Symposium 1

organized by Georgy Bakalkin

Dynorphins in neurological and mental disorders

Chaired by Georgy Bakalkin and Andreas Zimmer

Dynorphins in experimental Parkinson's disease revealed by imaging mass spectrometry

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Background and Aim: Dopamine replacement therapy with L-DOPA is the most effective pharmacotherapy for patients with Parkinson's disease (PD), however with PD disease progression and long-term L-DOPA treatment complications occur in many patients, including L-DOPA-induced dyskinesias.
Methods: One of the most challenging aspects of neurochemistry is the detection of endogenous neuropeptides due to their low *in vivo* concentrations, pico- to sub-femtomolar levels, which are well in the range of matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS detection limits. We use MALDI imaging mass spectrometry (IMS) for characterization, localization, and relative quantification of striatal neuropeptides in a rat model of LID and PD. MALDI IMS has the unique advantage of high sensitivity and high molecular specificity, allowing detection of molecular species (hundreds) in a single tissue section.
Results: Several dynorphin peptides were detected, including dynorphin A (1-17), dynorphin A (1-8), alpha-neoendorphin, dynorphin B and their metabolites Leu-Enk-Arg and Leu-Enk-Arg-Arg. The peak intensities of dynorphin B and alpha-neoendorphin were positively correlated to the dyskinesia score in both structures, but no association between dynorphin A peak intensities and dyskinesia was found. Two main findings dissociated the peptidomic changes in striatum from substantia nigra. In the substantia nigra, but not striatum, Leu-Enk-Arg peak intensity displayed a positive correlation to the dyskinesia score, suggesting release followed by bioconversion. By contrast, des-tyrosinated alpha-neoendorphin correlated strongly with dyskinesia in striatum but not in the substantia nigra.
Conclusion: The results, particularly the detection of des-tyrosine dynorphins by MALDI IMS highlights the advantage of unbiased analysis for the study of molecular dynamics in neurological diseases.

Acknowledgments:
This work was funded by the by Swedish Research Council grant#2006-6416 (MA), 2006-6414 (MA), 2007-5407 (MA), Swedish Parkinson Foundation (MA), Swedish Royal Academy of Sciences (MA), Swedish Research Council grant #342-2004-3944(JB), 621-2008-3562(JB).

Dynorphin mutations cause human neurodegenerative disorder spinocerebellar ataxia type 23: a novel non-receptor mechanism of cell signaling?

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Background and Aim: Neuropeptides have not been previously identified as causative factors for neurodegenerative disorders. The spinocerebellar ataxias (SCAs) are a genetically heterogeneous group of neurodegenerative disorders characterized by progressive cerebellar ataxia, dysarthria and loss of the Purkinje cells. The SCA23 locus has been previously located on chromosomal region 20p12.3-p13. We here report the identification of four missense mutations in prodynorphin (PDYN) located in this region.
Methods: SCA23 families and 1100 ataxia patients, and 500 control individuals were screened for *PDYN* mutations. In cellular experiments, expression and processing of mutant PDYNs and effects of wt- and mutant peptides on striatal neurons were analyzed. Cellular pathways were studied by shotgun proteomic and key-node analyses in autopsy samples.
Results: Three mutations were located in Dyn A, a peptide with non-opioid neurodegenerative actions. Two mutations resulted in excessive generation of Dyn A. Two Dyn A mutants induced toxicity above that of wild type peptide. The fourth mutation was located upstream of dynorphins and affected expression of components of the opioid and glutamate system in the cerebellum. PDYN and Dyn A were located in Purkinje cells.
Conclusions: Elevated non-opioid actions of Dyn A mutants or impairment of secretory pathways by mutant PDYNs may lead to glutamate neurotoxicity that underlies Purkinje cell degeneration and ataxia. This is the first demonstration of causative link between mutations in neuropeptides and neurodegenerative/neuropsychiatric disorders. Identification of such mutations will also provide further insight into neuropeptide functions.

Acknowledgments:
The Swedish VR and FAS; R. Franklin Fellowship, University of Groningen, the Netherlands.

A role for epigenetic mechanisms in the regulation of prodynorphin expression by alcohol

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Background and Aim: Alcohol alters several neurotransmitter systems within the brain and accumulated evidences indicate the endogenous opioid system as an important target of its action. We studied, *in vitro* and *in vivo* the molecular alterations occurring in the prodynorphin gene following different exposures to alcohol.
Methods: Human neuroblastoma SH-SY5Y cells were exposed to low, clearly not intoxicating, and high etha-

nol concentrations at different time points. Sprague Dawley rats received alcohol intragastrically trying to mimic human drinking that establishes tolerance and dependence conditions. Real-time RT-PCR was used to assess the abundance of mRNAs of interest. DNA methylation was analyzed by Methylation Specific-Real Time PCR and bisulfite-Pyrosequencing. Specific histone modifications at gene promoters were evaluated by Chromatin ImmunoPrecipitation.

Results: In the cellular model we demonstrated a temporal relationship between selective chromatin modifications induced by ethanol or acetaldehyde, and changes in prodynorphin gene expression were demonstrated. In the amygdala complex of alcohol-treated rats differential changes in prodynorphin gene expression changes were observed depending on the time of exposure; consistently, we propose potential epigenetic mechanisms responsible for these alterations, at least upong short ethanol exposure.

Conclusion: Our findings indicate a linkage between gene expression alterations and epigenetic modulation in prodynorphin promoter, thus adding novel information on how the opioid system can be affected by alcohol in several ways. Studies are ongoing to evaluate the chromatin remodelling in the neuroplasticity occurring in the progression of alcohol abuse. It will be also of value to study the ability of epigenetic modulators in reverting dynorphin genetic/epigenetic alterations and alcohol abuse-related behaviours. Moreover, opioid drugs already available in alcoholism treatment could also have possible epigenetic modulating properties.

