

# Symposia Presentations

## Monday 14 July

### Nitric oxide-oxidation and nitrosative stresses-PARP pathway (09.30–12.30)

#### S1.1

##### **Nitric oxide, nitrosative stress in the pathogenesis of diabetic complication** C Szabo<sup>a,b</sup> <sup>a</sup>University of Medicine and Dentistry of New Jersey, USA; <sup>b</sup>Semmelweis University, Hungary

Activation of poly(ADP-ribose) polymerase (PARP) plays a role in the pathogenesis of endothelial injury that underlies the etiology of various diabetic complications (vasculopathy, cardiomyopathy, retinopathy, neuropathy), which develop on the basis of chronically elevated circulating glucose levels in diabetes. Both during the pathogenesis of diabetes and during the pathogenesis of diabetic complications, free radical and oxidant production leads to DNA strand-breakage which activates the nuclear enzyme PARP and initiates an energy consuming, inefficient cellular metabolic cycle with transfer of the ADP-ribosyl moiety of NAD<sup>+</sup> to protein acceptors leading to cellular dysfunction. One of the key oxidant species that plays a role in this process is peroxynitrite (a reactive oxidant formed from the reaction of NO and superoxide). PARP also promotes the activation of various pro-inflammatory signal transduction pathways. During the last two decades, a growing number of experimental studies demonstrated the beneficial effects PARP inhibition in various models of diabetes and diabetic complications. The current lecture will provide an overview of the experimental evidence implicating PARP as a causative factor in the pathogenesis of diabetes and diabetic complications *in vitro* and *in vivo*, will show some of the key molecular mechanisms whereby PARP regulates multiple intracellular pathways relevant for the pathogenesis of diabetic complications, and will also present new data comparing the relationship between PARP activation and endothelial dysfunction in a rat model of well-insulin-controlled vs. poorly controlled diabetes and its vascular complications.

#### S1.2

##### **Modulation of NO production by tyrosine phosphatase inhibition in cardiovascular diseases**

V Richard, M Vercauteren, E Gomez, C Thuillez *Inserm U644, France*

Protection of vascular endothelium, and particularly maintenance of NO production, represents a major therapeutic goal in the context of prevention and treatment of cardiovascular diseases. We made the hypothesis that an increased phosphorylation of tyrosine residues, using pharmacological inhibitors of the dephosphorylating enzyme tyrosine phosphatases (PTP), might correct impaired NO production in disease states. This is based on the knowledge that physiological activation of eNOS involves a series of tyrosine phosphorylations, that lead to downstream serine phosphorylation of eNOS.

In support of this hypothesis, we showed that several structurally unrelated selective inhibitors of one particular PTP: PTP1B, acutely restored normal endothelial function (*ex vivo* flow-mediated vasodilatation in isolated small peripheral resistance arteries) and also corrected the altered eNOS phosphorylation in these arteries. Our initial demonstration came from studies in heart failure, but was recently extended to other diseases, especially diabetes and obesity. Results with pharmacological inhibitors of PTP1B have also been confirmed in PTP1B KO mice. PTP1B is mostly known for its negative influence on insulin sensitivity, and PTP1B inhibitors are currently tested as potential treatments of insulin resistance. Thus, our data point toward a novel approach for vascular (endothelial) protection based on PTP1B inhibition, but also suggest that, in many disease states, the beneficial effects of PTP1B inhibition may result from at least two mechanisms: the 'classical' increased insulin/leptin sensitivity, and the newly discovered endothelial protection, occurring through direct restoration of NO production.

#### S1.3

##### **Nitrosative stress and circulatory shock**

S Cuzzocrea *University of Messina, Italy*

A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially, superoxide and hydroxyl radical) and high energy oxidants (such as peroxynitrite) as mediators of shock and ischemia/reperfusion injury. ROS can initiate a wide range of toxic oxidative reactions. These include initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATP-ase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins. All these toxicities are likely to play a role in the pathophysiology of shock and ischemia and reperfusion. Moreover, various studies have clearly showed that treatment with either peroxynitrite decomposition catalysts, which selectively inhibit peroxynitrite, or with SODm's, which selectively mimic the catalytic activity of the human superoxide dismutase (SOD) enzymes, have been shown to prevent *in vivo* the delayed vascular decompensation and the cellular energetic failure associated with shock and ischemia/reperfusion injury.

#### S1.4

##### **Nitric oxide, nitrosative stress and PARP cascade in acute brain injuries**

V Besson *Paris Descartes University, France*

The deleterious pathophysiological cascade induced after acute brain injuries, such as stroke and traumatic brain injury (TBI), is initiated by an excitotoxic process triggered by excessive glutamate release. Activation of the glutamatergic NMDA

receptor, by increasing calcium influx, activates nitric oxide synthases leading to a toxic production of nitric oxide (NO). Moreover, after cerebral ischemia and TBI, free radicals are highly produced and contribute to a deleterious oxidative stress. Evidence has shown that the major toxic effect of NO comes from its combination with superoxide radical leading to peroxynitrite formation, a highly reactive and oxidant compound. Indeed peroxynitrite mediates nitrosative stress and is a potent inducer of cell death through its reaction with lipids, proteins, and DNA. Particularly, DNA damage caused by both oxidative and nitrosative stress, results in activation of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme implicated in DNA repair. In response to excessive DNA damage, PARP activation leads to energetic depletion and finally to cell death. During the last 10 years, accumulating data have showed that inactivation of PARP, either pharmacologically or using PARP null mice, induces neuroprotection in experimental models of brain injuries. In addition, inhibition of PARP mediates anti-inflammatory and anti-apoptotic effects. Thus acute brain injuries generating NO, oxidative and nitrosative stresses display PARP activation contributing to post-ischemic and post-traumatic neurological, histological, and behavioural consequences. Ten years of experimental research provided a rational basis for the development of inhibitors of PARP suggesting their pharmacological use for treatment of stroke and TBI.

#### S1.C001

##### **Nitric oxide-dependent oxidative changes in skeletal muscles from rats submitted to intestinal ischemia and reperfusion.**

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Contractile dysfunction (CD) of skeletal muscles is a common finding in the systemic inflammatory response syndrome (SIRS) caused by bacteria infection (i.e., sepsis; Krause *et al.*, 1998). However, there are no reports on the occurrence of CD in SIRS secondary to intestinal ischemia and reperfusion (IIR). We thus decided to analyse some inflammatory markers in skeletal muscles from rats under IIR. Male Wistar rats (250–300 g) were anesthetized with ketamine (80 mg/kg) and xylazine (1.6 mg/kg). After a medial abdominal laparotomy, the superior mesenteric artery was clamp-occluded during 45 min and followed by a 2 or 6 h-reperfusion period. Samples of both diaphragm and *tibialis anterior* muscles were collected for analysis of nitric oxide synthase (NOS) isoforms, superoxide dismutase (SOD), myeloperoxidase activity (MPO) and nitrotyrosine-containing proteins (NT). Significantly increased MPO (215%) and Ca-dependent NOS (262%) activities and 3-NT occurred in diaphragms from IIR animals after 2 h reperfusion in comparison to Sham rats, as well as reduced nNOS (78% for mRNA and 59% for protein expression), protein eNOS (52%) and SOD-1 (22%). Increased Ca-independent NOS activity (817%) and mRNA for both iNOS (213%) and nNOS (209%) were found in diaphragms after 6 h reperfusion, but the expressions of either SOD, nNOS or eNOS were unaltered. In addition, lowered Ca-dependent NOS activity (61%) and higher iNOS mRNA (512%) were measured in *tibialis anterior* samples from IIR animals after 2 h reperfusion. However, after 6 h reperfusion, decreased expression of eNOS mRNA (47%) and nNOS protein (32%) were observed, in addition to higher 3-NT. *Tibialis anterior* muscle MPO activity remained unchanged at the studied time-points. The results show that an oxidative response occurs in both diaphragm and *tibialis anterior* muscles from rats submitted to IIR. Whether this time- and tissue-dependent response involving NOS isoforms and protein modifications affects the muscle contractility, is currently under investigation.

##### **Reference:**

Krause *et al.* Am J Respir Crit Care Med. 1998; 157: 1277–1282.

#### S1.C002

##### **Pharmacological induction of heme oxygenase-1 protects hippocampal interneurons and inhibits inducible nitric oxide synthase following cerebral ischemia reperfusion injury**

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The reduction or the loss of blood flow to the brain results in neuronal damage, due to oxygen and nutrient deprivation, and induces a cascade of secondary mechanisms including oxidative stress leading to a wide range of cellular injuries which can also cause cellular death. A biochemical pathway involved in this process is the formation of peroxynitrite, resulting from the increased availability of both superoxide and NO, following the enhanced expression of the inducible nitric oxide synthase (iNOS) triggered by an ischemic insult. This study evaluated the effect of pharmacological regulation of heme metabolism in different brain regions in a rat model of ischemia/ reperfusion (I/R) injury. In male Wistar rats (180–200 g weight) we firstly evaluated the possibility of regulating heme-oxygenase-1 (HO-1), a heme metabolism limiting enzyme, in the brain by SnCl<sub>2</sub>. Following I/R rats were sacrificed and brains were analyzed for both HO enzymatic activity, protein content and immunohistochemistry (IH) of HO-1. Heme content was determined by HPLC analysis. SnCl<sub>2</sub> resulted in an increase in HO-1 protein expression which was also followed by increased activity in various brain regions. Additionally, IH showed that HO-1 induction was more evident in neuronal cells of the hippocampus (HP), cerebellum, hypothalamus and brainstem. The increased HO-1 expression was also associated with a reduction in heme levels. Furthermore, I/R strongly increased

inducible nitric oxide synthase (iNOS) expression throughout the brain, but principally in the HP, where a loss of interneurons and astrogliosis also occurred. Pre-treatment with SnCl<sub>2</sub> decreased both iNOS expression in I/R rats and loss of HP interneurons. The beneficial effects of SnCl<sub>2</sub> were prevented by concomitant treatment with SnMP, a strong inhibitor of HO activity. The effects of SnCl<sub>2</sub> were

further investigated in passive avoidance test, where rats showed an improvement in short term memory when treated with SnCl<sub>2</sub> ( $P < 0.01$ ). Our results showed that, following SnCl<sub>2</sub> administration, HO-1 is mainly expressed in neuronal cells of specific brain areas and that it can modulate heme metabolism and iNOS expression.

