase I and - occasionally - also phase II metabolites may contribute to the overall effect of a drug. This is not always apparent or revealed, since total concentrations of metabolites may be low in relation to parent drug concentrations. In the case of carvedilol, using a comparison of HPLC and β_1 -specific radioligand receptor binding assay (RRA), it was possible to demonstrate that regarding β -adrenoceptor blockade the effect appears directly linked to the serum compartment, which indicates instantaneous equilibrium between the blood compartment and the biophase, and that oxidative metabolites significantly contribute to the effect, particularly after oral administration. This drug serves as model compound in different approaches for evaluating the role of formation of active metabolites during first-pass when hepatic clearance varies.

Methods: A kinetic model, which includes the immediate transformation of a fraction of the dose into active metabolites during first-pass through the liver, i.e., before it reaches the systemic circulation (AM-FP model), was found superior to standard models.

Results: Overall, both systemic and oral clearance values were different for the two carvedilol enantiomers: 27.5 L/h (R) and 49.6 L/h (S) for i.v. administration, and 11.3 L/h (R) and 21.6 L/h (S) for p.o. administration. The hepatic extraction ratio was estimated to approximately 0.76 and 0.77 for the (R)- and (S)-enantiomer, respectively.

Conclusion: For carvedilol, the hepatic extraction ratio is considerable, and the oral availability (calculated assuming complete absorption and no intestinal elimination) amounts to 25-35% for the parent drug due to extensive first-pass

metabolism. In spite of their low serum levels, the contribution of the metabolites must not be neglected, especially for oral dosing. Elimination of the parent drug was found to be rate-limiting, i.e., the kinetics of the metabolites is formation-rate-limited, at least in healthy volunteers. The AM-FP model was most suitable, since parent drug and metabolites appear to enter the systemic circulation simultaneously. The new compartmental model is applicable for PK/PD simulation studies including situations where hepatic clearance is affected. It was used to simulate the contribution of active metabolites formed during first-pass to the overall effect, also under conditions of impaired liver function leading to reduced metabolic clearance and in case of drug-drug interactions that are hypothesized to induce absorption.

A39

Endogenous dynorphin in emotional control and stress response

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BMC Pharmacology 2009, 9(Suppl 2):A39

Background: Cerebral control of stress and anxiety involves several neurotransmitter systems. Beside serotonin, noradrenaline or catecholamines, also neuropeptide systems are considered to be involved in generating symptoms of anxiety and stress. These systems act in a circuit connecting amygdaloid and hypothalamic nuclei, the pituitary and adrenal glands, regulating the physiological response via ACTH and corticosterone release.

Methods: In this study, we investigated anxiety and stress-related behaviour of germ-line prodynorphin knockout (dynKO) mice. Behavioural data were complemented by in-situ hybridization analysis of neurotransmitter expression in anxiety-related brain areas and measurement of corticosterone serum levels.

Results: Male dynKO mice exhibited about 2-fold ambulation in the open field center. DynKO mice showed also more visits (2-fold) and more time (3-fold) spent on open arms of elevated plus maze test. Significantly higher numbers of entries, distance and time spent in open lit area (ca. 30% higher values) in light-dark test were observed in dynKO as compared to wild-type mice (WT). The anxiolytic phenotype of dynKO could be mimicked by injection of the selective κ antagonists norBNI (10 mg/kg, i.p.) or GNTI (3 nmoles, i.c.) in WT. Applying the specific κ agonist U50488H (2.5 mg/kg, i.p.) entirely reversed the anxiolytic phenotype of dynKO. These data are in line with reduced CRH expression in the hypothalamic paraventricular and central amygdaloid nuclei and attenuated basal corticosteron serum levels. Stress-induced increases in corticosterone levels were also less pronounced in dynKO mice; however, they did not translate into marked differences in stress-induced immobility.

Conclusion: Taken together, our data suggest anxiogenic effects of endogenous dynorphin. These effects are mediated by κ opioid receptors, however not in an immediate manner. Therefore, we propose a higher order controlling level for the

action of dynorphin, like regulating the expression of CRH and serum corticosterone levels, which in turn influence the behaviour of mice.

Acknowledgements

This project was supported by the Austrian Science Fund (P20107) and the Tiroler Wissenschaftsfonds.

A40

Bioactivation of nitroglycerin by the East Asian variant of aldehyde dehydrogenase-2

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BMC Pharmacology 2009, 9(Suppl 2):A40

Background: The East Asian variant of mitochondrial aldehyde dehydrogenase (ALDH2) exhibits significantly reduced dehydrogenase, esterase and nitroglycerin (GTN) reductase activities [1]. The small molecule Alda-1 was reported to partly restore low acetaldehyde dehydrogenase activity of this variant [2]. In the present study we compared the wild-type enzyme (ALDH2*1) with the East Asian variant (ALDH2*2) regarding GTN bioactivation and effects of Alda-1.

Results: Alda-1 increased acetaldehyde oxidation by ALDH2*1 and ALDH2*2 about 2- and 15-fold, respectively. The effects of this compound on the esterase activities of both enzymes were identical to that of NAD (6- and 10-fold stimulation with ALDH2*1 and ALDH2*2, respectively), but in the presence of the nicotinamide Alda-1 stimulated the esterase activity of ALDH2*1 only 1.7-fold, whereas the ALDH2*2-catalyzed reaction was increased 73-fold. ALDH2*1 exhibited a greater affinity for GTN than ALDH2*2 as well as a 7-fold higher maximal GTN denitration activity. However, bioactivation of the nitrate, measured as soluble guanylate cyclase (sGC) activation, was much more pronounced (8.8 ± 0.2 and $18.2 \pm 0.9 \mu\text{mol cGMP min}^{-1} \text{mg}^{-1}$ in the presence of $100 \mu\text{g}/100 \mu\text{L}$ ALDH2 and $100 \mu\text{M}$ GTN). Alda-1 caused 30-70% inhibition of GTN denitration by ALDH2*1 but had no effect on the ALDH2*2-catalyzed reaction and did not affect GTN-induced sGC activation in the presence of either variant.

Conclusion: The present results indicate that Alda-1 stimulates established ALDH2 activities by improving NAD binding but does not restore impaired GTN bioactivation by the East Asian variant. In addition, our data revealed an unexpected discrepancy between GTN reductase activity and sGC activation, suggesting that GTN denitration and bioactivation reflect independent reactions catalyzed by ALDH2.

Acknowledgements

Supported by Fonds zur Förderung der Wissenschaftlichen Forschung in Austria (W901 DK Molecular Enzymology and P20669) and Deutsche Forschungsgemeinschaft (KO1157/4-1).

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A41

A single neurotoxic dose of MDMA decreases BDNF expression in the frontoparietal cortex but not in the hippocampus

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BMC Pharmacology 2009, 9(Suppl 2):A41

Background: The popular recreational abuse drug 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') produces acute and long-lasting deficits in several markers of the serotonergic (5-HT) system. BDNF (brain derived neurotrophic factor) is a prominent trophic factor of serotonergic fibers. The aim of this study was to characterize the damage of serotonergic fibers in the frontoparietal cortex and hippocampus in parallel with the expression of BDNF.

Methods: Male Dark Agouti rats (7 weeks old) were treated with a single (15 mg/kg, i.p.) dose of MDMA. Density of tryptophan hydroxylase (Tph) immunoreactive (ir) fibers was quantified 3 days and 3 weeks after treatment. BDNF protein and mRNA levels were measured by ELISA and quantitative PCR method 24 hours and 3 weeks after treatment.

Results: Findings from Tph-ir fiber density revealed significant (30-50%) decrease, compared to baseline, both 3 days and 3 weeks after treatment in both hippocampus and the frontoparietal cortex. Results from ELISA showed that BDNF level in the frontoparietal cortex dropped significantly (30%) 24 hours and 3 weeks after treatment. This decrease in BDNF concentration was also confirmed with the quantitative PCR method. In contrast, no such a reduction was found in the hippocampus.