Dynorphins regulate the intensity of fear memory: from mice to men

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Background and Aim: The formation of fear memories and their extinction are necessary for the adaptation to a changing environment. Here with a translational approach we investigated the role of dynorphins in the dynamic change in fear memories in mice and in humans.

Results: In mice, genetic deletion of the dynorphin encoding gene prodynorphin (PDYN) in mice resulted in enhanced cue-dependent fear conditioning, as well as delayed extinction in contextual and cue conditioning/extinction paradigms. The pharmacological blockade of κ opioid receptors (KOR) produced a similar effect on fear extinction as the dynorphin deletion. The behavioral data are supported by the analysis of the induction of the immediate early gene c-fos, which demonstrated that the absence of dynorphin results in reduced neuronal activity in key limbic structures during extinction. Translating these findings into the human domain, we could demonstrate that a polymorphism in the dynorphin encoding gene PDYN impacts the activity of the amygdala, functional coupling between amygdala and the prefrontal cortex and the intensity of stress responses during extinction.

Conclusions: Our findings establish a role of PDYN/KOR signaling in fear extinction and suggest a biological mechanism for the success of trauma exposure therapy.

Symposium 2

organized by Helmut Schmidhammer

Novel ligands interacting with opioid receptors

Chaired by Helmut Schmidhammer and Masaaki Yoshikawa

Universal opioid receptor ligands – buprenorphine and related orvinols

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Background and Aim: The pharmacological profile of buprenorphine has some unique features, some of which are explicable by the slow onset and even slower offset of its interaction with μ opioid (MOP) receptors. As a MOP receptor partial agonist, buprenorphine would also be expected to show a ceiling to its opiate effects, in fact the level of effect from very high doses are lower than from intermediate doses. This has been suggested to be due to activation of the nociceptin/orphanin FQ (NOP) receptor at high buprenorphine concentrations. It was of interest to discover if analogues can be developed with even higher levels of NOP receptor activity.

Results: Binding data on the compounds synthesised suggest that it is the C20 substituent that occupies the putative lipophilic site in the NOP receptor, as required for high affinity binding. By varying this group, ligands with affinities from 8 nM–133 nM NOP receptors were generated (buprenorphine Ki-NOP 77 nM). Of the compounds with good NOP receptor affinity, efficacy at this receptor ranged from very low (5% of nociceptin) to moderate (58% of nociceptin) with buprenorphine being intermediate in this range (21% of nociceptin). One compound, BU08028, was found to have equal affinity at opioid and NOP receptors (all between 1.6–8.5 nM) and very similar activity to buprenorphine in the [35S]GTPγS assay, except having higher efficacy (48% of nociceptin) at NOP receptors. However, *in vivo*, BU08028 proved to be a MOP receptor agonist with morphine-like efficacy.

Conclusions: The first universal opioid receptor ligands have been developed, confirming that substantial NOP receptor affinity is possible within the orvinol series.

Acknowledgments:
NIDA grants DA020469 (SMH) and DA023281 (LT).

Discovery of novel κ opioid receptor ligands

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Background and Aim: The κ opioid receptor (KOP) plays a significant role in a broad range of physiological functions and there is evidence that receptor blockade might be useful for the treatment of depression, anxiety, addiction, psychosis and eating disorders. At present, few KOP antagonists are available. The search for new active ligands utilizing computational approaches became an important tool in drug discovery. In the absence of direct structural information of the KOP, a systemic study was initiated to develop a pharmacophore model that may serve as a powerful search tool to identify new chemical entities as potential KOP ligands. Herein, we present the application of a computer-aided drug design approach to discover new molecular scaffolds as novel KOP antagonists.
Methods: LigandScout and Catalyst softwares were used to generate and validate a ligand-based pharmacophore model. Virtual screening of different databases was performed and several hits were retrieved. Biological activities of the identified ligands were evaluated in *in vitro* opioid receptor binding and functional assays.
Results: An integrated computational screening strategy has led to the discovery of sewarine, as a novel KOP ligand. This phenolic alkaloid from the plant Rhazyastrictabinds with high selectivity to the KOP and shows antagonist activity towards the KOP. Synthetic phenolic compounds were also identified as novel selective ligands interacting with KOP and displaying agonist or antagonist properties.
Conclusion: This study uncovers a new class of ligands interacting with KOP and sharpens the understanding of ligand-receptor interactions, thus increasing the chance of developing useful clinical agents.

Advances in structure-activity studies of endomorphins

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Background and Aim: Since their discovery, endomorphins (H-Tyr-Pro-Trp-Phe-NH_2, H-Try-Pro-Phe-Phe-NH_2) [Zadina et al., 1997] are in focus as potential therapeutics in pain management. Recent published results, based on the mapping of metabolic pathway of endomorphins led to the application of alicyclic β-amino acids to obtain proteolytically stable, pharmacologically active compounds [Keresztes et al., 2010]. On the basis of our earlier research, dimethyltyrosine (Dmt), 2-aminocyclohexanecarboxylic acid (ACHC), βMePhe and pFPhe were used for substitution in the parent peptides to obtain highly potent and selective MOR ligands. SPPS was used for peptide synthesis.
Results: Radioreceptor assays using rat brain membrane preparation and selective radioligands for MOR and DOR receptors and functional [^{35}S]GTPγS binding assays were done for biological evaluation. In agreement with earlier findings, the derivatives manifested low to high potencies, selectivities and efficacies (all retained agonist properties) depending on the configuration of the incorporated amino acids and their position in the sequence. Multiple structural modifications of endomorphins were investigated. Combined application of Dmt[1], cis-(1S,2R)ACHC[2] and pFPhe[4] in endomorphin-2 resulted in the most potent analogue. NMR and molecular modeling studies of the endomorphin analogues confirmed the predominance of bend structures. It is apparent that bend structures are energetically more favored than random/extended structures for all studied compounds.