# Functional selectivity of GPCRs (09.30–12.30)

## S2.1

### Use of fluorescent probes to determine agonist-induced conformational changes in the $\beta$ -adrenoceptor

S Hill University of Nottingham, UK

## S2.2

### The multi-dimensional nature of efficacy

P Strange University of Reading, UK

Drug action at receptors depends on the binding of the drug to a receptor, characterised by affinity, and effects on signalling systems associated with the receptor, exhibited as efficacy. Agonists are compounds with positive efficacy. Agonist efficacy may be quantitated in several ways including the maximal agonist effect ( $E_{max}$ ), the ratio of agonist dissociation constant to agonist potency ( $K/EC50$ ) or the product of the two ( $E_{max}K/EC50$ ). The latter parameter has some favourable properties in terms of setting up scales of efficacy. It has become apparent that efficacy is not a fixed parameter but may be dependent on the assay system used. Thus if a range of compounds is tested in two assays linked to one receptor the pharmacological profile may not be the same. Efficacy may, therefore, be pathway dependent and we can describe efficacy as a multi-dimensional parameter. In this presentation I shall give some examples of pathway dependent efficacy and suggest some new ways of quantitating this pathway dependence.

## S2.3

### New parameters for functional pharmacology – from specific-drug pharmacology to drug-specific pharmacology?

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The ways in which drugs can interact with G-protein-coupled receptors (GPCRs) at the orthosteric site can be multiple (Jacoby *et al.*, 1999; Urban *et al.*, 2007), and some agents may interact with allosteric sites. Receptors may exist as homo- or hetero-dimers, with the potential for distinct pharmacology, and NC-IUPHAR has proposed stringent criteria for recognition of heterodimers. Furthermore, some drugs have the capacity for activating different signalling cascades from a single receptor (Urban *et al.*, 2007) indicating unique pharmacology. Tissue-selective accessory proteins, and tissue-selective (and state-selective) corepressors and coactivators markedly change the conformation of the orthosteric site in nuclear receptors, allowing distinct signalling profiles for individual drugs (Germain *et al.*, 2006). Thus although specific drugs were the main tool by which receptors were (and still can be, if appropriate precautions are taken) classified, drugs may also have distinct pharmacology at certain receptors depending on their chemical structure, showing drug-specific pharmacology. This has immense implications in drug screening, and development – which also entails much testing, and selection, in pathophysiological situations.

#### References:

- Jacoby E. *et al.* QSAR. 1999; 18: 561–572.  
Germain P. Pharm. Rev. 2006; 58: 685–704.  
Urban JD *et al.* J Pharm Exp Ther. 2007; 320: 1–13.

## S2.4

### Functional selectivity in GPCR desensitization

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We have investigated the molecular mechanisms underlying agonist-induced desensitization of  $\mu$ -opioid receptors (MOPr), in both heterologous expression systems and mature mammalian neurones. We find that different agonists trigger different molecular mechanisms of desensitisation, with DAMGO inducing desensitisation by the classical GRK/arrestin mechanism, whilst morphine induces desensitisation by a mechanism that requires, at least in part, PKC activation in the cell. We are currently attempting to identify the molecular mechanism for PKC involvement in morphine-induced desensitisation; recombinant MOPrs appear to be basally phosphorylated by PKC in HEK293 cells and using *in vitro* phosphorylation of GST-fusion proteins of MOPr, we have identified Ser261 in the third intracellular loop and Ser363 in the COOH-terminal tail of MOPr as sites of PKC phosphorylation. Basal PKC phosphorylation of these residues in the intact MOPr may underlie the ability of morphine to induce desensitisation. These results suggest that agonists such as DAMGO and morphine can induce different active conformations of MOPr (functional selectivity), which in turn drives desensitization by different mechanisms (morphine by PKC, and DAMGO by GRK/arrestin). Significantly, morphine has lower efficacy at MOPr compared with DAMGO, leading to the hypothesis that the different mechanisms of desensitisation we observe may be related to the efficacy of the agonist. To investigate this, we are beginning to

analyse the mechanisms of desensitisation of a range of MOPr agonists, to correlate this with the relative efficacies of the agonists, as well as their abilities to induce MOPr desensitisation and promote arrestin binding.

## S2.C001

### Functional selectivity at the human sphingosine-1-phosphate type 3 receptor

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The sphingosine-1-phosphate type 3 (S1P<sub>3</sub>) receptor is a G-protein coupled receptor which plays an important role in cardiovascular and pulmonary system. A detailed understanding of the S1P<sub>3</sub> signalling pathways is lacking due to the limited availability of S1P<sub>3</sub> selective ligands but some new pharmacological tools targeting S1P<sub>3</sub> receptors have become available recently. However, the pharmacological characterization of these compounds was based on a single functional read-out system (<sup>35</sup>S-GTP $\gamma$ S binding) only, whereas S1P<sub>3</sub> receptors can activate G<sub>i</sub> as well as G<sub>q</sub> and G<sub>12/13</sub>-mediated signal transduction pathways. In this study we, therefore, compared the pharmacological properties of the endogenous agonist S1P with those of VPC23019, VPC23153, VPC24191, FTY720-P on different human S1P<sub>3</sub> receptor-mediated functional responses. In CHO-FlpIn cells expressing the human S1P<sub>3</sub> receptor all compounds tested inhibited forskolin-induced cAMP accumulation, increased intracellular calcium concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) and induced S1P<sub>3</sub> receptor internalization but with different potencies and efficacies. S1P was the most potent and efficacious compound in all assays. FTY720-P was significantly less potent than S1P in all assays but more potent than the VPC compounds. Regarding the efficacy, all compounds except VPC23153, behaved as full agonists in the cAMP accumulation assay. Interestingly, in the calcium assay only S1P displayed full agonistic behaviour whereas FTY720-P, VPC23019 and VPC24191 behaved as partial agonists and VPC23153 even as a weak partial agonist. Surprisingly, the S1P-induced effects on [Ca<sup>2+</sup>]<sub>i</sub> seemed to be mediated via a different pathway than the FTY720-P and VPC-induced calcium effects. The G<sub>i</sub> inactivator pertussis toxin hardly affected S1P-induced responses but inhibited those to the other test compounds by more than 50%. We conclude that the endogenous agonist S1P differentially activates the human S1P<sub>3</sub> receptor compared to the other S1P<sub>3</sub> ligands. S1P seems to activate both G<sub>i</sub> and G<sub>q</sub>-coupled pathways, whereas FTY720-P and the VPC compounds predominantly activate G<sub>i</sub>-coupled pathways. This study thus indicates the presence of functional selectivity at the human S1P<sub>3</sub> receptor.

## S2.C002

### Ability of beta-2 adrenoceptor ligands to selectively induce receptor mediated cAMP formation and ERK phosphorylation

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It is now well established that a single type of receptor can couple to multiple signalling partners, which raised the interesting possibility that the signalling ability of a receptor through such partners might depend on the identity of the receptor ligands (termed functional selectivity). In the present study, we investigated signalling abilities of human beta-2 adrenoceptors through adenylate cyclase (mediated by G<sub>s</sub>) and ERK1/2 phosphorylation (mediated by G<sub>s</sub>, G<sub>i</sub> and b-arrestin) depending on the identity of the receptor ligand. As a model system we used HEK 293 or 2B2 cell clones (the latter of which does not express G<sub>s</sub> endogenously) that overexpress beta-2-AR permanently, and we measured cAMP accumulation and time dependent ERK phosphorylation in the presence of ligands having a broad spectrum of efficacy (adrenaline, alprenolol, cimaterol, clenbuterol, cyanopindolol, dobutamine, ICI-118551, ICI-89406, isoproterenol, pindolol, procaterol, pronethalol, propranolol, sotalol, terbutaline, timolol). We found that (i) there was an overall correlation between ligand efficacies for cAMP accumulation and ERK phosphorylation, however (ii) cimaterol, clenbuterol and procaterol which were strong agonists in terms of cAMP response, did not induce ERK phosphorylation, (iii) the time course of ligand-induced ERK phosphorylation was generally similar among ERK phosphorylation-competent ligands (i.e. a transient ERK phosphorylation that peaked at 5 min), with ICI-89406 and cimaterol being outliers, (iv) forskolin also induced ERK phosphorylation with a similar time course, suggesting that elevated cAMP alone could lead to the phosphorylation. The latter observation, together with the one mentioned in point-2 suggests that the actual cause of being an outlier might go beyond a simple mechanism of functional selectivity. Indeed, clenbuterol inhibited forskolin-induced ERK phosphorylation as expected, which indicates the presence of a clenbuterol-induced inhibitory signal for ERK phosphorylation that might also inhibit its own ability to activate ERK pathway. Hence, the absolute independence of different pathways seems to be essential for proper assessment of functional selectivity

# Reducing QT liability and proarrhythmic risk in drug discovery and development (09.30–12.30)

## S3.1

### Non-clinical strategies to address QT liability and proarrhythmic risk

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Since the primary molecular mechanism for drug-induced QT prolongation is inhibition of IKr, strategies to identify risk have focused on assays for the channel conducting IKr: hERG. Both electrophysiological and less direct methods to assess compound potency at hERG have evolved. Although these assays have different strengths and weaknesses, a common and essential feature is throughputs high enough to keep pace with the synthesis-screening cycle that is central to early drug discovery. There is genetic and pharmacological evidence suggesting that key compounds should also be tested in assays for other molecular mechanisms (e.g. inhibition of hERG trafficking or IKs). Since even a comprehensive *in vitro* strategy cannot replicate complicating factors seen only *in vivo*, QT data from an animal model are required to factor in: all possible mechanisms for the parent and metabolites; unexpected effects resulting from compound deposition in cardiac tissue and changes in autonomic tone. To determine whether a compound should be progressed to clinical development, an integrated risk assessment of all *in vitro* and *in vivo* QT-related data, the clinical indication, plus an estimate of the plasma concentration expected to give efficacy, is required. Since QT prolongation is not a perfect biomarker for the actual safety concern (Torsades de Pointes (TdP)), additional non-clinical models have been developed aiming to predict TdP risk. However, data from these models may only be useful to build internal confidence for the substantial additional investment needed to progress a QT-prolonging drug to market.