Conclusion: Our findings show that administration of a single dose of MDMA, a neurotoxic agent, leads to a significant drop in the Tph-ir axon density with a parallel decrease in BDNF levels 3 days and 3 weeks after treatment in the frontoparietal cortex. In contrast, in the hippocampus, the reduction of Tph-ir axon density is not accompanied by the decrease of BDNF level. These results highlight regional differences in the expression of BDNF, the main neurotrophic factor of serotonergic fibers, in the terminal serotonergic areas after a neurotoxic dose of MDMA.

Acknowledgements

Supported by the EC, LSHM-CT-2004-503474.

A42

Neuromorphological and functional effects of ecstasy during serotonergic damage and recovery

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BMC Pharmacology 2009, 9(Suppl 2):A42

Background: The ring-substituted amphetamine derivative (\pm)-3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) has become a widely abused psychoactive drug among young people. Studies indicate that MDMA produces long-term alterations of serotonergic parameters in the brain of rodents and primates, and MDMA was also found to be neurotoxic in humans. The aim of our work was to characterize the regional differences of the damage in the terminal and cellular areas, and the alterations during recovery. Serotonin (5-HT) plays a key role in the regulation of sleep, hence we used EEG recordings to measure functional and morphological effects in parallel during partial damage and recovery of brain serotonergic neurons by MDMA.

Methods: Our goal was to investigate the effects of a single dose (15 mg/kg) of MDMA on serotonin transporter and several sleep parameters 7, 21 and 180 days after MDMA administration in the Dark Agouti rat brain. The expression of 5-HT mRNA was compared in MDMA-treated and control animals in the dorsal and median raphe nuclei. The density of immunostained 5-HT fibers was quantified in several brain areas (e.g. cerebral cortex, hippocampus, hypothalamus and brainstem). Immunohistochemical measures and some general parameters (e.g. body weight and food intake) were also determined one year after the treatment.

Results: Seven and 21 days after MDMA treatment we observed significant (20-40%) reductions in 5-HT densities. 5-HT mRNA expressions were significantly elevated 7 days and decreased 21 days after MDMA treatment in the dorsal and median raphe nuclei. We also found alterations in several sleep parameters after drug treatment. Most of the above effects, except the decrease in hippocampal 5-HT densities, were transient; they recovered by 180 days after MDMA administration. One year after MDMA treatment we found some recovery in the hippocampus, but the 5-HT density was still significantly lower in CA2 and CA3 regions.

Conclusion: Our results indicate that a single dose of MDMA causes long-term damage in the terminal regions and also in neural functions of the serotonergic system. Interestingly, although the rate of the axonal damage shows little differences in the regions innervated by ascending axons, the process and speed of recovery differs markedly.

Acknowledgements

This work was supported by the 5th (QLG3-CT-2002-00809) and 6th Framework Program of the European Union LSHM-CT-2004-503474.

A43

Characterization of different G protein coupling properties of CB₁ and CB₂ cannabinoid receptors and GPR55 receptor using BRET

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BMC Pharmacology 2009, 9(Suppl 2):A43

Background: CB₁ and CB₂ cannabinoid receptors are G protein-coupled receptors which have been described to couple mainly to the G_{i/o} subfamily of G proteins. However, in some cell

types and upon stimulation with certain cannabinoid agonists, activation of other G protein subtypes has also been observed. GPR55 is an orphan G protein-coupled receptor which has been suggested to be a novel member of the cannabinoid receptor family.

Methods: In this study we wanted to characterize the G protein activation properties of the two known cannabinoid receptors and GPR55 following stimulation with different cannabinoid ligands, using bioluminescence resonance energy transfer (BRET). We monitored the activation of different G protein subtypes (G_o, G_q, G_s or G₁₂) using *Renilla* luciferase-tagged wild type or chimeric G α_o subunits (i.e. G α_o with the C-terminal 5 amino acids replaced with those of G α_q , G α_s or G α_{12} , respectively) co-expressed with EYFP-tagged $\alpha_1\alpha_1$ subunit and the receptor in CHO cells.

Results: We found that CB₁ was able to activate all four subtypes of G proteins, with different pharmacokinetic properties, following stimulation by non-selective (WIN55 and 2-AG) or CB₁-selective (ACEA) cannabinoid agonists. Basal activity of CB₁ could also be detected with G_o and G₁₂ subtypes, as the CB₁ inverse agonist AM251 caused significant BRET increase (i.e. G protein subunit association) when tested with these G proteins. In contrast, CB₂ showed no G protein activation other than G_o, upon either WIN55 or 2-AG stimuli. Stimulation of GPR55 with WIN55, 2-AG or AM251 did not alter the activity of the tested G proteins even at considerably high ligand concentrations.

A44

Electrophysiological characteristics of heart ventricular papillary muscles in diabetic histidine decarboxylase knockout and wild-type mice

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BMC Pharmacology 2009, 9(Suppl 2):A44

Background: Diabetes-induced action potential (AP) abnormalities have been studied mainly in rats where significant prolongation of repolarization and reduced maximum rate of depolarization (V_{max}) was detected. Histidine decarboxylase knockout (HDC-KO) mice lack endogenous histamine and they are characterized by impaired glucose tolerance. Furthermore, they have autoantibodies reactive to glutamic acid decarboxylase (GAD). These findings suggested that this model might have an increased susceptibility to autoimmune diabetes.

Methods: A standard microelectrode technique was used to characterise the cardiac electrophysiological parameters of control and streptozotocin (STZ)-induced diabetic HDC-KO mice compared with those of wild-type animals.

Results: With aging, blood glucose levels in HDC-KO mice were shifted towards values characteristic of diabetes. The electrophysiological changes relevant to diabetes, i.e. prolongation of repolarization and depression of V_{max} developed without any induction by STZ. In this group, STZ treatment caused no further significant AP changes.

Conclusion: One of the likely explanations may be that in the chain of events in HDC-KO mice on the one hand and in STZ-

induced diabetes on the other hand, leading to the alterations in the heart electrophysiological parameters, there is a common link. This link may be a similar shift in the expression/function of certain K⁺ channel populations.

Acknowledgements

This work was supported by Hungarian Health Science Council (ETT) grant 578/2006 (V.K.).

A45

Analysis of sleep EEG during rebound sleep after three days REM deprivation

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BMC Pharmacology 2009, 9(Suppl 2):A45

Background: Effects of selective rapid eye movement sleep (REMS) deprivation are usually studied with the single platform-on-water method (flower pot). In this protocol, animals are placed on a small platform (SP) surrounded by water, and as muscle atony is typical for REMS, they fall into the water and awaken immediately as they switch to REMS. A large platform (LP) is also used, in order to separate the effects of REMS deprivation from other stress factors caused by the procedure, because animals can curl and can reach REMS on LP. Our aim was to study quantitative-electroencephalography (Q-EEG) in different vigilance stages measured in the rebound period after three-day-long sleep deprivation.

Methods: In our study, male Wistar rats were equipped with EEG and electromyographic electrodes. After the recovery period rats were kept on SP or LP for 72 hours, and then animals were placed into the recording cages. Frontoparietal electroencephalogram, electromyogram and motility were recorded during the first hour of the rebound period beginning at the passive phase. Home cage (HC) recordings were also taken in the same one hour period. The EEG power spectra (1-60 Hz) were analyzed of the following sleep stages: active and passive wake (AW, PW), light and deep slow wave sleep (SWS-1, SWS-2) and REMS, and the results of SP, LP and HC groups were compared to each other.

Results: Our main results show that in sleep stages SWS-1 and SWS-2, both LP and SP enhanced the EEG power in a wide frequency range (5-25 Hz), but only the LP group decreased the delta frequency power compared to HC. A shift in theta frequency power in PW and REM was also caused by both LP and SP.