Acknowledgments:
This work was supported by the Hungarian OTKA-PD 73081 funding and FP-6 European Normolife grant (LSHC-CT-2006-037733)

A novel family of minimalist opioid peptides deprived of the classic pharmacophores

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Background and Aim: Recent data support the evidence that some molecules can activate opioid receptors (ORs) though they lack of the protonated amine, generally regarded to as the fundamental pharmacophore. Among such unusual ligands, we discovered the agonist c[YpwFG], having a good MOR affinity (Ki 34 nM), that is an effective and potent analgesic for visceral pain when administered peripherally (*ip* ED50 1.25 mg/kg, *sc* ED50 2.7 mg/kg), and retains moderate central analgesic effects (tail-flick, 20 mg/kg).
Methods: Starting from the bioactive structure of c[YpwFG], determined by molecular docking, we designed and synthesized (SPPS or in-solution) cyclic or linear peptides containing the sequence D-Trp-Phe. Their affinities for cloned human MOR, DOR, and KOR were determined by displacement binding assays. The agonism was assessed by the inhibition of forskolin-stimulated cAMP formation in MOR-expressing HEK-293 cells. Conformational analysis was performed by VT-NMR, 2D-gCOSY, 2D-ROESY, and molecular docking by Autodock and Hybrid QM/MM calculations.
Results: Among the cyclopeptides, c[YAwFA] revealed a modest DOR affinity (Ki 170 nM), c[YGwFA] a significant KOR affinity (Ki 29 nM), while c[YGwFG] and c[d(1-NH2)β-AwF] turned out to be MOR-selective ligands with excellent affinity (Ki 3.6 nM, 5.9 nM, respectively), and a (partial) agonist behavior (inhibition of cAMP: IC50 31 nM, and 37 nM). The linear peptides N-Ac-wFG-HN2 and N-Ac-wF-HN2 were MOR agonists with nanomolar receptor affinity (5.6 nM, and 15 nM). Conformational analysis and molecular docking provided insights into ligand-receptor interaction.
Conclusion: Peptides including the D-Trp-Phe pharmacophoric motif constitute atypical MOR agonists characterized by a bioactive conformation having the indolyl NH of D-Trp H-bonded to Asp147 of TMH VI, and a clear inverse type II beta turn centered on D-Trp-Phe.

Acknowledgments:
MIUR (PRIN08), "Fondazione del Monte di Bologna e Ravenna", the Italian Minister for Foreign Affairs (bilateral proj. Italy-Mexico).

Symposium 3

organized by Halina Machelska-Stein

Opioids and immune system

Chaired by Halina Machelska-Stein and Marzia Malcangio

Opioids and immune system

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Background and Aim: Opioid drugs remain the mainstay treatment for acute and chronic pain conditions beside still being common drugs of abuse. *In vivo* administration of morphine induces a decrease of multiple immune parameters, affecting almost all cells of innate and acquired immunity. The immunological effects of opioids are receiving considerable attention because of concerns that opioid-induced changes of the immune system may affect the outcome of surgery or of variety of disease processes, including bacterial and viral infections and cancer. The opioid-induced immunomodulation can therefore have a deep impact on health.

Methods: Our research in the field is focussed mainly on two topics:

1) to understand the mechanism at the basis of morphine induced immunosuppression;

2) to evaluate whether all opioid drugs share the same immunosuppressive properties.

Results: Our results demonstrate that the macrophage is mostly involved in opioid-induced immunosuppression, and a signalling cascade that starting from TLR4 activates NF-κB and ends up in proinflammatory cytokine production represents an intracellular pathways that is a preferential target for morphine. From a clinical point of view, significant differences among opioid drugs are present when considering their ability to modulate the immune function. Not all opiate drugs exert the same immunosuppressive properties. From experimental studies it is emerging that opioid drugs such as tramadol and buprenorphine do not exert a negative impact on the immune system.

Conclusion: The possibility to reach adequate and equivalent pain control choosing either immunosuppressive drugs or drugs without effect on immune responses could represent an important point in opioid therapy.

Analgesic effects of immune cells in neuropathic pain

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Background and Aim: Neuropathic pain results from injury to nerves and can seriously affect a patient's quality of life. Although it has been recognized that neuronal damage can involve inflammation, it is generally assumed that immune cells predominately enhance neuropathic pain. However, recently we showed that leukocytes containing opioid peptides can alleviate pain following neuropathy.
Methods: After chronic constriction injury (CCI) we examined the expression of opioid peptides and receptors by immunofluorescence, quantified leukocytes by flow cytometry and measured nociception using von Frey filaments in wild-type and severe combined immunodeficiency (SCID) mice.
Results: We found opioid receptors in sensory fibers and opioid peptides (beta-endorphin, Met-enkephalin and dynorphin) expressed with corticotropin-releasing factor (CRF) receptors in immune cells in injured nerves. CRF applied at the nerve injury (CCI) site produced antinociception that was reversed by opioid peptide antibodies, opioid receptor antagonists and by intercellular adhesion molecule-1 (ICAM-1) antibody, which attenuated the accumulation of opioid peptide-containing leukocytes at damaged nerves. In SCID mice lacking opioid-containing T lymphocytes, CRF-induced antinociception was substantially diminished and it could be restored with T cell transfer. Furthermore, antinociception following exogenous opioid application at the CCI site was substantially decreased by anti-ICAM-1.
Conclusions: Selective activation of opioid-containing leukocytes promotes endogenous pain control following nerve damage. Moreover, opioid cells appear important for satisfactory exogenous opioid antinociception. Targeting of opioid-containing immune cells might represent a new disease-modifying approach utilizing beneficial effects of neuro-inflammation in painful neuropathies.