## S3.2

### The thorough QT study: a review three years after its implementation

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The ICH E14 guidance on how to clinically assess a new drug's QT liability was adopted in May 2005. A centre piece of the guidance was the establishment of one single trial, the 'thorough QT study', intended to definitively identify drugs, which may cause QT prolongation, proarrhythmias and sudden death in susceptible patients. Initially perceived as a great challenge, this study has rapidly become a standard component of all development programs. The study is normally conducted in healthy volunteers, includes both a positive and a negative (placebo) control and is stringently powered to exclude an effect on the QTc interval exceeding 10 ms. The E14 guidance was not very prescriptive and allowed sponsors and service providers to explore new methodologies. This has allowed for a rapid development of new methods, such as automated algorithms for QT measurements, during the first years after the guidance's implementation. Regulators have worked in close collaboration with pharmaceutical industry to set standards for the design and conduct of the 'thorough QT study', which therefore has evolved as a key component of cardiac safety assessment of new drugs. The presentation summarizes the requirements on the 'thorough QT study' and discusses the further development of this trial since the implementation of the ICH E14 guidance.

## S3.3

### Drug-induced QT shortening – is it an issue?

R Shah Pharmaceutical Consultant, UK

New drugs are now routinely evaluated for their potential to prolong QT interval. Our understanding of and concerns regarding drug-induced prolongation of QT interval have evolved gradually, beginning with recognition of potentially fatal ventricular tachyarrhythmias associated with congenital long QT syndromes followed by an ever increasing number of drugs that also prolong QT interval and induce equally malignant proarrhythmias. Since the first description of idiopathic short QT interval as a new clinical syndrome, a number of genetically heterogeneous variant forms of short QT interval have been described, each variously associated with syncope, tachyarrhythmias or sudden death. Additionally, there is an over-representation of short QT interval values among patients with idiopathic ventricular fibrillation. Not surprisingly, the potential of drugs to shorten QT interval is emerging as another issue of concern. These concerns are heightened by drugs such as pinacidil and levromakalim that shorten action potential duration and are proarrhythmic in non-clinical models. A number of small molecules have recently been identified that significantly shorten action potential duration. Whether or not, following a normal QT interval at baseline, drugs can induce shortening of QT interval such that it is proarrhythmic remains to be seen. Nevertheless, since proarrhythmic safety of drugs is a matter of great concern, arrhythmias associated with congenital short QT syndromes have important implications for the development of new drugs that are found to accelerate cardiac repolarization, thereby shortening the action potential duration or the QT interval. Such drugs will almost certainly attract close regulatory scrutiny.

## S3.4

### Integrated risk assessment and predictive value to humans of non-clinical repolarisation assays

R Wallis Pfizer Global Research & Development, UK

The potential for drugs to be associated with the life threatening arrhythmia, Torsades de Pointes (TdP), continues to be an area of regulatory, academic and industrial concern. Despite being an imperfect biomarker, prolongation of the QTc interval of the surface ECG is used to assess the risk of a drug being associated with

TdP such that a thorough examination of drug effects on the QTc interval is required for all new chemical entities. Given the importance of understanding the potential for drugs to prolong the QTc interval most pharmaceutical companies are testing for this liability early in the drug discovery process. This study investigates the correlation between the non-clinical and clinical assays for QTc prolongation. The non-clinical data for 19 compounds has been compared with the outcome in human clinical QTc studies. All clinical studies were designed to detect a 7–10 ms change in QTc. A positive effect in the non-clinical assays were defined as 10% inhibition of hERG channel activity, 10% prolongation of action potential duration in the canine Purkinje fibre assay and 10 ms prolongation of QTc *in vivo* (primarily conscious Beagle dogs). 11 of the 19 compounds were positive in the human QT study. Based on the outcome of the non-clinical assays the sensitivity (true positives divided by the sum of the true positives and false negatives) and specificity (true negatives divided by the sum of the true negatives and false positives) of each assay and an integrated assessment was determined (sensitivity, specificity): hERG (0.82, 0.75), Purkinje (0.20, 1.00), *in vivo* (0.83, 0.86) and integrated assessment (0.90, 0.88). Based on this data set the non-clinical assays accurately predict the outcome of the human QTc study 90% of the time. One compound was positive in the human study that was not detected pre-clinically and clearly prolongs QTc through a non-hERG mechanism of action. This compound is the subject of further studies in order to further refine the understanding of the relationship between non-clinical and clinical QTc assays.

## S3.C001

### Value of non-clinical QT-related assays to support drug discovery, development, and regulatory approval: case studies

J-P Valentin AstraZeneca, Safety Assessment, Mereside, Alderley Park, Macclesfield, Cheshire, UK

Non-clinical QT-related assays aligned to the drug discovery and development phases are used in several ways. During the early discovery phases assays are used for hazard identification and wherever possible for hazard elimination. The data generated enable to (i) establish structure activity relationship and therefore influence the medicinal chemistry design and provide tools for effective decision-making (e.g., hERG potency), (ii) solve problems earlier, or (iii) provide reassurance to progress. For compounds progressing into non-clinical development, the 'core battery' QT-related data enable to build an integrated risk assessment used to (i) fulfil regulatory requirements, (ii) influence Phase I design, and (iii) contribute to the clinical plans. Once a compound progresses into clinical development, QT-related data can be applied in the context of risk management and risk mitigation. The data can be used to: (i) investigate discrepancies that may have emerged within and/or between non-clinical and clinical data, (ii) understand the mechanism of an undesirable pharmacodynamic effect, (iii) provide reassurance for progression into multiple dosing in humans and/or large scale clinical trials, (iv) assess drug-drug interactions, (v) identify novel clinical biomarker and ultimately (vi) support regulatory approval. Based on emerging data, the integrated risk assessment is reviewed and the benefit-risk for compound progression re-assessed. Project examples will be provided to illustrate the impact of non-clinical data to support compound progression throughout the drug discovery and development phases, and regulatory approval.

## S3.C002

### Artificial neural network for automatic detection of arrhythmia from electrocardiogram

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Numerous heart diseases can be detected by means of analyzing electrocardiograms (ECGs). Due to the large number of patients in intensive care units and the need for continuous observation of them, several methods for automated arrhythmia detection have been developed in the past few decades in an attempt to simplify the monitoring task. Recently, artificial neural network (ANN) models become powerful forms concurrent to statistical ones for ECG signal classification. In the present study, the proposed ECG processing cascade has two main stages: (i) feature extraction from the QRST zone of ECG signals; and (ii) pattern classification for heart diseases diagnosis using NN. The features are used to classify among three cardiac cases: normal sinus rhythm (NSR) case, premature ventricular contraction (PVC) case, and atrial premature contraction (APC) case. These features were used as the input for a feed forward back propagation NN to classify the ECG signal. The NN was designed using five nodes at the input layer, one hidden layer with four nodes and three nodes at the output layer. The NN system was trained using 95 ECG samples (NSR: 45, PVC: 25, APC: 25). The system was tested by 45 ECG samples (NSR: 15, PVC: 15, APC: 15). The prediction performance of the system was evaluated by measuring the false rate. The false rate for training and test phases for NSR case was 4.65% and 20%, respectively. Therefore, at least 75% prediction accuracy for NSR was obtained in both phases. For PVC case the false rate for training and test phases was 0% and 25%, respectively. Therefore, also at least 75% prediction accuracy for PVC was obtained in both phases. The false rate for training and test phases for APC case was 0% and 7.14%, respectively. Therefore, at least 93% prediction accuracy for APC was obtained in both phases. The classifier acquires arrhythmia properties from the underlying dynamics of the system, even when the dataset includes incomplete information, such as missing feature values and unclassified classes. In conclusion, this approach is potentially useful for generating a pattern recognition model based on given {input, output} sets to classify future input sets for detection of arrhythmia

# Calcium-activated K<sup>+</sup> channels and endothelial cell signalling – therapeutic target? (09.30–12.30)

## S4.1 Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels and release of EDHF

G Edwards *University of Manchester, UK*

The vascular endothelium plays an important role in controlling vessel tone. In addition to releasing the vasorelaxants prostacyclin and nitric oxide, endothelium-dependent myocyte hyperpolarization (previously attributed to a 'factor', EDHF) also dilates small arteries. EDHF as a single factor does not exist. Rather, agonist-induced endothelial cell activation increases [Ca<sup>2+</sup>]<sub>i</sub> which stimulates the opening of two types of Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel, SKCa and IKCa. Efflux of K<sup>+</sup> ions from these endothelial channels and the resultant hyperpolarization induces myocyte hyperpolarization (and relaxation) by two distinct pathways. One involves the electrotonic transfer of endothelial cell hyperpolarization to the myocytes via gap junctions. The other involves the K<sup>+</sup> clouds originating from the endothelium and which form in the myo-endothelial space. The resulting increase in [K<sup>+</sup>]<sub>o</sub> (i) stimulates types 2 and/or 3 Na<sup>+</sup>/K<sup>+</sup>-ATPases and (ii) partially relieves the block of inwardly-rectifying K<sup>+</sup> channels (Kir). Both these actions hyperpolarize the myocytes. In myograph experiments, spasmogens are used to generate the tone that allows EDHF responses to be observed. The associated increase in myocyte [Ca<sup>2+</sup>]<sub>i</sub> triggers the opening of Ca<sup>2+</sup>-sensitive K<sup>+</sup> channels (BKCa) on the myocytes. The resulting K<sup>+</sup> cloud can itself fully activate Na<sup>+</sup>/K<sup>+</sup>-ATPases and Kir, effectively ensuring (i) that endothelial cell-derived K<sup>+</sup> clouds are no longer effective and (ii) that gap junctions play the more important role in linking endothelial hyperpolarization to myocyte relaxation. *In vivo*, however, evidence suggests that such electrotonic coupling makes little contribution to endothelium-dependent vasodilatation.