Conclusion: Using the same setup and time interval, a marked REM rebound in SP compared to both LP and HC groups was described by us. In spite of that we found here that Q-EEG analysis provided evidence for differences between LP and HC groups, while the SP group data resembled those of LP or HC using different measures. These findings suggest that the effects of stress and REM deprivation could be differentiated using sleep and Q-EEG analysis. Decrease in REM latency in depressed patients and the general REM-reducing effects of antidepressants underline the significance of these results.

Acknowledgements

This work was supported by the 6th Framework Program of the EU, LSHM-CT-2004-503474.

A46

Regulating and regulated role of the orphanin FQ/nociceptin system

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BMC Pharmacology 2009, 9(Suppl 2):A46

Background: There is a permanent need for improvement in effective pain management that calls for implication of recent advances in the understanding of endogenous morphine-like substances. Using the technique of “reverse physiology” identification of an orphan, opioid-receptor-like 1 receptor (ORL1, recently named as OP4/NOP), and isolation of its endogenous agonist orphanin FQ/nociceptin (NC) opened a new era in opioid pharmacology. In recognition of the involvement of NOP-NC system in a wide range of physiological, and pathophysiological processes, the nociceptinergic system is considered an important new target for drug development in pain control, morphine-withdrawal, anxiety, depression, cognitive impairment, body mass control, cough suppression, hypertension, heart failure and ischemic brain injury.

Methods: We conceptualized that elucidation of (patho) physiological conditions/human disorders with changes in endogenous levels of NC can give significant contribution to drug targeting. [¹²⁵I]NC-RIA and HPLC methods were used for measurements.

Results: In Wistar rats both in the central nervous system (CSF) and the periphery (blood plasma) NC levels reached the adult level by the age of 12 weeks. Plasticity of unmaturing NOP receptors was evidenced in neonatal hormonal imprinting studies with NC. In patients with chronic liver disorders of different etiology (Wilson's disease, primary biliary cirrhosis) plasma NC was found to be elevated, and extremely high levels were observed in hepatocellular carcinoma patients compared to age-matched healthy controls. The functional role of circulating NC in primary neurovascular headaches (migraine and cluster headache patients) was also evidenced. Plasma NC levels in acute stroke and transient ischemic attack patients were found to be elevated compared to healthy controls; however, significantly lower NC levels were observed in atherosclerotic patients with chronic limb ischemia in both the plasma and blood vessels.

Conclusion: The observed changes in endogenous NC levels designate the NOP-NC system as a potential new target in therapy.

Acknowledgements

This work was supported by a grant of the Hungarian Ministry of Health (ETT 353/2006).

A47**Behavioural sensitisation of rat dams treated with morphine pre- and postpartum**Melinda Sobor^{1,2}, Julia Timár¹, Susanna Gyarmati¹ and Susanna Fürst^{1,3}¹Department of Pharmacology and Pharmacotherapy, Semmelweis University, 1089 Budapest, Hungary²National Institute of Pharmacy, 1051 Budapest, Hungary³HAS-SE Neuropsychopharmacology Research Group, 1089 Budapest, Hungary

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BMC Pharmacology 2009, 9(Suppl 2):A47

Background: In our previous experiments a trend to sensitisation to maternal behavioural disruptive and conditioned place preference (CPP)-inducing effects of morphine (MO) was observed when rat dams were treated chronically with a constant medium dose of MO during pregnancy and lactation. The aim of the present work was to perform more detailed studies how chronic MO treatment of dams influences behavioural effects of subsequent MO challenge.

Methods: Pregnant Wistar rats, from the day of mating were treated daily with MO (10 mg/kg, s.c.) or saline (SAL) until weaning (postpartum day, PD, 21). In both treatment groups maternal behaviour (active nursing, passive nursing, littering and behaviours out of the nest) was observed after an acute challenge with saline, 3 mg/kg naloxone s.c. (NX), 10 mg/kg MO s.c. and 10 mg/kg MO plus 3 mg/kg NX s.c. on PD2, PD3, PD5 and PD7, respectively. In a different population of dams, a pup retrieval test was performed after the same challenge. Experiments started 30 min after MO and 10 min after NX injection. In a further population of MO- and SAL-treated dams, NX aversion test was made using the place preference paradigm.

Results: (1) Acute challenge with MO significantly impaired the maternal behaviour in both groups; this effect of MO could be antagonised completely by NX in the SAL-treated group, but only partially in the MO-treated one. This appeared both in observational and pup retrieval tests. (2) MO treatment significantly potentiated the ability of naloxone to produce place aversion.

Conclusion: The data indicate that constant medium dose MO treatment during pregnancy and lactation results in sensitisation to the place aversion-inducing effect of NX similarly to MO on CPP, but it attenuates the ability of NX to antagonise the effect of MO on MB.

Acknowledgements

This work was supported by the Hungarian grants OTKA K-60999 and ETT-441/2006.

A48**Spinal interaction between μ and δ opioid receptors in naive and morphine-tolerant rats**Pál Riba¹, Kornél P Király¹, Tamás Friedmann¹, Mahmoud Al-Khrasani¹, Melinda Sobor³ and Susanna Fürst^{1,2}¹Department of Pharmacology and Pharmacotherapy, Semmelweis University, 1089 Budapest, Hungary²Hungarian Academy of Sciences-Semmelweis University Neuropsychopharmacological Research Group, 1089 Budapest, Hungary³National Institute of Pharmacy, 1051 Budapest, Hungary
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BMC Pharmacology 2009, 9(Suppl 2):A48

Background: The role of δ opioid receptors in opioid antinociception and tolerance development is still unclear. In the spinal cord of morphine-tolerant mice δ receptor ligands given intrathecally (i.t.) differently influenced the antinociceptive effect of the μ agonist D-Ala²-methyl-glycinol (DAMGO). The δ_1 agonist D-Pen^{2,5}-enkephalin (DPDPE) inhibited, the δ_2 agonist deltorphin II did not alter, and the δ antagonist cha-TIPP ψ potentiated the effect of DAMGO. We hypothesized that during the development of morphine tolerance the formation of μ - δ heterodimers may contribute to the spinal μ opioid tolerance. Delta ligands may affect the dimer formation differently. Those, like DPDPE may facilitate the dimer formation, hence inhibit the antinociceptive effect of DAMGO by causing virtual μ receptor down-regulation. Ligands that do not affect the dimer formation do not influence antinociception but ligands with the presumed capability of disconnecting the dimers may decrease the spinal tolerance to DAMGO. The δ ligand profile in morphine-tolerant rats, were also studied.

Methods: Male Wistar rats (150-200 g) were treated with subcutaneous (s.c) morphine twice daily for four days with increasing doses (50, 100, 200, 200 μ mol/kg). On the fifth day the antinociceptive effect (rat tail flick test) of DAMGO was measured alone and combined with a fixed dose of δ ligands given i.t.: DPDPE, Ile^{3,5}-deltorphin II, cha-TIPP ψ and naltrindole, respectively.

Results: The repeated treatment with morphine resulted in approximately three to six-fold shift of the ED₅₀ value of DAMGO compared to that of naive rats. Both in naive control and morphine-tolerant rats all ligands except naltrindole potentiated the antinociceptive effect of i.t. DAMGO (two to five-fold). In the tolerant rats the potentiation restored the potency of DAMGO to the control level.

Conclusion: Delta ligands behave differently in rats than in mice. One possible explanation could be a higher basal density of the μ - δ heterodimers in rats. The inhibitory action of naltrindole on the antinociceptive effect of DAMGO could be explained by its relatively low μ/δ selectivity as well as by the different effect on the μ - δ heterodimer. The difference in the DPDPE effect in morphine-tolerant rats and mice requires further clarification.

Acknowledgements

This work was supported by the Hungarian grants OTKA K-60999 and ETT-441/2006, and a Bolyai Fellowship of the Hungarian Academy of Sciences and Faculty of General Medicine, Semmelweis University.