Acknowledgments:
Supported by Deutsche Forschungsgemeinschaft (MA 2437/1-4; MA 2437/4-1). No conflict of interest exists.

Glial activation and opioid analgesia in neuropathic pain

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Background and Aim: Development of nerve injury-induced neuropathic pain is accompanied by many changes in biosynthesis of neuropeptides in the spinal cord and DRG. These changes correspond to immune and glial activation that have been shown to be responsible for the development of hyperalgesia and allodynia in neuropathy. The aim of our study was to examine the inhibition of glia activation by minocycline on injury-induced neuropeptide changes and the effectiveness of opioid receptor ligands in a rat model of neuropathic pain.
Methods: The experiments were carried out according to IASP recommendations and were approved by the local Bioethics Committee. The chronic constriction injury (CCI) of the sciatic nerve was performed. Opioid receptor ligands and minocycline were injected intrathecally or intraperitoneally. For behavioral studies the allodynia/hyperalgesia were measured. For biochemical studies the RT-PCR and immunohistochemistry methods were used.
Results: We provide evidence that chronic intraperitoneal administration of minocycline not only diminishes neuropathic pain-related behavior and C1q-positive cell activation in the spinal cord and DRG, but also reduces the injury-enhanced expression of

prodynorphin in the DRG, and in consequence the level of dynorphin, a peptide that is known to exhibit non-opioid pronociceptive activity is increased. Moreover, antiallodynic effects of MOR and KOR agonists (morphine and U50,488H, respectively), but not DOR (DPDPE, deltorphine II), were significantly potentiated in rats with microglia inhibited by repeated injection of minocycline.

Conclusions: We demonstrated that sciatic nerve injury-induced changes in the expression of opioid neuropeptides, parallel to significant microglia, macrophages and leukocytes activation. Our results support the idea that targeting microglial activation represents a novel and clinically promising method for enhancing analgesic effects of opioids in neuropathic pain.

Acknowledgments:
Supported by grants NN405375937 and Demeter 1.1.2. from MNiSzW.

Morphine and Botulinum neurotoxin A: a successful pharmacological combination against pain?

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Background and Aim: The clinical utility of opioids is limited by important side effects, including the development of tolerance during repeated use. A role of NMDA receptors has been suggested in morphine tolerance, as well as in the analgesic action of botulinum neurotoxin serotype A (BoNT/A). The aim of the present research was to verify if BoNT/A can exert a synergistic action with morphine and eventually influence the development of tolerance after the opioid's chronic administration.

Methods: CD1 male mice were intraplantarly injected with BoNT/A (2–15 pg/paw) and systemically with morphine (1–4 mg/kg) and their single or combinatorial effects on formalin-induced inflammatory pain were tested. Moreover, the effects of BoNT/A (15 pg/paw) on the tolerance induced by morphine (20 mg/kg along 8 days) were evaluated. Behavioral responses were correrlated with immunofluorescence staining of glial cells.

Results: BoNT/A and morphine exert a synergistic action on licking response induced by formalin during both early and late phases characterizing the formalin test. Tolerance induced by a chronic administration of morphine is inhibited by a previous intraplantar injection of BoNT/A. BoNT/A results also in a modulatory action on the astrocytes' expression at the spinal cord level.

Conclusion: The present results, showing that BoNT/A potentiates the analgesic effects of morphine and prevents tolerance induced by the chronic treatment of the opiate, could have a tremendous impact in terms of clinical application of BoNT/A as innovative pharmacological treatment for treating pain.

Acknowledgments:
Research grant from Filas Regione Lazio Funds for "Sviluppo della Ricerca sul Cervello".

Symposium 4

organized by Volker Höllt

Molecular mechanism of drugs of abuse

Chairmen: Volker Höllt and Ryszard Przewłocki

Functional selectivity of μ-opioid receptor ligands

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Background and Aim: Agonists of the μ-opioid receptor (MOPr) are the most appropriate therapy for moderate to severe pain, but their clinical use is limited by the development of tolerance and physical dependence. Receptor phosphorylation and subsequent internalization are thought to play a critical role in the development of opioid tolerance and dependence. Therefore, in the present study, we have generated phosphosite-specific antibodies, which allowed us to selectively detect the S363-, T370- and the S375-phosphorylated form of the MOPr.
Results: Using these antibodies, we provide evidence for distinct agonist-selective patterns of MOPr phosphorylation following activation by internalizing or non-internalizing agonists. Moreover, we have recently shown that the agonist-selective activation of phospholipase D2 (PLD2) plays a key role in the induction of MOPr internalization. Therefore, we tested here the functional selectivity of opioids in receptor-mediated PLD2 signaling pathways such as ROS (reactive oxygen species)-synthesis and p38 MAPK-activation. We found that receptor-internalizing agonists (like DAMGO, β-endorphin, Sufentanil, Fentanyl, and Etonitazene) strongly induce ROS-synthesis *via* PLD-dependent pathways, whereas agonists that do not induce MOPr endocytosis and PLD2-activation (like morphine, buprenorphine, hydromorphone, and oxycodone) failed to activate ROS-synthesis in transfected HEK293 cells. Moreover we demonstrate that the opioid-selective activation of PLD2 is a requirement for the activation of p38 MAPK, which is involved in the induction of receptor endocytosis.