## S4.2 Endothelial KCa-channels, EDHF-signalling, and arterial blood pressure.

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Several candidate molecules/mediators have been shown to act as EDHF(s) in different tissues and species. These include e.g. K<sup>+</sup>, cytochrome P450 metabolites, lipoxigenase products, NO itself and reactive oxygen species (e.g. H<sub>2</sub>O<sub>2</sub>), cAMP, C-type natriuretic peptide, and electrical coupling through myoendothelial gap junctions. At the endothelial level, Ca<sup>2+</sup>-activated K<sup>+</sup> channels (KCa), IK (KCa3.1) and SK (KCa2.3) are thought to play an important role in the initiation of EDHF-signalling. In this overview, we will focus on the functional consequences of a genetically encoded loss of one and of both endothelial KCa for EDHF-mediated dilation in mice. Moreover, we will show that suppression of one and of both endothelial KCa causes significant alterations in arterial blood pressure. The data indicate that endothelial KCa are fundamental determinants of EDHF-signalling and thereby crucial effector proteins in the control of vascular tone and overall circulatory regulation.

## S4.3 K<sup>+</sup> channels and release of nitric oxide

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KCa channels are involved in endothelium-derived hyperpolarizing factor (EDHF) type vasodilatation which is most pronounced in small arteries. In a series of studies we addressed whether KCa channels also contribute to nitric oxide-mediated vasodilatation in large arteries. In rat superior mesenteric arteries acetylcholine caused hyperpolarization of the endothelial cell layer by activation of apamin and charybdotoxin-sensitive K channels. In rat superior mesenteric arteries acetylcholine relaxation was NO-mediated, since acetylcholine-evoked increase in NO concentration and relaxation were inhibited in the presence of an inhibitor of NO synthase, asymmetric dimethylarginine (ADMA); further addition of oxyhaemoglobin reversed the persisting increases in NO concentration and relaxation. Blockers of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, apamin (0.5 μM) and of intermediate- and large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, charybdotoxin (0.1 μM), added individually did not change NO concentration and relaxation, but the combination of the blockers reduced ACh-induced increases in NO concentration by 85% without changing S-nitroso-N-acetylpenicillamine relaxation. In the presence of indomethacin and ADMA, the addition of apamin and charybdotoxin abolished the persisting ACh relaxation and increase in NO concentration. An opener of SKCa and IKCa, NS309 evoked endothelium dependent relaxations and NO release, which were inhibited in the presence of ADMA.

Our findings suggest endothelial cell Ca<sup>2+</sup>-activated K<sup>+</sup> channels are involved in ACh-evoked NO release in rat superior mesenteric artery, and responses, which might be ascribed to EDHF can in part be explained in terms of residual NO release.

## S4.4 K<sup>+</sup> channels and endothelial dysfunction – therapeutic option?

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EDHF-mediated responses involve an increase in the endothelial intracellular calcium concentration via the activation of transient receptor potential channels, the opening of calcium-activated potassium channels of small and intermediate conductance and the hyperpolarization of the endothelial cells. This results in an endothelium-dependent hyperpolarization of the smooth muscle cells, which can be evoked by direct electrical coupling through myo-endothelial gap junctions and/or the accumulation of potassium ions in the intercellular space. Potassium ions hyperpolarize the smooth muscle cells by activating inward rectifying potassium channels and/or Na<sup>+</sup>/K<sup>+</sup>-ATPase. The endothelium can also release epoxyeicosatrienoic acids, which diffuse and hyperpolarize the smooth muscle cells by activating

large conductance calcium-activated potassium channels. Additionally, NO, prostacyclin and other endothelium-derived factors such as lipoxigenase derivatives or H<sub>2</sub>O<sub>2</sub> can produce smooth muscle hyperpolarization by activating various populations of potassium channel. These different mechanisms are not necessarily exclusive and can occur simultaneously. EDHF-mediated responses are altered by aging and various pathological conditions. Therapeutic or adjuvant interventions can restore these responses, suggesting that the improvement of the EDHF pathway contributes to the observed beneficial effect. Therefore, the activation of endothelial potassium channels directly or indirectly could present some therapeutic interest. Some of these manoeuvres would also increase endothelial intracellular calcium concentration and eNOS activity. Furthermore, activation of smooth muscle potassium channels can also reduce the endothelial dysfunction since the activity and expression of smooth muscle NADPH-oxidase, and therefore the generation of superoxide anion, are regulated by membrane potential.

## S4.C001 Role of calcium-activated potassium channels with small and intermediate conductance in bradykinin-induced release of NO in porcine retinal arterioles

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Endothelial dysfunction leads to changed release of vasodilating agents from the endothelium, and may be involved in the pathogenesis of retinal vascular diseases. In this study, we investigated the role of nitric oxide (NO) synthase, cyclooxygenase (COX), and a NO scavenger, oxyhaemoglobin, in the relaxation induced by bradykinin and NS309, an opener of calcium-activated potassium channels of small (SKCa) and intermediate (IKCa) conductance in porcine retinal arterioles. Furthermore, we investigated the role of SKCa and IKCa channels in NO-mediated bradykinin- and NS309-evoked relaxation. Retinal arterioles (diameter ~112 μm, n = 122) were mounted in wire myographs for isometric tension recordings. The arterioles were contracted with the thromboxane analogue, U46619, and concentration-response curves for bradykinin and NS309 were obtained. In U46619-contracted arterioles bradykinin and NS309 induced concentration-dependent relaxations. Bradykinin relaxation was abolished in vessels without endothelium. Inhibition of NO synthase with asymmetric dimethylarginine and/or COX with indomethacin markedly reduced bradykinin- and NS309-induced relaxation. NO synthase and COX inhibition together with oxyhaemoglobin abolished bradykinin relaxation. This treatment only attenuated the NS309-evoked relaxation, indicating no involvement of an endothelium-derived hyperpolarizing (EDHF) type relaxation. In the presence of indomethacin, inhibition of SKCa-channels with apamin markedly reduced NO-mediated bradykinin- and NS309-relaxation, whereas inhibition of IKCa-channels with charybdotoxin had less effect. Opening of SKCa- and IKCa-channels with NS309 potentiated bradykinin relaxation. In porcine retinal arterioles, mainly NO and to a lesser degree prostaglandins were found to be involved in bradykinin and NS309 relaxation. These findings suggest that modulation of SKCa and IKCa channels can lead to an increased release of NO from the endothelium and increase retinal blood flow.

## S4.C002 Evidence in favour of an intermediate-conductance calcium-activated potassium channel in cortical astrocytes

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The intermediate-conductance calcium-activated potassium channel (IKCa) is expressed in various cell types where it has roles in processes as diverse as cell division and the control of vascular tone. In the vasculature, endothelial IKCa channels contribute to the production of a hyperpolarizing potassium cloud that has a relaxant effect on adjacent vascular myocytes (Edwards and Weston, 2004). In the brain, astrocytes possess large-conductance calcium-activated potassium channels on end-feet, which contact intracerebral arterioles, and these have been implicated in the local control of vascular tone in the process of functional hyperaemia (Filosa *et al.*, 2006). However, no studies have thus far investigated the possible involvement of IKCa in this process. Thus, in the present study, a combination of immunofluorescence and whole-cell patch clamp electrophysiology was used in an attempt to identify IKCa in astrocytes. In immunohistochemical studies, antibody staining for IKCa was seen in GFP+ astrocytes in brain slices taken from mice expressing green fluorescent protein (GFP) under the control of the glial fibrillary acidic protein promoter (Lalo *et al.*, 2006). In electrophysiological studies, rat cultured cortical astrocytes exhibited increased current in the presence of 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO; 100 μM), an opener of IKCa channels. This DC-EBIO-evoked current was markedly inhibited by the application of 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; 1 μM), a selective-blocker of IKCa channels. Further data was gathered from a U373 astrocytoma cell line that indicated the presence of an IKCa channel, which was also DC-EBIO and TRAM-34 sensitive. In conclusion, this study presents strong evidence in favour of the presence of IKCa channels in astrocytes, where it is hypothesised that it may play a role in functional hyperaemia.