A49**The electrophysiological and hemodynamical effects of pyruvate on diabetic and control rat hearts**Gábor Bárdi, Katarina Csoltkova, László Károlyi, Andrea Szebeni and Valéria Kecskeméti
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BMC Pharmacology 2009, 9(Suppl 2):A49

Background: Pyruvate, as an end-product of glycolysis, might influence the electrophysiological parameters of the heart by several mechanisms: Pyruvate enhances the phosphorylating potency of the cytosol; it can facilitate the reduction of inorganic

phosphate and finally can influence the cytoplasmatic redox state by decreasing the H^+ concentration. According to the literature, pyruvate enhances the amplitude of the action potential (APA) and also reduces the duration of the action potential (APD); hereby, it might be used to enhance the contractility in case of heart failure. Our aim was to determine the electrophysiological and hemodynamical effects of pyruvate on diabetic and control rat hearts.

Methods: Using conventional microelectrode techniques and the Langendorff system we examined the effects of sodium pyruvate (1, 3, 10 and 30 mmol/L) on electrophysiological and hemodynamic parameters of control ($n = 28$) and streptozotocin-induced diabetic ($n = 29$) rat hearts.

Results: Similarly to our previous electrophysiological results, the APD of the right ventricular papillary muscles of diabetic rats' hearts was significantly longer compared to the control animals. In the control group only the highest pyruvate concentration caused significant APD reduction while in the diabetic groups the second and third pyruvate concentrations already significantly reduced the APD. In our Langendorff system both the mean left ventricular pressure (LVP) and the diastolic left ventricular pressure (LVP_D) were significantly higher in the control group. Pyruvate treatment induced significant increase only in the control group (LVP_D at 3, 10 and 30 mmol/L pyruvate; LVP at 10 and 30 mmol/L pyruvate).

Conclusion: Our electrophysiological result mainly correlates with the previously published results. Corresponding to our hemodynamic results we can conclude that in case of diabetic animals higher pyruvate concentrations would be required to achieve the same favorable effect. On account of its beneficial effect on the heart muscle, pyruvate can become a potential drug in the future, one which can open new possibilities in the therapy of cardiogenic shock.

Acknowledgements

This work was supported by the Hungarian Health Science Council (ETT), grant 578/2006 (V.K.).

A50

Investigation of the PtdIns(4,5)P₂ dependence of plasma membrane receptor endocytosis in living cells

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BMC Pharmacology 2009, 9(Suppl 2):A50

Background: Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) plays an important role in various cellular processes: not only in calcium signalling as a precursor for the second messenger Ins(1,4,5)P₃, but also in the regulation of ion channels, cytoskeletal dynamics and many other events connected to the plasma membrane. Since many of the molecules participating in the process of endocytosis can bind PtdIns(4,5)P₂, a role of this lipid in the regulation of the internalization of plasma membrane receptors seemed possible.

Methods: In this study we focused on the investigation of the lipid dependence of the internalization of plasma membrane receptors, and we used the highly sensitive method of bioluminescence resonance energy transfer (BRET), which allows the detection of molecular closeness between two

proteins labeled by bioluminescent and fluorescent markers. By fusing various plasma membrane receptors (e.g. angiotensin II AT₁ receptor, serotonin 5-HT_{2C} receptor and EGF receptor) to *Renilla* luciferase and applying YFP-tagged proteins as components of the endocytic machinery (β -arrestin, clathrin, β -adaptin, PM-targeted YFP, Rab proteins) we could follow the process with high temporo-spatial resolution in HEK cells. To decrease the plasma membrane PtdIns(4,5)P₂ level we used the previously developed rapamycin-induced heterodimerization system, in which PtdIns(4,5)P₂ depletion was achieved by the recruitment of 5-phosphatase enzymes to the plasma membrane.

Results: To check whether the PtdIns(4,5)P₂ depletion was sufficient we measured the BRET signal between the PH domain of PLC δ_1 - which binds specifically to PtdIns(4,5)P₂ - fused to either *Renilla* luciferase or YFP. To follow receptor internalization we measured the BRET ratio between the receptors and plasma membrane-targeted YFP, which decreased upon stimulation with the appropriate agonist. After optimizing our system we were able to show that the internalization of EGF receptor was significantly reduced after depletion of the lipid, and the same was noticed in the case of AT₁ and 5-HT_{2C} receptors.

Conclusion: These data suggest that PtdIns(4,5)P₂ level is an important factor in the regulation of plasma membrane receptor endocytosis.

A51

Transactivation within the AT₁ angiotensin receptor homodimer: the role of the conserved DRY motif

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BMC Pharmacology 2009, 9(Suppl 2):A51

Background: The concept of 7TM receptor dimerization has been well established. However, the mechanism and the functional consequences of dimerization are not known in detail. Recent data showed that interaction within the receptor dimer depends on G protein coupling.

Methods: In this study we examined the possible functional consequence of transactivation within angiotensin AT₁ receptor homodimers. To do this, we used the S109Y mutant of the AT₁ receptor, in which the binding site of the nonpeptid AT₁ receptor antagonist, candesartan, was destroyed. Expressing this mutant receptor together with the wild-type in CHO cells, in the presence of candesartan, we could examine the interaction between them by stimulating the S109Y mutant receptor, and following the activation of the wild-type receptor. To monitor the signaling of the receptor two parameters were measured: the conformational change of the receptor using an intramolecular sensor, and the binding of β -arrestin-2 to the activated receptors. In both cases the highly sensitive method of bioluminescence resonance energy transfer (BRET) was applied. Additionally, we performed a radioactive ligand binding assay to examine the cooperativity between receptor monomers.

Results: Under these conditions, we were able to record the activation of the wild-type receptor, which can be explained by the occurrence of the receptors in the form of di- or multimers, with a functional interaction (transactivation) within the complex. This transactivation did not depend on the phosphorylation

of the donor receptor, because using the DRY/AAY mutant of the receptor, which is known to inhibit the G protein coupling, resulted in the disappearance of this phenomenon. The radioactive ligand binding data showed increased binding of angiotensin II when the DRY/AAY mutant was expressed compared to the wild-type.

Conclusion: The results also suggest that the interaction between the monomers depends on the presence of the DRY/AAY mutation.

A52

Search for novel antipsychotic drugs: dopamine forever?

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BMC Pharmacology 2009, 9(Suppl 2):A52

During the history of antipsychotic medications, the dopamine D₂ receptor has been the crucial main target of drug action. Typical antipsychotics, which dominated the first 25 years of antipsychotic treatment as well as atypical antipsychotics, medications of the last three decades, all retained dopamine D₂ receptor antagonism as the basic mode of action. Whether the superior side-effect profile (fewer extrapyramidal symptoms) of atypical antipsychotic agents is due to their serotonin 5-HT_{2A} receptor antagonist activity or to their looser binding to the dopamine D₂ receptor is still a matter of debate. Brain imaging studies demonstrating a strong relationship between dopamine D₂ receptor occupancy and clinical effects and side effects of antipsychotics gave fundamental support for a central role of dopamine D₂ receptors in the pathology and therapy of schizophrenia. The clinical failure of alternative approaches lacking the D₂ component, such as selective serotonin 5-HT_{2A} and dopamine D₄ receptor antagonists further pointed at the indispensable role of D₂ antagonism. The recently developed atypical antipsychotic agent, aripiprazole, while preserving the predominant D₂ action, introduced a new pharmacological approach: dopamine D₂ receptor partial agonism. An appropriate degree of partial agonism presumably results in effective blockade of overstimulated dopamine D₂ receptors and improvement in psychotic symptoms, while it prevents the induction of extrapyramidal side effects or secondary negative symptoms by avoiding complete silencing of dopaminergic transmission. In recent years, the glutamatergic hypothesis for the pathology of the disease has gradually gained acceptance. Beside theoretical considerations, this concept was initially fuelled by some successful trials with the glutamate NMDA receptor co-agonists glycine, D-cycloserine and D-serine on the negative symptoms of the disease. However, their efficacy was modest and these compounds were still applied as adjunct therapy to the standard D₂ dopaminergic antipsychotics. The most recent and, perhaps, most promising "challenge" to the D₂ centred therapy of schizophrenia has been the successful proof of concept trial with the selective metabotropic glutamatergic receptor mGlu₂/mGlu₃ agonist prodrug compound, LY2140023. However, confirmation of the clinical efficacy of the compound is still awaited, and it has been raised that the compound may eventually affect - though indirectly - dopaminergic mechanisms.