Acknowledgments:
This work was supported by the Deutsche Forschungsgemeinschaft (KR1740/10-1) to T.K. and (SCHU924/101) to S.S., by the Land Sachsen-Anhalt from the "Europäischen Fond für regionale Entwicklung" (EFRE 2007-2013) and the Doktor Robert Pfleger-Stiftung.

A global view of drug-induced gene expression alterations in the striatum

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Background and Aim: The biological basis of addiction and other neuropsychiatric disorders is hidden in disturbed signal transmission between neuronal cells in brain regions controlling motivation, mood and emotions. Psychoactive drugs stimulate neuroplasticity and readaptation of the target neural systems. It is believed that these alterations are dependent on changes in genome expression and translation of new proteins.
Methods: To reveal the transcriptional networks activated by different classes of drugs we compared the effects of opioids (morphine, heroin), psychostimulants (cocaine, methamphetamine), antidepressants (imipramine, fluoxetine), anxiolytics (diazepam, buspirone) and antipsychotics (haloperidol, clozapine) on gene expression profile in the mouse striatum. We applied whole-genome microarray profiling to evaluate time-course (1, 2, 4 and 8 h after injection) of transcriptome alterations following acute drug administration.
Results: The present study elucidated all major drug-regulated expression patterns in the brain that are formed by inducible transcriptional networks. The regulation of gene transcription is related to various drug-receptor interactions and pharmacological effects of the drugs. Moreover, we identified novel candidate genes with drug-specific gene expression profiles.
Conclusions: Our results indicate the networks of drug-regulated genes that share common regulatory elements and functional properties.

Acknowledgments:
This work was supported by MSHE grants: POIG DeMeTer 3.1, IUVENTUS Plus and NN405274137. The authors declare no competing financial interests.

Glutamate inputs to catecholamine pathways shape behavioral effects of addictive drugs

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Background and Aim: The persistence of drug-conditioned behaviors is associated with adaptations in glutamate signaling on catecholamine pathways in the mesolimbic system. It was observed that even a single exposure to a drug of abuse leads to long-term strengthening of glutamatergic synapses on dopaminergic neurons of the ventral tegmental area (VTA), and that the changes the VTA subsequently lead to plasticity in the nucleus accumbens.
Methods: In order to elucidate the roles of glutamate signaling on different components of the catecholamine systems, we have generated a Cre/loxP mouse models in which the essential NMDA receptor subunit NR1 is ablated in dopaminergic, noradrenergic or dopaminoceptive neurons.
Results: While none of the mutations affected drug-induced reinforcement as measured in the conditioned place preference (CPP) paradigm, they had specific effects in either psychomotor sensitization or reinstatement of extinguished preference. Thus, ablation of functional NMDA receptors on dopaminergic cells prevented reinstatement of CPP, but did not alter the psychomotor effects. Deletion of NMDA receptors on dopaminoceptive (dopamine D1 receptor expressing) neurons caused motor hypoactivity and diminished reinstatement of CPP, but did not prevent development of sensitization. Finally, ablation of NMDA receptors on noradrenergic cells did not affect reinstatement of CPP, but prevented development of morphine-induced psychomotor sensitization.

Conclusions: Taken together these results dissect the functions of NMDA-receptor dependent signaling in the mesolimbic system, and reveal an interesting complementarity between dopamine and noradrenaline signaling in development of drug-conditioned behaviors.

Acknowledgments:
Support: statutory funds of the Institute of Pharmacology PAS as well as grants N405 143238 from the Polish Ministry of Science and Higher Education and grant POIG.01.01.02-12-004/09 „Depresja–Mechanizmy–Terapia". The author declares no conflicts of interests.

Alterations of the mGluR5, vasopressin and oxytocin receptor systems induced by chronic opioid administration and withdrawal

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Background and Aim: Drug addiction is a chronic relapsing disorder characterised by a transition from occasional, controlled drug use, which is driven by the positive reinforcing properties of the drug, to the compulsive drug taking, which is driven by the emergence of a negative emotional state when access to the drug is prevented. Understanding the neuromolecular mechanisms which are involved in triggering this transition will not only provide us with important information about the neurobiology of drug addiction but also will provide us with novel targets for the treatment of drug addiction and especially for the prevention of relapse. We recently investigated the effect of chronic morphine or acute or chronic withdrawal from morphine administration on the mGluR5 metabotropic glutamate receptor density as well as vasopressin and oxytocin receptor density with the use of quantitative receptor autoradiographic mapping of mouse brains.

Methods: Male C57BL/6J mice were treated with a chronic intermittent escalating dose morphine administration paradigm or withdrawn from morphine for 1 day (acute withdrawal) or 7 days (chronic withdrawal). Quantitative autoradiographic mapping of mGluR5, vasopressin and oxytocin receptors was carried out in brain sections of mice treated with the aforementioned paradigm with the use of [^{3}H]MPEP, [^{3}H]Arginine Vasopressin (AVP) and [^{125}I]OVTA respectively. To discriminate between V1a and V1b receptors, a selective V1b receptor antagonist (SSR149,514) was used in displacement studies.

Results: Chronic morphine administration caused a small but significant overall increase in mGluR5 binding which persisted during acute withdrawal in many brain regions. A dramatic 2-3 fold increase in mGluR5 binding was found in almost all the brain regions of chronically morphine withdrawn mice compared to controls. Chronic morphine administration significantly increased SSR149,514-resistant [^{3}H]AVP binding in the piriform cortex, nucleus accumbens, lateral septum and amygdala, which persisted following acute and protracted withdrawal. Chronic morphine increased oxytocin receptor binding in the olfactory nuclei which persisted following acute withdrawal while long-term withdrawal triggered the increase of oxytocin receptor binding in the piriform cortex, septum, nucleus accumbens, amygdala, ventromedial hypothalamus and periventricular thalamic nucleus. No detectable SSR149,514-sensitive [^{3}H]AVP binding was observed in any of the brain regions analysed.