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# The histamine H3 and H4 receptors: drug targets for the anti-histamines of the 21st century

(09.30–12.30)

## S5.1

### Histamine H3 receptors in psychiatric diseases and Parkinson's disease

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Histamine H3 receptor (H3R) regulates neurotransmitter release in the brain, which renders H3R a potentially important receptor in CNS diseases. H3R ligand binding is higher in the substantia nigra of Parkinson's disease patients than control subjects, whereas mRNA expression is higher only in the pallidum externum. Histamine levels and turnover are also higher in PD patients than normal control subjects. Schizophrenic patients also have abnormally high histamine turnover. We determined the expression and binding patterns of H3R in normal post-mortem human thalamocortical system, and ligand binding patterns of H3R in post-mortem psychiatric patients. H3R mRNA was expressed highly in the anterior, medial and lateral thalamic areas, but not in the ventral thalamus. In the prefrontal cortex, H3R mRNA expression was highest in lamina V, whereas binding was highest in laminae III and IV. It thus seems that the thalamocortical projection from anterior and dorsal thalamus is regulated by H3R. In a post-mortem study on 60 subjects (15 schizophrenics, 15 depressive, 15 bipolar and 15 age-matched controls), H3R binding was significantly higher in the prefrontal cortex of schizophrenic patients than in the other groups. No such difference was seen in the temporal cortex. Although medication may contribute to the finding of high H3R binding in prefrontal cortex, the finding may also be specific of the disease since no difference was seen in temporal cortex. The H3R may be upregulated in the prefrontal cortex, or the higher binding may be due to differences in cell density between normal and schizophrenic brains.

## S5.2

### Histamine receptors: from the human genome to new therapeutic options for inflammatory disorders

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Until 2000 histamine has been thought to act via three different G-protein coupled receptors. All three receptors have attracted considerable interest as therapeutic targets. Antagonists for the H1- (e.g. fexofenadine in Allegra® or l-cetirizine in Xyzal®) or H2 receptor (e.g. cimetidine in Tagamet® or ranitidine in Zantac®) have been successfully used for many years for the treatment of allergic conditions and gastric ulcers, respectively. Moreover, currently a lot of attention in the pharmaceutical industry is directed towards the therapeutic use of H3 antagonist for e.g. cognitive disorders and obesity. And then there were four..... Following the sequencing of the human genome, data mining efforts have revealed the existence of a new histamine receptor with high expression levels in mast cells and leukocytes. Using the sequence information of the human H3 receptor, several groups independently identified a homologous GPCR sequence in the human genome sequence databases. The new GPCR is expressed predominantly in bone marrow, eosinophils and mast cells and this histamine H4 receptor shows a clearly distinct pharmacological profile. This moment the first selective compounds have now been reported and are being made available for pharmacologists/immunologists. Initial *in vivo* experiments with H4 receptor antagonists suggest a role for the H4 receptor in several inflammatory conditions.

## S5.3

### The H4 Receptor and potential cancer therapy

A Falus, R. Kiss *Semmelweis University, Hungary*

Based on earlier data human and murine dendritic cells (DC) express both histamine H4 receptors and histidine decarboxylase resulting in production and potential autocrine action of histamine. The locally available histamine influences DCs' gene expression profile and function, including antigen processing and presentation. This situation suggests that histamine may influence antigen presentation through H4 receptors raising the potential relevance of molecules with ability to bind to H4 receptors to act as adjuvants in vaccination. Following *in silico* screening and selection procedures 255 molecules were selected by radioligand binding. The best 24 moieties were further investigated and proved to act on H4 receptor in 5  $\mu$ M concentration. The best hits reveal new basic structures which might serve to develop entirely new H4 receptor agonists and antagonists acting as adjuvants in cancer vaccination.

## S5.4

### Histamine H3 receptor antagonists: from target identification to clinical candidate

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The histamine H3 receptor is an inhibitory auto and heteroreceptor with the highest densities of receptors found in the central nervous system. Activation of the H3 receptor leads to a decrease in the synthesis and release of histamine whereas antagonism, or inverse agonism, of the H3 receptor affords a general activation of histaminergic neurotransmission in the brain and consequently an increase in waking, improved cognition and suppression of food intake. Therefore, potential therapeutic applications for an H3 antagonist include a broad range of neuropsychiatric diseases, metabolic diseases and sleep disorders. The successful cloning and functional expression of the histamine H3 receptor by our group at Johnson & Johnson in the late 1990's has greatly facilitated drug discovery and, indeed, prompted our efforts to identify small molecule, non-imidazole based compounds to

permit the evaluation of H3 antagonists in models of CNS disorders. High throughput screening identified several series of lead compounds, including a series of imidazopyridines, which led to JNJ-6379490 a compound with high affinity and excellent selectivity for the human H3 receptor. Analysis of the structural features common to the series of non-imidazole H3 receptor ligands resulted in a pharmacophore model. This model led to the design of JNJ-5207852 a diamine-based H3 antagonist with good *in vitro* and *in vivo* efficacy but with an undesirably long-half life. However further modification of the template provided an understanding of the effect of structural modifications on pharmacokinetic properties, ultimately affording several additional series of compounds including JNJ-10181457, a compound with improved pharmacokinetic properties. H3 antagonists promote wakefulness but unlike modafinil and classical psychostimulants, they do not increase locomotor activity or produce any alteration of the EEG power spectral activity in rats. H3 antagonists increase extracellular acetylcholine and norepinephrine but not dopamine level in rat frontal cortex. In addition, cFos immunoreactivity studies show that H3 antagonists activate neuronal cells in restricted rat brain regions in contrast to a widespread activation after modafinil or amphetamine treatment. Therefore, H3 antagonists are promising clinical candidates for the treatment of excessive day time sleepiness (narcolepsy, sleep apnea, shift work sleep disorder) and/or cognitive disorders.

## S5.C001

### Effect of the H<sub>1</sub>R antagonist JNJ777120 on the cartilage histamine content in rats with adjuvant arthritis

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Histamine (HI) may play a role in chondrocyte behaviour in rheumatoid arthritis (Tetlow and Woolley, 2005). This study aimed to examine the effects of the H<sub>1</sub> receptor antagonist JNJ777120 (Thurmond *et al.*, 2004) in a rat model of adjuvant arthritis. Male Wistar rats of 200–250 g bw were divided into four groups (*n* = 5–7). Group A received normal saline, groups C and D received complete Freund's adjuvant (CFA) i.d. groups B and D received 10 mg/kg JNJ777120 i.p. Blind daily scoring of abnormal paw signs was performed. At day 20, animals were sacrificed, #9–10 ribs dissected medial to the costochondral junction and their HI levels quantified fluorophotometrically (Tiligada *et al.*, 2000). Differences were located by non-parametric statistical analyses and Anova. Contrary to groups A and B, groups C and D developed arthritis signs (score 16). Cartilage HI content increased in groups B and C (*P* < 0.05 vs. A), while it was comparable among groups D, B and C (*P* > 0.05). The increased cartilage HI levels and the clinical signs were indicative of systemic inflammation. The JNJ777120-induced HI increases in the absence of clinical signs raise questions on the pharmacological target(s) of the antagonist in the rat. These data present first evidence for the contribution of HI in the arthritic phenotype and provide the lead for the elucidation of the H<sub>1</sub>R actions in the cartilage.

#### References:

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Thurmond R.L. *et al.* *J Pharmacol Exp Ther.* 2004; 309: 404–413.  
Tiligada E. *et al.* *Pharmacol Res.* 2000; 41: 667–670.

## S5.C002

### The H<sub>4</sub> histamine receptor is functionally expressed on neurons in the mammalian CNS

PL. Chazot<sup>a</sup>, W. Connolly<sup>b</sup>, FC Shenton<sup>a</sup>, R. Leurs<sup>c</sup>, H. Waldvogel<sup>d</sup>, G. Lees<sup>b</sup> <sup>a</sup>University of Durham, Durham, UK; <sup>b</sup>University of Otago, Dunedin, New Zealand; <sup>c</sup>Vrije Universiteit Amsterdam, Amsterdam, the Netherlands; <sup>d</sup>University of Auckland, Auckland, New Zealand

The histamine H<sub>4</sub> receptor (H<sub>4</sub>R) is the latest of the G-protein coupled histamine receptor family to be discovered and displays prominent expression in a number of immune cell types which highlights its importance in inflammatory conditions (Dijkstra *et al.*, 2007; Baumer *et al.*, 2008; Dijkstra *et al.*, 2008). Herein, we report, for the first time, that the H<sub>4</sub>R polypeptide, labelled with our novel previously validated anti-H<sub>4</sub> receptor antibody (Van Rijn *et al.*, 2006) is also expressed in both human, rat and mouse brain. We provide the first evidence that the mouse, rat and human brain H<sub>4</sub> receptor exists as a robust dimer analogous to the H<sub>4</sub> receptor expressed in human lymphocytes (Van Rijn *et al.*, 2006). The H<sub>4</sub>R is prominently expressed in murine thalamus, entorhinal cortex, hippocampal CA4 stratum lucidum and layer IV of the cerebral cortex. Electrophysiological and pharmacological studies in layer IV of the mouse somatosensory cortex demonstrate that the H<sub>4</sub>R agonist 4-methyl histamine (4-MH 20  $\mu$ M) directly hyperpolarises neurons, an effect which is occluded by the selective H<sub>4</sub> antagonist JNJ 10191584 (1  $\mu$ M), and promotes outwardly rectifying currents in these cells, a functional property distinct from all the other histamine receptors expressed in the CNS. Therefore, we provide the first pharmacological evidence for the presence of functional H<sub>4</sub> receptors on neurons in the mammalian brain. This further demonstrates that the H<sub>4</sub> receptor subserves roles in other cell types in addition to its well characterised roles in haematopoietic cells (Dijkstra *et al.*, 2007; Baumer *et al.*, 2008; Dijkstra *et al.*, 2008). This work was funded in part by the Wellcome Trust.