It seems, despite several burial attempts and attractive alternative hypotheses and tremendous drug development efforts, the therapy of schizophrenia cannot - so far - detach itself from the dopaminergic system.

A53

The role of P2X₇ ATP receptors in the nervous system: potential implications in inflammatory and depression-like diseases

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BMC Pharmacology 2009, 9(Suppl 2):A53

Background: The P2X₇ receptor is a ligand-gated ion channel expressed in neuronal, glial and immune cells and is implicated in a wide range of pathological conditions, including ischemia, and inflammation. The P2X₇ receptor can modulate the maturation and release of the proinflammatory cytokine, interleukin-1β (IL-1β). IL-1β is suggested to be involved in the pathophysiology of depression and sickness behaviour, elicited by peripherally administered bacterial lipopolysaccharide (LPS).

Methods: The levels of IL-1β production were quantified in the hippocampi of rodents, using an ELISA kit. In order to identify genes involved in LPS-induced changes in P2X₇ receptor knock-out (KO) and wild-type (WT) mouse amygdala we performed whole mouse genome microarray analysis of mRNA extracted after six hours of intraperitoneal LPS injection.

Results: We showed that *in vivo* LPS challenge elevated IL-1β levels in the rodent hippocampus. Antagonists of P2X receptors inhibited LPS-induced IL-1β levels with a pharmacological profile similar to that of P2X₇ receptors and their inhibitory effect was attenuated in the absence of P2X₇ receptors. In WT mice, LPS overexpressed mRNA encoding P2X₄ and P2X₇ receptors in the hippocampus and also caused a remarkable increase in the levels of IL-1β in the blood serum. The hippocampal increase of IL-1β was substantially alleviated when contamination by circulating blood cells was excluded by transcardial perfusion, indicating the peripheral origin of hippocampal IL-1β elevation. Six h after i.p. injection of LPS, the expression of 74 transcripts (41 upregulated and 33 downregulated) was significantly altered two-fold or more in mouse amygdala. These genes can be classified according to their biological function as follows: inflammatory response: Il4ra, Ccl21b; depression-associated genes: Slc17a7, Nfatc1, Creb3l3. Our microarray studies have identified 8,165 transcripts that were significantly affected by the deficiency of P2X₇ receptors indicating that the deletion of P2X₇ receptors causes genome-wide alterations of gene expression including depression-related genes in mouse amygdala (GABA_A, GABA_C receptors, AMPA and NMDA_{2B} ionotropic and mGlu₅, mGlu₇ metabotropic glutamate receptors were downregulated in KO mice).

Conclusion: These results point to the key role of the endogenous activation of P2X₇ receptors in the level of IL-1β and in the regulation of individual protein which could be of potential interest for the study of the neurobiological basis underlying psychiatric diseases like depression.

A54**Role of sensory neurons on pancreatic beta cell function and on development of insulin resistance**

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BMC Pharmacology 2009, 9(Suppl 2):A54

Background: To investigate the role of capsaicin-sensitive sensory afferent nerves on the pancreatic beta cell function and on the development of insulin resistance in genetically obese, insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

Methods: At the age of 6 weeks, OLETF rats were divided into two groups. The control group was treated with the vehicle for capsaicin, and the capsaicin group was treated with a single subcutaneous dose of 50 mg/kg capsaicin. The next 19 weeks, the metabolic variables (body weight gain, ingested food and water, stool and urine production) were measured by means of metabolic cages. At the end of the treatment period, the glucose-stimulated insulin response was determined by an oral glucose tolerance test (OGTT), whole body insulin sensitivity was determined by means of hyperinsulinaemic euglycaemic glucose clamping, and the hepatic glucose production (HGP) as well as insulin-stimulated peripheral glucose uptake (PGU) were determined by means of [³H]glucose infusion. Fasting plasma insulin levels were determined by RIA and fasting blood glucose values by the glucose oxidase method. Pancreatic beta cell function was characterized by the HOMA-B index based on fasting insulin and glucose levels.

Results: The body weight of the capsaicin-treated group was significantly lower than that of the control group. There were no changes in the other metabolic parameters. During the OGTT, the control group had a reduced glucose-stimulated response compared to the capsaicin-treated group and the area under the curve values were 1844 ± 124, and 1287 ± 87, respectively (p < 0.5). The whole body insulin sensitivity improved (from 9.4 ± 1.8 to 15.6 ± 2.1 mg/kg/min) significantly according to the improvement in HGP (from 7.5 ± 1.5 to 12.9 ± 3.1 mg/kg/min) and PGU (from 6.7 ± 1.2 to 2.8 ± 1.1 mg/kg/min). There was no difference in pancreatic beta cell function between the two treatment groups.

Conclusion: Capsaicin-sensitive sensory afferents play role in the development of obesity and insulin resistance in OLETF rat. To explore the interaction between the CCK₁ and TRPV1 receptor in the vagal afferents, further experiments are needed.

Acknowledgements

The work was supported by the Hungarian Scientific Research Fund (No. 74162).

A55**Cardioprotective effects of bilberry extract on ischemia-reperfusion-induced injury in isolated rat heart**

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BMC Pharmacology 2009, 9(Suppl 2):A55

Background: Bilberries (*Vaccinium myrtillus* L.) are recognized as a good source of flavonoids, especially anthocyanins, which have strong antioxidative activity. Therefore, they may have a strong potential as cardioprotective agents in ischemic-reperfusion injury.

Methods: Anthocyanins from the prepared bilberry extract were analyzed using the HPLC-DAD system and were expressed as a standard of cyanidine-3-glucoside (mg/L). Experiments were carried out on the isolated hearts from Wistar rats of both sexes according to the Langendorff method. Post-ischemic myocardial injuries during reperfusion were determined by changes in coronary flow rate, lactate dehydrogenase (LDH) release rate, electrocardiogram analysis, incidence and duration of arrhythmias.

Results: Bilberry extract (0.01-5 mg/L) increased coronary flow and decreased LDH release rate during reperfusion. Coronary flow was increased up to 2.5-fold (p < 0.001) at 0.1 mg/L and up to 2.0-fold (p < 0.01) at 1 mg/L compared to the control values. The LDH release rates were decreased 3.7-fold (p < 0.001) at 0.1 mg/L and 6.7-fold (p < 0.001) at 1 mg/L compared to the control. Furthermore, the application of bilberry extract was also effective in the prevention of arrhythmias. The duration of arrhythmias was maximally shortened at 0.1 mg/L to 3.2 ± 0.2% (p < 0.001) and at 1 mg/L group to 4.4 ± 0.3% (p < 0.001) of the control value from the untreated group. However, the bilberry extract had no significant effect on heart rate and on left ventricular developed pressure.

Conclusion: Our results show that bilberry extract has anti-ischemic and anti-arrhythmic activity on ischemia-reperfusion-induced injury in isolated rat hearts.

A56**Presence of bilirubin translocase in the vascular system and its role in the vasodilatation activity of anthocyanins**

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BMC Pharmacology 2009, 9(Suppl 2):A56

Background: Flavonoids are well known for their vasodilatation activity. Their mechanism of action is still to be elucidated. Bilirubin translocase is a bilirubin-specific membrane carrier that is also responsible for the ATP-independent transport of flavonoids across the cell membrane [1]. Recently, the expression of bilirubin translocase in the endothelium has been characterized [2]. The aim of the study was to examine the possible role of bilirubin translocase in the vasodilatation activity of flavonoids. As a

source of flavonoids we used bilberries, which are abundant in anthocyanins that show strong affinity for bilitranslocase.