Discussion: These results indicate a prolonged dysregulation of the mGluR5, vasopressin and oxytocin systems by chronic morphine administration which persists or triggered after long-term withdrawal, suggesting that the mGluR5, vasopressin and oxytocin systems might play an important role in the mechanisms underlying opioid addiction and opioid craving after abstinence. These and other sudies will impact on the future developments of novel relapse prevention pharmacotherapy for opioid addiction. Recent preclinal studies will be discussed in this context.

Acknowledgments:
Funded by EC (GENADDICT:LSHM-CT-2004-005166), RCUK and the British Pharmacology Society

Symposium 5

organized by Vincenzo Di Marzo

Opioid-cannabinoid interactions

Chaired by Vincenzo Di Marzo and Aron Lichtman

A general introduction to the endocannabinoid system and its interactions with opioid receptor signalling

Vincenzo Di Marzo

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The most studied endocannabinoids, anandamide and 2-arachidonoylglycerol, are arachidonic acid-containing, phospholipid-derived mediators produced "on demand" that act mostly by stimulating the activity of the two G-protein-coupled receptors for Δ^9-tetrahydrocannabinol, the cannabinoid receptors of type 1 and 2 (CB_1 and CB_2). This "endocannabinoid signaling system" is emerging as a key player in the control, on the one hand, of synaptic function and, on the other hand, of metabolism, reward and nociception, three physiological functions in which opioid receptor signaling is deeply involved. Both cannabinoid CB_1 receptors and proteins controlling endocannabinoid tissue levels, such as the biosynthesizing enzymes, diacylglycerol lipase-α and NAPE-PLD, and the inactivating enzymes, monoacylglycerol lipase and fatty acid amide hydrolase, are expressed in brain areas controlling metabolism, reward and pain, areas which very often also co-express μ-opioid receptors (MORs). On the other hand, functional interactions at the level of down-stream signaling or of the potential formation of heterodimers, were shown to occur between MORs or μ-opioid receptors and CB_1 receptors. Furthermore, an alternative molecular target for anandamide and the less studied endocannabinoid, *N*-arachidonoyl-dopamine, was identified as the transient receptor potential of vanilloid type-1 (TRPV1) channel, which plays a key role in inflammatory and chronic pain and is also often co-expressed with μ-opioid receptors (MORs) in both spinal and supra-spinal CNS neurons that control nociception. Recent aspects of the cross-talk between CB_1/TRPV1 receptors and MORs in neurons, with emphasis on their role in the framework of pain control, will be discussed in my presentation.

Involvement of the cannabinoid system in the antinociception induced by opioids

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Background and Aim: Interactions between the cannabinoid and the opioid systems are reciprocal for pain modulation. However, the role of the cannabinoid system in the antinociceptive effects of opioids remains uncertain. We aimed at highlighting cannabinoid system involvement in pain modulation during activation of the opioid system.
Methods: Male C57BL/6, cnr1WT, cnr1KO, cnr2WT and cnr2KO mice (25–30 g) received saline or morphine 1 μg in the dorsal surface (subcutaneous; *sc*) of the left hind paw or intrathecal (*it*) saline or morphine 0.1 μg administered 5 min before formalin (n = 6 per group). Inflammation was induced by intradermal injection of 5% formalin (10 μl) into the hind paw. Nociception was assessed behaviourally over the next 60 min. The level (Bmax) of mu opioid receptors (MOPR) for each four genotypes was measured using saturation binding assays on mice spinal cord (n = 3 per group).
Results: In the late phase of the formalin test, *sc* and *it* morphine both produced a significant analgesic effect in C57BL/6 and wild type mice *vs*. their respective controls. The antinociceptive effects of morphine were decreased by 87% (*sc*) and 100% (*it*) in cnr1KO *vs*. cnr1WT mice and by 76% (*sc*) and 90% (*it*) in cnr2KO *vs*. cnr2WT mice. The level of MOPR did not differ between groups.
Conclusions: The loss of analgesic effectiveness of peripheral and spinal morphine in cannabinoid receptors knockout mice reveals the existence of an interaction between the cannabinoid and the opioid systems which is not caused by a decrease in MOPR expression within the different genotypes.

Acknowledgments:
This work was supported by a Vanier Canada Graduate Scholarships from the Canadian Institutes of Health Research (CIHR) 204685 (JD) and a 'Quebec Pain Research Network' (QPRN) grant (PB-LG-JFB). We would also like to thank Dr Beat Lutz for providing cnr1KO mice.

Endocannabinoid catabolic enzyme inhibitors reduce opioid withdrawal responses

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Background and Aim: In 1889, Birch (The Lancet, 133:625) published a case report in which an extract from Cannabis sativa alleviated withdrawal symptoms in an opium addict. Since then, several preclinical studies that the primary psychoactive constituent of this plant, Δ^9-tetrahydrocannabinol (THC) reduced naloxone-precipitated withdrawal in morphine-dependent rodents. Although THC produces the bulk of its pharmacological actions through the activation of CB1 and CB2 cannabinoid receptors, the role of these receptors in reducing opioid withdrawal symptoms remains unknown. The endogenous cannabinoids, N-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonylglycerol (2-AG), activate both cannabinoid receptors, but are rapidly metabolized by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. The objective of the present study was to test whether increasing AEA or