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# Symposia Presentations

## Tuesday 15 July

### G12/13-dependent signalling of G protein-coupled receptors: mechanistic insights and therapeutic impact (09.30–12.30)

#### S6.1

##### G-protein-mediated signalling pathways in vascular physiology and pathology

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Many hormones and factors which regulate cardiovascular functions act through G-protein-coupled receptors which control the activities of G-proteins of the Gq/G11- and G12/G13-families. Activation of Gq/G11 leads to the stimulation of  $\beta$ -isoforms of phospholipase C resulting in an IP<sub>3</sub>-mediated release of intracellularly stored Ca<sup>2+</sup> and the diacylglycerol-mediated activation of protein kinase C. G12/G13 have been shown to activate the small GTPase RhoA by direct regulation of a subgroup of Rho guanine nucleotide exchange factors including LARG, PDZ-RhoGEF and p115-RhoGEF. In recent years we have generated mouse mutants in which the genes coding for the  $\alpha$ -subunits of these G-proteins as well as of some of their effectors have been inactivated constitutively as well as conditionally. This has opened the possibility to systematically analyze the role of G-protein-mediated signalling pathways in physiological and pathological processes of the cardiovascular system. Data will be presented which describe the roles of Gq/G11- and G12/G13-mediated signalling in platelet activation as well as in the regulation of vascular smooth muscle tone under normal and hypertensive conditions.

#### S6.2

##### Involvement of G12 proteins in signaling processes impacting oncogenesis and metastasis

J Juneja, P Kelly, PJ Casey Duke University Medical Center, USA

Metastatic disease continues to contribute significantly to the morbidity and mortality of cancer. We observed that G $\alpha$ 12 and G $\alpha$ 13, two closely-related  $\alpha$  subunits of heterotrimeric G proteins, are significantly upregulated in breast and prostate cancers in the earliest stages of the cancers, implying that amplification of G12 signaling may be an early event in cancer progression. When activated, this family of G proteins leads to the activation Rho and inactivation of the cell adhesion molecule E-cadherin; both of these processes are known to play crucial roles in cancer metastasis. We investigated the role of G12 proteins in breast and prostate cancer metastasis. Blockade of G12/13 signaling had a profound inhibitory effect on *in vitro* invasion of both cancer types. Further, inhibition of G12/13 signaling markedly impaired *in vivo* metastasis in a 4T1 murine breast cancer model. These findings indicated that signaling through G12 proteins can critically impact on the metastatic process. In recent studies, c-Jun NH2-terminal kinase (JNK) was found to be a key downstream effector of G12 on this pathway. Stimulation of G12 signaling led to increased JNK and c-Jun phosphorylation in breast cancer cells. Pharmacologic inhibition or RNAi-mediated knockdown of JNK significantly decreased G12-induced JNK activation as well as *in vitro* invasion of breast cancer cells. These data suggest that JNK activation is required for G12-induced invasion of breast cancer cells. We are continuing to explore the molecular mechanisms through which activation of G12 proteins impacts on processes important in cell migration and invasion.

#### S6.3

##### Regulation of RhoGEF Proteins by Galpha12/13-coupled receptors, and disease-related context

BH Meyer, F Freuler, S Siehler Novartis Institutes for BioMedical Research (NIBR), Switzerland

G12/13-dependent signalling of GPCRs is relevant to many cellular events, and an impaired regulation can cause various disease conditions such as hypertension, cardiovascular disease, stroke, impaired wound healing, cancer metastasis, or asthma. Activation of G12/13 proteins by GPCRs causes activation of RhoGEFs, which in turn activate the small GTPase RhoA. A novel subcellular imaging technique was developed to measure G12/13 signalling in a kinetic fashion using EGFP-tagged RhoGEF proteins. p115-RhoGEF rapidly translocated from the cytosol to the plasma membrane upon co-expression of constitutively active mutants of Galpha12/13, or upon activation of various G12/13-linked GPCRs. Plasma membrane translocation of p115-RhoGEF stimulated by a GPCR agonist could be either blocked by pre-treatment of cells with an antagonist, or could be reversed by subsequent application of a GPCR antagonist. GPCR activation of Galpha12 versus Galpha13 can be dissected through recombinant expression of wildtype Galpha12/13, and PAR-1 was found to selectively activate p115-RhoGEF through Galpha12, but not via Galpha13. Immunodetection of endogenous p115-RhoGEF can be applied to primary cells. In HUVEC, S1P and FTY720-P induced activation of the G12/13-RhoGEF pathway with potencies of 1.6 and 5.1 nM, respectively. The G12/13-RhoGEF pathway assay enables the development of GPCR drugs active in a G12/13-linked disease context.

#### S6.4

##### Genetic screens for serotonin signaling mutants reveals competing G-proteins acting to regulate synaptic vesicle priming

S Nurrish MRC Laboratory for Molecular Cell Biology, University College, London, UK Serotonin reduces levels of acetylcholine (ACh) release at the *Caenorhabditis elegans* neuro-muscular junction causing a decrease in locomotion. Screens for mutants defective in their serotonin response define competing G protein pathways that act

within the same neuron to facilitate or inhibit the release of ACh and thus alter locomotion. Two G protein alpha subunits, Gq and G12, stimulate ACh release by increasing levels of the second messenger diacylglycerol (DAG) at synaptic release sites, whereas a third, Go, decreases ACh release by decreasing DAG levels. DAG, in turn, stimulates release of synaptic and dense core vesicles via the DAG binding proteins UNC-13 and PKC-1. UNC-13 is thought to have a role in activating syntaxin, which an essential component of the synaptic vesicle release machinery, thus, these experiments define a genetic pathway leading from G-protein-coupled receptors to the general synaptic release machinery. Gq acts to increase DAG via two effectors: the first is a phospholipase C, which hydrolyses the phospholipid PIP2 to form both DAG and IP3, the second is a RhoGEF that activates the small GTPase Rho. Activated Rho binds to and inhibits a DAG kinase that removes DAG. Thus, Gq both stimulates the production of DAG and inhibits the removal of DAG. G12 activates a subset of the Gq effectors as G12 also activates Rho via another RhoGEF. Interestingly, G12 activates DAG signaling via UNC-13 but not PKC-1. Thus, G12 reveals that DAG effectors can be selectively activated within the same cell.

#### S6.C001

##### Isoflavone attenuates vascular contraction through inhibition of RhoA/Rho-kinase signaling pathway

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Isoflavones decrease blood pressure, improve lipid profiles, and restore vascular function. We hypothesized that isoflavone attenuates vascular contraction by inhibiting RhoA/Rho-kinase signaling pathway. Rat aortic rings were denuded of endothelium, mounted in organ baths, and contracted with U46619, a thromboxane A2 analogue or KCl in the presence of genistein or daidzein (10, 30 and 100  $\mu$ M) for 30 min or not. We determined the phosphorylation level of myosin light chain (MLC<sub>20</sub>), myosin phosphatase targeting subunit 1 (MYPT1) and protein kinase C (PKC) -potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI17) by the Western blot. We also measured the amount of GTP RhoA as a marker for RhoA activation. Cumulative addition of U46619 and KCl increased vascular tension in a concentration-dependent manner, which were inhibited by pretreatment with genistein or daidzein. U46619 (30 nM) and KCl (50 mM) increased phosphorylation level of the MLC<sub>20</sub>, which were inhibited by genistein or daidzein. Furthermore, both genistein and daidzein decreased the amount of GTP RhoA activated by U46619 or KCl. U46619 (30 nM) increased Rho-kinase-dependent phosphorylation of the MYPT1<sup>Thr855</sup> and CPI17<sup>Thr38</sup>, which were also inhibited by genistein or daidzein. However, neither genistein nor daidzein inhibited PDBu-induced vascular contraction and CPI17 phosphorylation. Isoflavone attenuates vascular contraction at least in part through inhibition of RhoA/Rho-kinase signaling pathway. Supported by BK21 Project.

#### S6.C002

##### Sphingosine-1-phosphate signalling in rat bladder smooth muscle cells

PB van Loenen, MC Hendriks-Balk, MC Michel, SLM Peters, AE Alewijnse Academic Medical Center, Amsterdam, the Netherlands

Sphingosine-1-phosphate (S1P) receptors, most importantly the S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> receptor, are G-protein coupled receptors which play an important role in smooth muscle cell biology. In this study the signalling of S1P receptors in rat isolated bladder smooth muscle cells was investigated using pharmacological tools which have recently become available. These included SEW2871 (S1P<sub>1</sub> agonist), FTY720-P (S1P<sub>1</sub>, S1P<sub>3-5</sub> agonist) and JTE013 (S1P<sub>2</sub> antagonist). The endogenous agonist S1P concentration-dependently induced an increase in intracellular calcium concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) in bladder smooth muscle cells. Interestingly, SEW2871 and FTY720-P did not induce such increases in [Ca<sup>2+</sup>]<sub>i</sub> suggesting that the effects observed for S1P are most likely S1P<sub>2</sub>-receptor mediated. Further indications for a S1P<sub>2</sub> receptor-mediated effect came from experiments using the S1P<sub>2</sub> antagonist JTE013. JTE013 (1  $\mu$ M) did not affect endothelin-mediated calcium responses but significantly decreased the S1P-induced increases in [Ca<sup>2+</sup>]<sub>i</sub>. In addition, the S1P-induced calcium response was significantly inhibited by pre-treatment with Pertussis Toxin, indicating that it is for an important part G<sub>i</sub>-mediated. Because G<sub>i</sub>-activation generally is associated with an inhibition of cAMP production the effects of S1P on cAMP accumulation were subsequently investigated. Remarkably, S1P did not decrease but rather increased basal and forskolin-induced cAMP production, indicating a possible additional coupling to G<sub>s</sub>. FTY720-P also increased basal and forskolin-induced cAMP accumulation but the effects were rather small and required much higher concentrations of FTY720-P compared to S1P. In conclusion, our data show that S1P receptors, most importantly S1P<sub>2</sub> receptors, are involved in the modulation of [Ca<sup>2+</sup>]<sub>i</sub> and cAMP production in bladder smooth muscle cells. In contrast to the effects observed in vascular smooth muscle cells and in contrast to our general expectations for the G<sub>i</sub>-coupled S1P receptors, cAMP levels were not decreased but increased upon activation by S1P. Based upon these results we conclude that S1P receptors may play an important role in bladder smooth muscle cell biology although the specific relationship between the observed signalling alterations and effects on bladder function awaits further investigation.