Methods: A bilberry extract, of which the anthocyanin content was quantified by HPLC-DAD analysis, was used. Anthocyanins were expressed as a standard of cyanidin 3-glucoside (mg/L). Thoracic aortic rings obtained from male Wistar rats were mounted in standard organ baths filled with Krebs-Henseleit solution, maintained at 37°C with a 95% O₂ / 5% CO₂ mixture. Rings were divided into four groups: control group (intact aortic rings), endothelium denuded rings, rings with nitric oxide synthase (NOS) activity inhibited by application of L-NNA (0.1 mmol/L) and rings with inhibited bilitranslocase-mediated membrane transport. In the last group, aortic rings were pre-incubated for 30 min with mono-specific polyclonal bilitranslocase antibodies (0.24 µg/mL). After inducing submaximal contraction (60 mmol/L KCl) in all studied groups, a chemically characterized bilberry extract was applied in increasing concentrations (0.5-20 mg/L). Vascular tone was measured isometrically by a mechano-electrical transducer. Further tests were done to check the expression of bilitranslocase in the vascular system (endothelial cell line EA.hy 926 and vascular smooth muscle cell line A7r5) by Western blot analysis using bilitranslocase antibodies.

Results: Western blot analysis showed the presence of bilitranslocase on both endothelial and smooth muscle cells. Bilberry extract relaxed aortic rings in a concentration-dependent manner in the control group, but neither in endothelium-denuded aortic rings nor in rings with inhibited NOS. The maximum relaxation (19.00 ± 2.01%, n = 5) observed at 20 mg/L in the group with inhibited bilitranslocase activity was significantly lower (p < 0.001) compared to the control group (34.95 ± 3.11%, n = 10).

Conclusion: Our results show that the vasodilatation activity of anthocyanins in bilberry extract is partly dependent on the bilitranslocase-mediated transport of flavonoids into the endothelium, followed by the activation of NOS. However, even though the bilitranslocase is also expressed on smooth muscle cells, its role in the vasodilatation activity on these cells remains negligible.

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A57

Cellular antioxidant activity of bilirubin in the human endothelial cell line EA.hy 926 is mediated by bilitranslocase

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BMC Pharmacology 2009, 9(Suppl 2):A57

Background: Oxidative stress plays an important role in the pathogenesis of cardiovascular degenerative diseases. Bilirubin is known to be a potent endogenous antioxidant, both *in vitro* and *in vivo*. The latter can be ascribed to an efficient amplification cycle whereby bilirubin, acting as antioxidant, is itself oxidized to biliverdin and then recycled back to bilirubin by NADPH-dependent biliverdin reductase. Bilitranslocase, a bilirubin-specific membrane carrier that mediates cellular uptake of bilirubin, has recently been found in the vascular endothelium. However, the levels of albumin-free bilirubin in plasma and tissues are only 10 to 50 nM. Therefore, the objective of this study was to elucidate the antioxidant activity of low concentrations of bilirubin and the involvement of bilitranslocase-mediated plasma membrane transport in human endothelial cells.

Methods: In this study we used the cellular antioxidant activity (CAA) assay developed by Wolfe and Liu [1]. Briefly, the assay is designed to trigger an acute oxidative stress into cells (by adding the radical initiator ABAP to the cell medium) and to fluorimetrically follow the subsequent increase of an intracellular radical-sensitive fluorescent dye. Substances with antioxidative properties that have free oxygen radical scavenging properties have been found to decrease the formation of fluorescence. By using the CAA assay, we have quantitatively evaluated the antioxidant activity of bilirubin in the endothelial cell line EA.hy 926. In our experiments, the cells were pre-incubated with anti-bilitranslocase antibodies (studied group) or bovine IgG (control group) before starting the CAA assay with bilirubin (0.5-100 nM).

Results: The intra-cellular antioxidant activity of bilirubin was concentration-dependent with an apparent saturation obtained at higher concentrations. The half-maximal effect was obtained at concentrations as low as 5 nM. The pre-incubation of the cells with anti-bilitranslocase antibodies reduced the antioxidant activity by about 50%.

Conclusion: Bilirubin has a potent intracellular antioxidant activity if applied to cells at concentrations close to its albumin-free plasma levels. The observed cellular antioxidant activity depends on bilitranslocase-mediated plasma membrane transport and therefore confirms the functional role of bilitranslocase as a membrane transporter in the endothelium.

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A58

Sertraline and amitriptyline enhance histamine metabolism in guinea-pig tissues

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BMC Pharmacology 2009, 9(Suppl 2):A58

Background: Aside from their typical use to treat depression, many antidepressants are also used to treat anxiety disorders and chronic pain disorders. Moreover, it has been proved that they show anti-inflammatory effects. We believe that a part of this

effect of antidepressants can arise as a consequence of histamine elimination from the site of inflammation. In mammals, histamine is mainly metabolised by diamine oxidase (DAO) and histamine-N-methyltransferase (HNMT). Therefore, we studied the effects of two antidepressants, amitriptyline and sertraline, on histamine metabolism in guinea-pigs.

Methods: Guinea-pigs were treated with amitriptyline (4 mg/kg, i.p.) and/or histamine (10 µg/kg, i.v.). Tissue and plasma histamine and methylhistamine concentrations were then measured using high performance liquid chromatography. In the animals treated only with amitriptyline (or saline), DAO and HNMT tissue mRNAs were detected by PCR. In the same tissues, specific enzymatic activities of DAO and HNMT were measured by radiometric assays. In addition, DAO and HNMT activity was measured after *in vitro* incubation with different concentrations of sertraline and amitriptyline.

Results: Five minutes after i.v. histamine application, plasma histamine concentration reached its maximum and thereafter slowly decreased. Meanwhile, histamine was distributed into several tissues, where concentrations of histamine and methylhistamine significantly increased. This distribution was faster in animals pre-treated with amitriptyline, reflecting also faster decreases in plasma histamine concentrations. In some tissues of the amitriptyline-treated animals the amount of DAO and HNMT mRNA as well as enzyme activity increased. In these tissues, we detected lower histamine concentrations and higher methylhistamine concentrations, indicating a faster histamine metabolism in the amitriptyline-treated animals. Our *in vitro* results showed that both antidepressants change DAO and HNMT activity also at the molecular level.

Conclusion: Our results clearly show that the metabolism of histamine is enhanced when antidepressants are present. Amitriptyline induced enzyme mRNA synthesis and increased enzyme activity, and consequently lowered tissue histamine concentration. At some concentrations, sertraline increased DAO activity, but had no effect on HNMT activity. Due to the fact that we managed to decrease the histamine concentration in the tissues, we expect the effects of histamine in antidepressant-treated animals to be less dramatic.

A59

The role of nonneuronal neurotrophins in the brain

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BMC Pharmacology 2009, 9(Suppl 2):A59

Background: Neurotrophins (nerve growth factor, NGF; brain-derived neurotrophic factor, BDNF; neurotrophin-3, NT-3) are involved in various CNS functions from differentiation and neuron survival to synaptogenesis and synaptic plasticity. They are synthesized in neurons and also in nonneuronal cells (astrocytes), which therefore represent an important local source of trophic support in normal and diseased brain. Their synthesis in astrocytes is susceptible to up-regulation by cytokines, hormones, drugs and synaptically released neurotransmitters. They all show significant but diverse regulatory

effects on neurotrophin levels in astrocytes with differences in dose-dependency and short-term kinetics. Cytokines are the most effective stimulators of NGF synthesis and/or secretion, whereas the monoaminergic neurotransmitters noradrenaline, adrenaline, 5-HT, dopamine and histamine differentially affect synthesis of all three neurotrophins. Their stimulatory effect is a specific receptor-mediated process involving either cytokine (IL-1), adrenergic (α_1 , β_1/β_2), dopaminergic (D₁) or histamine (H₁, H₂, H₃) receptors and corresponding intracellular mechanisms.