2-AG, *via* inhibition of their respective catabolic enzymes, reduces naloxone-precipitated morphine withdrawal symptoms in *in vivo* and *in vitro* models of opioid dependence.
Method: Mice were implanted subcutaneously with 75 mg morphine pellets and challenged 72 h later with naloxone (1 mg/kg) to precipitate a profound withdrawal syndrome consisting of jumping, paw tremors, head shakes, diarrhea, and weight loss.
Results: THC and the MAGL inhibitor, JZL184 dose-dependently reduced the intensity of most withdrawal measures through the activation of CB1 receptors. The FAAH inhibitor, PF-3845 reduced the intensity of naloxone-precipitated jumps and paw flutters, but not diarrhea or weight loss in morphine-dependent mice. In the final series of experiments, we investigated whether JZL184 or PF-3845 would attenuate naloxone-precipitated contractions in morphine-dependent ilea.
Conclusions: Both enzyme inhibitors attenuated the intensity of naloxone-induced contractions. Taken together, these findings suggest that targeting endocannabinoid catabolic enzymes represents a promising strategy to treat opioid withdrawal.

Regulation of cannabinoid receptors Type 1 and μ opioid receptors by micro RNA

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μ opioid receptors (MOR) mediate the effects of most of the clinically used opioids such as morphine. Most effects of cannabinoids are mediated by either type 1 (CB1) or type 2 cannabinoid receptors. Both, MOR and CB1 are expressed constitutively in many neuronal cells, including human neuroblastoma SH SY5Y cells, and are inducible in T lymphocytes. In the last decade it has been found that small RNA species termed microRNA (μRNA) regulate genes at the posttranscriptional level. In general, μRNA binds specificly to a gene's mRNA resulting in translational repression and gene silencing. We investigated the effects of μRNA on the expression of MOR and CB1 in SH SY5Y cells and in human Jurkat T lymphocytes by assaying two functions typically mediated by these receptors: (I) the phosphorylation of p42/44 MAP kinase induced by morphine and methanandamide and (II) the decrease in the intracellular cAMP content of the cell induced by morphine and methanandamide. Overexpression of μRNA let7A, let7D and mir98 in transiently transfected cells resulted in decreased MOR activity in both cell types, while mir23B had no effect. On the other hand, overexpression of let7D and mir23B resulted in decreased CB1 activity in both cell types, while let7A and mir98 had no effect. In summary, these data demonstrate regulation of MOR and CB1 by μRNA. Since it was demonstrated that, vice versa, drugs of abuse regulate μRNA expression, this may offer novel approaches to the understanding of phenomena such as tolerance and addiction.

Symposium 6

organized by Barbara Przewlocka

Different mechanisms of opioid action

Chaired by Maria Victoria Milanés and Ian Kitchen

Morphine withdrawal regulates phosphorylation of heat shock protein 27 through extracellular-signal-regulated kinase in the heart

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Background and Aim: Heat shock protein 27 (Hsp27) is a well-known stress response protein that becomes phosphorylated through interaction with extracellular signal-regulated kinase (ERK). Different drugs of abuse, such as morphine and/or its withdrawal, induce severe stress situations. In this study, we have investigated phospho-Hsp27 expression during morphine dependence and withdrawal and have evaluated the interaction between Hsp27 and ERK in the rat right ventricle.

Methods: Dependence on morphine was induced by a 7-days *sc* implantation of morphine pellets. Morphine withdrawal was precipitated on day 8 by injection of naloxone (2 mg/kg, *sc*). Phospho-Hsp27 at serine 15 was determined by quantitative blot immunolabelling using a specific antibody.

Results: Naloxone-precipitated morphine withdrawal increased the phosphorylation of Hsp27 at serine 15 30, 60, 90 and 120 min after the injection of the opioid antagonist. However, there were not changes in Hsp27 phosphorylation in the morphine dependent group injected with saline. In addition, pretreatment with SL327, an inhibitor of ERK phosphorylation, decreased the activation (phosphorylation) of Hsp27, suggesting that ERK activation triggers Hsp27 phosphorylation.

Conclusions: The present findings demonstrate that morphine withdrawal is capable of inducing the activation of Hsp27 in the heart and suggest that phosphorylation of Hsp27 is closely linked and also dependent on the ERK pathway.

Acknowledgments:
Supported by Ministerio de Educación y Ciencia (Grant SAF/FEDER 2007-62758), Fundación Séneca. Comunidad Autónoma de la Región de Murcia (Grant 05679/PI/07) and Instituto de Salud Carlos III (RD06/0001/1006).

μ-opioid receptor (MOR) expression and opioid-mediated intracellular signalling are altered in glial cells exposed to TNF-α

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Background and Aim: Glial cells are activated at multiple sites along the pain pathway following peripheral trauma or inflammation, thus inducing production and release of several pro-inflammatory cytokines which act in a autocrine/paracrine fashion, drive pain amplification and maintain chronic pain states like neuropathic pain. Toll-like receptor 4 (TLR4) engagement mediates the initial release of pro-inflammatory cytokines, such as TNF-α, which in turn sustain prolonged glial activation. TLR4-mediated modulation of glial activity as well as opioid-induced glial activation have been extensively investigated; however, mu-opioid receptor (MOR) expression and function in glial cells have been little explored. Aim of this study has been to investigate MOR expression upon exposure of U87-MG human glial cells and rat primary microglia to TNF-α 24 – 72 h), and to evaluate any effect elicited by different opioid agonists on MOR intracellular signalling.