# Pharmacological modulators of endocannabinoid tone: their therapeutic potential (09.30–12.30)

## S7.1

### Molecular bases of the therapeutic use of antagonists of endocannabinoid action against metabolic disorders

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The endocannabinoid system participates at several levels in the control of lipid and glucose metabolism, with the possible ultimate end of accumulating energy into fat. Endocannabinoids, mostly by activating cannabinoid CB1 receptors in an autocrine or paracrine way, stimulate food intake after brief food deprivation, slow down intestinal motility and maximize lipid accumulation in the adipose tissue, possibly at the expense of energy expenditure by the skeletal muscle. Following chronic unbalanced energy intake, however, the EC system becomes dysregulated (in most cases overactive) in several organs participating in energy homeostasis, including the hypothalamus, the pancreas and, particularly, the adipose tissue. This dysregulation might contribute to: hyperphagia, excessive accumulation of fat in visceral adipose tissue and reduced adiponectin release from this depot, insulin resistance and the onset of several cardiometabolic risk factors that are associated with obesity and type 2 diabetes. This phenomenon might be at the basis of the mechanism of action of CB1 antagonists/inverse agonists, recently developed by several pharmaceutical companies as adjuncts to life-style modification for weight reduction, glycemic control and dyslipidemia in obese and type 2 diabetes patients. It also helps explain why some of the beneficial actions of these new therapeutics appear to be partly independent from weight-loss.

## S7.2

### The endocannabinoid system as a target for the treatment of motor dysfunction

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Recent studies have provided support to the idea that cannabinoid-based medicines, with selectivity for different targets (e.g. receptors, inactivation mechanism, enzymes) of the cannabinoid signaling system, might be beneficial in basal ganglia disorders, namely Parkinson's disease (PD) and Huntington's disease (HD). These benefits would include not only the alleviation of specific motor symptoms (e.g. choreic movements in HD with CB1/TRPV1 agonists; bradykinesia in PD with CB1 antagonists) but also the possible delay of disease progression based on the neuroprotective properties of cannabinoid compounds (e.g. CB1 agonists to reduce excitotoxicity; CB2 agonists to limit the toxicity of reactive microglia, and antioxidant cannabinoids to attenuate oxidative damage). The rationale for this proposal is based on extensive biochemical, anatomical, physiological and pharmacological work that has demonstrated: that the different elements (ligands, cannabinoid receptors, enzymes) of the cannabinoid signaling system are abundant in basal ganglia structures and have been reported to be markedly altered in basal ganglia disorders;

– that the cannabinoid signaling system plays a prominent role in basal ganglia function exerted through modulating the activity of those neurotransmitters that operate at the basal ganglia circuit, not only in healthy conditions but also in the pathologies;

– that the activation and/or inhibition of the cannabinoid signaling system is associated with important motor effects that were maintained and even enhanced in conditions of basal ganglia malfunctioning and/or degeneration. The present lecture will review the anatomical, neurochemical, electrophysiological and pharmacological bases that sustain the importance of the cannabinoid system in basal ganglia function, trying to collect the present information and the future lines for research on the therapeutic potential of this system in PD, HD and other basal ganglia disorders.

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## S7.3

### Inhibition of endocannabinoid metabolism and dual-action COX/FAAH inhibitors as a strategy for the treatment of pain

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Cannabinoids have long been of interest with respect to their medical potential, and a standardized cannabinoid extract formulated as a spray has been licensed in Canada for the alleviation of pain in patients with multiple sclerosis. A major issue with cannabinoids as therapeutic drugs is of course the separation between their beneficial effects on the disorder in question and their unwanted psychotropic effects resulting from the activation of central CB1 receptors. CB2 receptor agonists will not produce such effects, and such compounds are active in models of inflammatory and neuropathic pain. An alternative approach is to target the endocannabinoid metabolic pathways. Compounds inhibiting the metabolism of anandamide by the enzyme fatty acid amide hydrolase (FAAH) are active in models of inflammation and inflammatory pain, and do not produce cannabis-like effects in behavioural studies. Similarly, mice with genetic deletions of FAAH show a blunted nociceptive response to an inflammatory stimulus. Locally administered anandamide acts synergistically with non-steroidal anti-inflammatory drugs in a model of inflammatory pain, raising the possibility that compounds inhibiting both FAAH and cyclooxygenase may be useful analgesic compounds. Other approaches include the development of compounds with combined FAAH-inhibitory and transient receptor potential (vanilloid) 1 antagonistic properties, and compounds selectively inhibiting monoacyl glycerol lipase, an enzyme which contributes to the metabolism of the endocannabinoid 2-arachidonoylglycerol.

## S7.4

### Targeting peripheral cannabinoid 1 (CB1) receptors for chronic pain

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Evidence is growing that activation of peripheral CB1 receptors may reduce allodynia and hyperalgesia in chronic pain states (Fox *et al.*, 2001; Agarwal *et al.*, 2007) without mediating CNS adverse effects. The antinociceptive profile of compound A, a potent CB1/CB2 agonist ( $EC_{50}$ ,  $hCB1 = 94$  nm and  $hCB2 = 3.5$  nm) which has low CNS penetration (brain:plasma ratio = 0.02) was examined. Mechanical allodynia was determined using von Frey filaments in male Wistar rats (151–296 g) following ligation of the L5 spinal nerve. Thermal hyperalgesia was measured using the plantar test. Nociceptive responses to intraplantar injection of formalin (5% solution) were determined in male ICR mice (23–29 g) using an automated device and responses summed (0–5 min; Phase 1 and 20–30 min; Phase 2). Catalepsy was measured in rats (male Wistar 122–155 g) as described by Fox *et al.* (2001).

Compound A significantly attenuated thermal hyperalgesia by  $54 \pm 20\%$  inhibition in rats following spinal nerve (L5) ligation (SNL) following an oral dose of 30  $\mu\text{mol/kg}$  and by  $113 \pm 26\%$  inhibition following a dose of 0.75  $\mu\text{mol/kg}$  s.c. ( $n = 6-12$ ,  $P < 0.05$ , Kruskal-Wallis, followed by Dunn's test). Catalepsy was not observed in rats following doses up to 60  $\mu\text{mol/kg}$  s.c. (duration of catalepsy = 0 s,  $n = 6$ ,  $P > 0.05$ , one-way ANOVA). In addition, Compound A significantly attenuated mechanical allodynia in SNL rats by  $58 \pm 14\%$  and  $86 \pm 11\%$  following 30 and 40  $\mu\text{mol/kg}$  p.o., respectively ( $n = 10$ ,  $P < 0.05$ , Kruskal-Wallis, followed by Dunn's test). A higher dose of 100  $\mu\text{mol/kg}$  p.o. was required to inhibit the 2nd phase response in the mouse formalin paw test ( $52 \pm 13\%$  inhibition,  $n = 6$ ,  $P < 0.05$ , Kruskal-Wallis, followed by Dunn's test); no effect was observed on the 1st phase. Compound A is a peripherally restricted, potent, orally active CB1/CB2 receptor agonist that possesses anti-nociceptive and anti-allodynic activity in models of inflammatory and neuropathic pain and is devoid of CNS side-effects. Targeting CB1 receptors in the periphery may provide analgesia without causing unwanted side-effects mediated by central CB1 receptors.

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## S7.C001

### Inhibitors of endocannabinoid hydrolases and cyclooxygenases modulate mesenteric relaxation to anandamide but not 2-arachidonoylglycerol in spontaneously hypertensive rats

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Using the third order branches of the superior mesenteric artery in normotensive, male Wistar rats, we have shown that relaxations to the endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG) are limited by local degradation via fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) respectively, and cyclooxygenases (COX; Ho and Randall, 2007). Here, we have examined mesenteric relaxations to endocannabinoids in male Spontaneously Hypertensive Rats (SHR; 200–350 g). Vessels were precontracted with 10  $\mu\text{M}$  methoxamine. Data are expressed as mean $\pm$ SEM ( $n \geq 4$  rats) and analysed by two-way analysis of variance of the whole data set. The FAAH inhibitor, URB597 (3'-carbamoyl biphenyl-3-yl-cyclohexylcarbamate) significantly potentiated anandamide-induced relaxation (control:  $pEC_{50} = 6.7 \pm 0.3$ ;  $E_{max} = 67 \pm 7\%$ ; +1  $\mu\text{M}$  URB597:  $pEC_{50} = 6.8 \pm 0.3$ ;  $E_{max} = 84 \pm 5\%$ ;  $?P < 0.01$ ). Combined additions of COX inhibitors, indomethacin and nimesulide (10  $\mu\text{M}$  for both), but not indomethacin alone, slightly potentiated anandamide responses (control:  $pEC_{50} = 6.0 \pm 0.1$ ;  $E_{max} = 61 \pm 9\%$ ; +indomethacin:  $pEC_{50} = 6.3 \pm 0.3$ ;  $E_{max} = 85 \pm 6\%$ ; +indomethacin+nimesulide:  $pEC_{50} = 6.5 \pm 0.4$ ;  $E_{max} = 79 \pm 7\%$ ;  $P < 0.05$ ). On the other hand, relaxation to 2-AG was not affected by URB597 or methylarachidonyl fluorophosphate (MAFP; an inhibitor of FAAH and MGL) (control:  $pEC_{50} = 6.2 \pm 0.3$ ;  $E_{max} = 38 \pm 6\%$ ; +URB597:  $pEC_{50} = 6.4 \pm 0.3$ ;  $E_{max} = 43 \pm 7\%$ ; +MAFP:  $pEC_{50} = 6.3 \pm 0.3$ ;  $E_{max} = 56 \pm 12\%$ ). Indomethacin, applied alone or together with either MAFP or nimesulide, also had no effect (data not shown). To conclude, relaxation to anandamide, but not 2-AG, is limited by endocannabinoid hydrolases and cyclooxygenases in small mesenteric arteries from SHR, suggesting that vascular metabolism of the two endocannabinoids might be differentially altered in some forms of hypertension.