Summary: In conclusion, the studies on the mechanisms of astrocytic neurotrophin regulation suggest the importance of positive cooperation between the excitatory monoaminergic neuronal activity and astrocytic neurotrophic support (neuron-astrocyte crosstalk) in the developing, mature and diseased brain.

A60

Monoamine neurotransmitters modulate NT-3 levels in astrocytes

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BMC Pharmacology 2009, 9(Suppl 2):A60

Background: Neurotrophin-3 (NT-3), a member of the neurotrophin family of neurotrophic factors, displays profound neuromodulatory functions in the normal and in the diseased brain. Under physiological conditions, NT-3 is produced by neuronal cells and also by local glial cells. We focused our investigation on the ability of astrocytes to synthesize NT-3 and, additionally, on the active involvement of the neurotransmitters noradrenaline, adrenaline, dopamine, histamine and serotonin (5-HT) in the regulation of NT-3 production in neonatal rat cortical astrocytes.

Results: Our study confirms the ability of neonatal rat cortical and cerebellar astrocytes in primary culture to express and synthesize significant amounts of NT-3. The examined monoamines, with the exception of 5-HT are able to potently and transiently increase NT-3 mRNA and NT-3 protein cell levels; their action is dose- and time-dependent. Screening different activators of basic intracellular second messenger systems which can participate in the possible monoamine receptor mediated stimulation of NT-3 by examined monoamines revealed that forskolin, dibutyryl cAMP (dBcAMP), as well as calcimycin (Ca²⁺ ionophore A23187) and phorbol 12-myristate 13-acetate (TPA), markedly increase the cellular level of NT-3 protein. Neurotransmitter-induced NT-3 is susceptible (to varying degrees) to inhibition by H-89 (an inhibitor of protein kinase A, PKA) or staurosporin (an inhibitor of protein kinase C, PKC), which led us to conclude that downstream signaling responsible for the stimulation of NT-3 synthesis by monoamines in astrocytes is a receptor-mediated process consisting of multiple, complex intracellular mechanisms involving the cAMP/PKA pathway, activation of PKC, as well as mobilization of Ca²⁺ ions.

Conclusion: In conclusion, our study indicates for the first time that monoaminergic neurotransmitters play an important role in the regulation of neurotrophic NT-3 activity in cultured rat astrocytes.

A61**Histamine-neurotrophin-3 interactions in cultured rat astrocytes**

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BMC Pharmacology 2009, 9(Suppl 2):A61

Background: Neurotrophin-3 (NT-3) is produced by astrocytes, in addition to neurons, and monoamine neurotransmitters play a role in controlling NT-3 synthesis [1]. The impact of histamine on the regulation of NT-3 synthesis in cultured astrocytes has not been studied in detail. Therefore, we focused our present study on the active involvement of multiple histaminergic receptor and intracellular mechanisms in the regulation of NT-3 production by histamine.

Results: Histamine (1 μ M) significantly and transiently elevates NT-3 mRNA levels by 2.2-fold after 30 min of incubation following by 2.1-fold increase in NT-3 intracellular levels after 6 h. Its stimulation was partly inhibited by the H₁ antagonists triprolidine and mepyramine, the H₂ antagonists famotidin and cimetidin, and by the H₃ antagonist ciproxifan. NT-3 levels in astrocytes were increased by specific and selective H₁, H₂ and H₃ agonists, but none of the tested agonists was able to reach the level of histamine's stimulatory effect. Different activators of basic intracellular histamine receptor second messenger systems (forskolin, dibutyl cAMP, dBcAMP; as well as calcimycin, i.e. Ca²⁺ ionophore A23187) and phorbol 12-myristate 13-acetate, TPA) markedly increase the cellular level of NT-3 protein. Histamine-induced cellular levels of NT-3 were significantly reduced by H-89 (an inhibitor of protein kinase A, PKA) and by staurosporin (an inhibitor of protein kinase C, PKC). Studying histamine receptor subtype expression in astrocytes using quantitative RT-PCR we confirmed that the expression levels of histamine H₁ and H₂ receptors (already shown in radioligand binding) as well as of H₃ receptors are high.

Conclusion: Our study confirmed that the synthesis of NT-3 in astrocytes is regulated by the histaminergic system and indicate the possible involvement of multiple, complex histamine H₁, H₂ and H₃ receptors and corresponding intracellular mechanisms involving the cAMP/PKA pathway, as well as mobilisation of Ca²⁺ ions and activation of PKC.

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A62**Cardiotoxic activity of polymeric 3-alkylpyridinium salts from the marine sponge *Reniera sarai***

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BMC Pharmacology 2009, 9(Suppl 2):A62

Background: Marine sponges are a rich source of novel biologically active substances such as haemolytic proteins, hemagglutinins and proteins with antimicrobial, antiviral and antifungal activity. Toxic water-soluble polymeric 3-alkylpyridinium salts (poly-APS) have been isolated from the marine sponge *Reniera sarai*. Poly-APS has pore-forming properties expressed in a dose-dependent manner. These interactions with cell membranes results in increased Ca²⁺ permeability. In *in vivo* experiments performed in rats, poly-APS caused transient bradycardia, prolongation of expiration, lowered blood pressure, formed numerous thrombocyte aggregates and caused death. The aim of the present study was to investigate the direct cardiotoxic effects of poly-APS on isolated rat hearts.

Methods: Poly-APS isolated from the marine sponge *Reniera sarai* was purified and lyophilized. The experiments were carried out on the isolated rat hearts (Wistar rats, n = 30, weight 250-290 g) of both sexes according to Langendorff. The isolated rat hearts were perfused with different poly-APS concentrations (1, 10 and 100 nmol/L and 1 μ mol/L). During the experiments, the coronary flow rate, lactate dehydrogenase (LDH) release rate, left ventricular pressure, heart rate and duration of arrhythmias were measured.

Results: The most evident activity in all of the studied parameters was observed in the group perfused with poly-APS in a concentration of 1 μ mol/L. Only the lowest concentration of poly-APS that was used (1 nmol/L) did not influence the measured parameters. Poly-APS in a concentration of 1 μ mol/L diminished the coronary flow 7.6-fold, increased LDH release rate 12.7-fold, increased the pressure in left ventricle 2.6-fold and diminished heart rate 2.9-fold compared to the control group (all p < 0.001). Poly-APS also showed proarrhythmogenic activity.

Conclusion: Poly-APS salts isolated from the marine sponge *Reniera sarai* showed potent cardiotoxic activity on isolated rat hearts.

A63**The preventive cardiovascular effect of a combination of statin and angiotensin receptor blocker at sub-therapeutic doses in middle-aged healthy volunteers**

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BMC Pharmacology 2009, 9(Suppl 2):A63

Background: Endothelial dysfunction is supposed to be a key event in the development of several cardiovascular diseases. In the middle-aged, apparently healthy population, the endothelial function is frequently already impaired, representing the first step in the development of cardiovascular diseases. It seems reasonable to try to improve endothelial dysfunction in the mentioned population also by pharmacological approaches. It can be assumed that therapies that could improve the function of the

vascular endothelium would lead to diminishing or slowing the onset of cardiovascular diseases. The aim of our study was to evaluate the protective pleiotropic effects of a combination of statin and angiotensin receptor blocker at sub-therapeutic doses in middle-aged volunteers with low risk for cardiovascular disease on the function of endothelium.