Results: We found that TNF-α determined a significant, concentration- and time-dependent up-regulation of MOR mRNA and protein levels; as expected, morphine and DAMGO induced ERK phosphorilation in untreated glial cells, but not in TNE-α-pre-treated glial cells. Our findings show that MOR expression and function in glial cells are significantly altered following prolonged exposure to TNF-α, as MOR-mediated activation of MAPK pathway in glial cells seems to be desensitized after TNF-α treatment, albeit the significant up-regulation of MOR expression.
Conclusions: These results suggest that MOR may play a relevant role in glial cells which is altered by prolonged exposure to TNF-α: any influence on glial modulation of neuronal functions and opioid effectiveness in chronic pain states needs further investigations.

Peripheral and central analgesic effects of novel opioid peptides

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Background: Malignant, long-lasting pain is an imminent component in patients with advanced cancer, which decreases the quality of life of patients. Novel opioid peptide analogs offer new possibilities in cancer pain relief *via* binding to peripheral opioid receptors as well as reducing cancer cell proliferation. Analgesia produced by the stimulation of peripheral opioid receptors results in a reduction of undesirable centrally-mediated side effects and enables long-term administration.

Methods: B16T0 melanoma cells were inoculated *sc* into the left hindpaw footpad of male C57BL6 mice. Nineteen days after cell implantation animals were injected with biphalin, biphalin analog or morphine. Pain thresholds were assessed in the plantar and tail flick tests.
Results: Tumor growth resulted in an onset of pain behavior in the tumor-bearing hindpaw Analgesia produced by biphalin in the tumor bearing paw was greater than after morphine injection in the plantar test.

Conclusions: The difference in effectiveness between biphalin and morphine in cancer pain may result from differential BBB of both drugs. Biphalin produces stronger peripherally-mediated analgesia than morphine due to a slower uptake into the CNS. Also the change in the specific milieu in pathologically changed tissue may affect opioid receptor expression and binding properties in the periphery.

Acknowledgment:
6FP STREP Normolife grant (LSHC-CT-2006-037733)

Homer proteins regulate the heroin-induced rewarding effect under neuropathic pain

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Background and Aim: Group1 metabotropic glutamate receptors and Homer proteins regulate glutamate transmission modulating behavioral sensitivity of chronic pain and drugs of abuse. Using a combination of behavioral genetics and immunoblotting approaches we delineated the relative roles played by different Homer isoforms, including their interactions with mGluR5, in the development of neuropathic pain and consequent effects on heroin-induced place-conditioning (CPP).
Method: *Homer1a*, *Homer1* and *DOH1/2* knock-out (KO) and transgenic (Tg) mice with reduced mGluR-Homer binding (*mGluR5*^{F1128R}, *mGluR5*$^{T1123A/S1126A}$) were subjected to sciatic nerve ligation and tested for pain symptoms and heroin-induced CPP. Homer protein levels were modulated by intra-accumbens virus-mediated gene transfer of *Homer1c* cDNA or siRNA.
Results: Injury elevated accumbens Homer1c level that was accompanied by increased expression of mGluR5 and NR2a. Compared to uninjured WT controls, nerve injury increased pain symptoms in WT mice. Relative to WTs, *Homer1a* KO and all Tgs exhibited increased allodynia. Heroin elicited robust CPP in uninjured WT mice and conditioned place-aversion (CPA) in neuropathic WTs. Interestingly, with the exception of the *mGluR5*^{F1128R}, all uninjured mutant lines exhibited heroin CPP, but neuropathic mice failed to develop heroin CPA. Additionally, AAV-Homer1c produced, while shRNA-Homer1c prevented against, heroin CPA in neuropathic mice.
Conclusion: These indicate that different Homer isoforms appear to play distinct roles in regulating the processing of chronic pain, which may have relevance to our understanding of its etiology and treatment. Moreover, accumbens Homer proteins and Homer-mGluR5 interactions are important for the development of opioid-dependent neuroplasticity of relevance to opioid addiction.

Acknowledgment:
Supported by UCSB funds to KKS.

Metabotropic mGluR5- and adenosine A_{2A}-receptor interactions in opioid addiction

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Background and Aim: Substantial evidence shows that mGluR5 receptors play an important role in opioid addiction and chronic morphine treatment and long-term withdrawal from that treatment up-regulates mGluR5 binding in brains of mice. Functional interactions between mGluR5 receptors and striatal adenosine A_{2A}-D_2 dopamine receptor heterodimers have been implicated and are hypothesised to play important roles in opioid addiction.

Method: To further investigate mGluR5-A_{2A} receptor interactions in the brain, we carried out quantitative autoradiographic mapping of the mGluR5 receptor ([^{3}H]MPEP) in brains of naive male CD-1 (8–12 week) wild-type (WT) and adenosine A_{2A}-receptor knockout mice (KO). To mimic the human pattern of opioid abuse, an age-matched treatment group were treated with a chronic intermittent saline or an escalating dose morphine protocol (20–100 mg/kg, *ip* twice-daily, 8 days). Horizontal locomotor activity was recorded daily.

Results: In naive mice, two-way ANOVA showed a significant genotype effect with no significant genotype x region interaction effect. A significant decrease (~50%) in [^{3}H]MPEP binding was observed solely in ventral striatal areas of KO mice *vs*. WT. Three-way repeated measures ANOVA showed significant genotype x time x treatment interaction on post-treatment locomotor activity at higher (80–100 mg/kg) but not lower doses (20–40 mg/kg) of morphine. Diminished morphine-stimulated locomotor response was observed in KO *vs*. WT mice 60–120 minutes post-injection on Day 7.

Conclusion: This data clearly adds to the evidence for the presence of an mGluR5-A_{2A} receptor interaction in the ventral striatum which might be involved in opioid addiction; whether mGluR5-A_{2A} interactions are involved in behavioural effects of opioids remains to be determined.