Methods: Ten healthy volunteers (males) aged from 30 to 50 years without history of cardiovascular disease, diabetes mellitus, hypertension or hypercholesterolemia were recruited for the study. Subjects took a combination of statin (fluvastatin 10 mg) and angiotensin receptor blocker (valsartan 20 mg) daily for 30 days. Ultrasound measurements (Aloka Alfa 10 ProSound echomachine) of flow-mediated dilatation (FMD) were repeated three times: at the beginning of the study, after 14 days, and at the end of the study. FMD was measured on the right brachial artery after reactive hyperemia, which was induced by inflation of a pneumatic blood pressure cuff placed around the widest part of the forearm to a systolic pressure of 190 mmHg for 4 minutes. Blood samples were taken at the beginning and at the end of the study in order to make laboratory measurements.

Results: In almost all subjects involved in our study (9 out of 10) FMD was initially impaired. FMD was significantly improved at the end of the study compared to values at the beginning of the study ($3.98 \pm 0.43\%$ vs. $5.87 \pm 0.72\%$; $p < 0.01$). The values of diastolic blood pressure were significantly lower at the end of the study (83.2 ± 7.4 mmHg vs. 71.1 ± 7.0 mmHg; $p < 0.05$). The combination of the drugs used in the study did not affect serum cholesterol levels.

Conclusion: In middle-aged healthy volunteers exposed to a combination of sub-therapeutic doses of fluvastatin and valsartan for 30 days, endothelium-dependent vasodilation was significantly improved.

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Endothelin-I increases osteoclastic bone resorption via endothelin A receptors during orthodontic tooth movement in rats

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BMC Pharmacology 2009, 9(Suppl 2):A64

Background: The involvement of the endothelin signaling system during orthodontic tooth movement has not been explained yet. Therefore, the aim of this study was to determine the role of endothelins ET-1, ET-2 and ET-3 and both receptor subtypes ET_A and ET_B during all the three phases of orthodontic tooth movement in a rat model.

Methods: The study was performed on male Wistar rats (n = 85). Orthodontic tooth movement was induced by a closed

coil spring (F = 25 cN), which was placed between the upper left first molar and the upper incisors. The effects of the endothelin system were investigated using tezosentan, a non-selective endothelin antagonist, and TBC3214, a highly selective ET_A antagonist. Measurements of the distance between the upper left first molar and the ipsilateral incisor were performed on a weekly basis for 6 consecutive weeks. After that, the animals were sacrificed and tissue samples of the maxilla were taken for further biochemical and histological evaluations.

Results: Tezosentan increased tooth movement ($p < 0.01$). The opposite effect was shown using TBC3214, which decreased tooth movement ($p < 0.01$). On day 14, gene expression levels for ET-1 ($p < 0.05$) and ET-3 ($p < 0.001$) were increased compared to day 0. On day 28, a down-regulation of ET-3 was observed when compared to day 0 ($p < 0.001$). On day 42, ET-1 ($p < 0.001$) and ET-3 ($p < 0.01$) gene expression levels were strongly up-regulated, while ET-2 gene expression level was down-regulated ($p < 0.01$) when compared with day 0. The immunoreactivity of ET_A and ET_B significantly decreased on day 14 ($p < 0.001$) and increased on day 28 ($p < 0.001$). Alveolar bone volume was significantly higher in the TBC3214 group compared to the appliance only group ($p < 0.001$). Osteoclast volume was significantly lower in the TBC3214 group compared to the appliance only group ($p < 0.05$).

Conclusion: ET-1 and ET-3 are the endothelin isopeptides, which are involved in all three phases of orthodontic tooth movement. However, ET-1 is the predominant physiological form functioning during the late phase of orthodontic tooth movement. Gene and protein expression levels indicate that the major signaling pathway during the late phase of orthodontic tooth movement mainly involves ET_A receptors. During this phase ET-1 increases osteoclastic bone resorption via ET_A.

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The effect of cetirizine, an H₁ receptor antagonist, on bone modeling during orthodontic tooth movement in rats

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BMC Pharmacology 2009, 9(Suppl 2):A65

Background: H₁ receptor antagonists are widely used drugs for treatment of allergic conditions. While histamine is involved in bone remodelling [1, 2, 3], the aim of this study was to determine the effects of cetirizine, an H₁ receptor antagonist, on bone modeling processes during orthodontic tooth movement.

Methods: We used three groups of Wistar rats: control group (n = 16), orthodontic appliance only group (n = 16) and

cetirizine group (n = 16). Animals of the last two groups were fitted with a super-elastic closed coil spring appliance (F = 25 cN) between the upper first left molar and the upper incisors. Animals of the appliance only group were treated daily with saline and animals of the cetirizine group with 3 mg/kg of cetirizine, respectively. Tooth movement was measured weekly from day 0 to day 42. Animals of each group were sacrificed on day 42 and tissue samples were prepared for further analysis. Gene expression levels for bone turnover markers cathepsin K and osteocalcin were determined by means of RT-PCR. Alveolar bone volume, osteoblast and osteoclast volume were determined histomorphometrically.

Results: Cetirizine decreased the amount of tooth movement from day 28 onwards ($p < 0.01$) and it also decreased osteoclast volume ($p < 0.001$). The increase in the alveolar bone volume was observed in the cetirizine group ($p < 0.01$) compared to the appliance only group. No significant difference was observed in osteoclast activity, osteoblast volume and osteoblast activity between the cetirizine and the appliance only groups.

Conclusion: Cetirizine influences bone modeling, mainly by inhibiting bone resorption. Therefore, H_1 receptor antagonists therapy is supposed to interfere with orthodontic treatment.

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A66

Pharmacogenetics of thiopurine therapy: from thiopurine S-methyltransferase to S-adenosylmethionine

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BMC Pharmacology 2009, 9(Suppl 2):A66

Background: The efficiency and safety of the thiopurine therapy rely on the concentration of patient's cytotoxic

thioguanine nucleotides (TGN), which in turn depend on the deactivation of thiopurine drugs by thiopurine S-methyltransferase (TPMT). The activity of TPMT largely depends on the presence of genetic polymorphisms. Determination of mutations in the TPMT gene before starting 6-mercaptopurine (6-MP) therapy represents a quick, simple and cost-effective strategy to individualize thiopurine dosing. However, TPMT phenotype-to-genotype correlation is not complete, indicating a need for identification of novel biomarkers. The prime candidate is S-adenosylmethionine (SAM) which by binding into the active site of TPMT stabilizes its structure and consequently influences the metabolism and toxicity of thiopurine drugs [1].

Methods: 6-MP-induced cytotoxicity was studied in MOLT cells. Metabolic activity of cells was determined by the CellTiter Aqueous One Proliferation Assay. Cytosolic TGN, SAM and methylthioinosine monophosphate (MeTIMP) levels as well as TPMT activity were measured by the modified reverse-phase HPLC method. Intracellular ATP levels were determined by the CellTiter Glo Luminescent Cell Viability Assay (Promega). Apoptotic cells were detected by the Annexin V-FITC Apoptosis Detection Kit (Sigma) and visualized by fluorescence microscopy. Caspase-3 activity was measured using labeled DEVD substrate.

Results: We herein present evidence of a novel TPMT-mediated mechanism of SAM-specific effects on 6-MP-induced cytotoxicity [2]. Our results show that exogenous SAM rescues cells from the toxic effects of 6-MP by restoring cell proliferation and delaying the onset of apoptosis. This is achieved by altering the dynamics of 6-MP metabolism, resulting in lower production of TGNs and MeTIMP. We prove that the extent of MeTIMP-induced inhibition of *de novo* purine synthesis (DNPS) determines the concentrations of intracellular ATP, and consequently SAM, which acts as a positive modulator of TPMT activity. This leads to a greater conversion of 6-MP to inactive 6-methylmercaptopurine, and lower availability of thioinosine monophosphate for the biotransformation to TGNs and MeTIMP. By acting as a TPMT-stabilizing factor, the availability of SAM contributes to the extent of 6-MP cytotoxicity.

Conclusion: By identifying SAM as an important modulator of TPMT activity and consequently thiopurine toxicity, novel rationalization of the therapy may apply.

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