This study was supported by the British Heart Foundation and Anne McLaren Fellowship

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## S7.C002

### Role of endocannabinoids in retrograde synaptic signaling in the human cerebral cortex

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The CB<sub>1</sub> cannabinoid receptor is typically localized on axon terminals and its activation leads to presynaptic inhibition of neurotransmission (Szabo and Schlicker, 2005). During the process of retrograde signaling, the presynaptic CB<sub>1</sub> receptor is activated by endogenous cannabinoids (endocannabinoids) synthesized

by postsynaptic neurons (Chevalyere *et al.*, 2006). The aim of the present experiments was to study endocannabinoid-mediated retrograde signaling in the human brain. Cortical tissue removed during tumor and epilepsy surgery was used. 250  $\mu\text{m}$ -thick slices were cut with a vibratome and superfused. Spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) were recorded in pyramidal neurons with patch-clamp techniques. In order to enhance the activity of cannabinoid-sensitive presynaptic axons, muscarinic acetylcholine receptors were continuously stimulated by carbachol ( $2.5\text{--}5 \times 10^{-6}$  M). Under these conditions, depolarization of postsynaptic pyramidal neurons [by 9 depolarizing pulses at a frequency of 1 Hz (from  $-70$  mV to  $0$  mV for 100 ms)] led to suppression of GABAergic sIPSCs. This depolarization-induced suppression of inhibition (DSI) was  $18 \pm 3\%$  during the 10 s following the depolarization. DSI was abolished by the CB<sub>1</sub> receptor antagonist rimonabant ( $10^{-6}$  M), verifying involvement of endocann-

abinoids and CB<sub>1</sub> receptors. The CB<sub>1</sub> receptor agonist WIN55212-2 ( $5 \times 10^{-6}$  M) also inhibited sIPSCs (maximally by  $32 \pm 5\%$ ). In the presence of WIN55212-2, the depolarization no longer elicited suppression of sIPSCs - probably because the presynaptic CB<sub>1</sub> receptors were saturated by the exogenous cannabinoid agonist. This is the first demonstration of endocannabinoid-mediated retrograde synaptic signaling in the human brain. This kind of short-term synaptic plasticity, together with long-term synaptic plasticity, is thought to be important for memory and learning. The interference of exogenous cannabinoid agonists and antagonists with retrograde synaptic signaling in human brain tissue suggests that these compounds will interfere with memory and learning in man.

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# L-Arginine metabolism and arginase in health and disease (09.30–12.30)

## S8.1

### Recent advances in L-Arginine metabolism

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Arginine is metabolically very versatile, being interconvertible with the amino acids proline and glutamate and also serving as precursor for synthesis of protein, nitric oxide, creatine, polyamines, agmatine, and urea. These processes do not all occur within every cell but are differentially expressed according to cell type, age and developmental stage, diet, and state of health or disease. Although our knowledge of mammalian arginine metabolism has increased greatly in recent years, it is still imperfect and incomplete. There is growing interest in the arginases, which are expressed as two independently regulated isozymes whose expression can change rapidly and dramatically in health and disease states. Regulation of arginase expression is complex; e.g., the induction of arginase expression by certain combinations of stimuli is synergistic and stochastic. The consequences of elevated arginase expression are not simply metabolic but can include altered expression of specific genes at translational and pretranslational steps. It has become clear that a more complete understanding of arginine metabolism in health and disease will require integration of information obtained from genomic, proteomic, and metabolomic approaches.

## S8.2

### Regulation of cationic amino acid transport

E. Closs *Johannes Gutenberg University, Mainz, Germany*

Cationic amino acids (CAA), such as arginine, lysine, and ornithine, share the same transport proteins. Most mammalian cells express two types of CAA transport proteins, mediating activities of the so called systems y<sup>+</sup> (specific for CAA) and y<sup>+</sup>L (CAA and neutral AA), respectively. Although only distantly related, the two transporter types belong to the same gene family: SLC7. The family members SLC7A1–A3 correspond to the CAT proteins (CAT for CAA transporter) mediating system y<sup>+</sup> activity. They seem to be the major entry path for CAA in most cells. SLC7A6 and 7 encode for system y<sup>+</sup> transporter y<sup>+</sup>LAT2 and 1, respectively. They catalyze the exchange of CAA against NAA plus Na<sup>+</sup> and thus seem to be rather CAA exporters under physiological conditions. The physiological role of each individual system y<sup>+</sup> and y<sup>+</sup>L transporter has not yet been fully elucidated. However, besides providing cells with CAA for protein synthesis and energy supply, the CAA transporters seem to be involved in important signal pathways such as nitric oxide, mTOR and neurotransmission. CAT-1 and CAT-2 are extensively regulated on the level of both, transcription and translation. In addition, the activity of both, system y<sup>+</sup> and y<sup>+</sup>L is down-regulated upon activation of protein kinase C. The talk will attempt to give an update of our current knowledge on the regulation and physiological function of these transport proteins.

## S8.3

### Role of arginase in the immune system

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The arginine-degrading enzyme arginase is expressed in human neutrophil granulocytes and is released during inflammatory reactions. The ensuing extracellular arginine depletion severely impairs key functions of human T lymphocytes and might be central to the immune defects associated with chronic inflammatory processes or cancer.

(i) Human T lymphocytes were activated in the presence or absence of arginine. Mammalian homologues of lower eukaryote amino acid response pathways were analysed by Western blotting. (ii) Human NK cells and granulocytes were activated and the influence of arginine on cell viability, proliferation, granule exocytosis, cytokine production, gene transcription, phagocytosis, production of reactive oxygen species and killing activity was analysed.

(i) Human T lymphocytes respond to extracellular arginine depletion by phosphorylation of the kinase GCN2. Other mammalian homologues of eukaryotic stress elements (eIF2 $\alpha$ , p-ATF-2 and ATF-4) are less stringently regulated upon arginine depletion. (ii) Arginine depletion does not influence NK cell viability, granule release and the killing of target cells. In contrast, NK cell proliferation and cytokine secretion crucially depend on the presence of extracellular arginine. All tested functions of human granulocytes are completely impaired in the context of arginine deficiency. The immunosuppression in granulocyte-dominated inflammation might have evolved as a homeostatic immunoregulatory mechanism that limits excessive immune activation. While adaptive immunity (T lymphocytes) is severely suppressed, innate immune functions are partially (NK cells) or completely (granulocytes) preserved. This study supports development of pharmacological tools to revert unwanted arginase-induced immunosuppression.

## S8.4

### Arginase: a novel key enzyme in asthma

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Arginase converts L-arginine into L-ornithine and urea and is constitutively expressed in the airways, particularly in bronchial epithelial cells, endothelial cells, (myo)fibroblasts and alveolar macrophages. Increased expression and activity of arginase have been observed in asthmatic airways, both in animal models and in humans. Asthma is a chronic disease characterized by early and late asthmatic

reactions (EAR and LAR), airway hyperresponsiveness (AHR), airway inflammation and airway remodelling. Increased arginase may contribute to these features by altering the L-arginine homeostasis, leading to decreased production of bronchodilating nitric oxide (NO) by NO synthases (NOS) and increased formation of peroxynitrite, polyamines and L-proline. Using a guinea pig model, we demonstrated that increased arginase activity contributes to the AHR after the EAR by inducing a strong deficiency of neuronal and non-neuronal NO by limiting the L-arginine availability to constitutive NOS isoforms. Moreover, arginase-induced L-arginine limitation to inducible NOS leads to the simultaneous production of NO and superoxide by this enzyme, which underlies the peroxynitrite-mediated AHR after the LAR. Inhibition of arginase diminished the AHR after both the EAR and LAR by restoring the production of bronchodilating NO and preventing peroxynitrite formation. Increased arginase activity also underlies peroxynitrite-mediated AHR in chronic asthma. Moreover, increased arginase could be involved in airway remodelling via increased production of L-proline and polyamines, downstream products of L-ornithine. In conclusion, arginase activity may be considered a key enzyme in the pathophysiology of asthma and arginase inhibitors clearly have therapeutic potential.

## S8.C001

### The effect of PI3K inhibition on IL-13-induced arginase I expression in mice tracheal segments

H. Farghaly, B. Causton, I. Blagbrough, M. Watson *University of Bath, Bath, UK*

Interleukin (IL)-13 is a critical cytokine in the development of airway hyperresponsiveness, a process that depends on phosphoinositide 3-kinase (PI3K) activity (Farghaly *et al.*, 2008). A potential mechanism of IL-13-induced hyperresponsiveness is the induction of arginase, which can compete with nitric oxide synthase for the common substrate arginine, and remove the modulatory effects of NO on airway smooth muscle contraction (Meurs *et al.*, 2003). The aim of the present study was to determine whether IL-13-induced airway hyperresponsiveness depends on a PI3K-dependent induction of epithelial arginase. Tracheae were isolated from humanely killed male CD1 strain mice (25–30 g). Reactivity of tracheal rings to carbachol (CCh) was assessed in organ baths and recorded via Powerlab data collection apparatus. Exposure of tracheal rings to IL-13 (100 ng/mL, 24 h) resulted in enhanced responsiveness to CCh, to give an Emax value 188  $\pm$  9% of control ( $n = 7$ ), whereas responsiveness was reduced to 146  $\pm$  3 ( $n = 4$ ,  $P < 0.01$ ) when contraction was assessed in the presence of the arginase inhibitor L-norvaline (10 mM). Under the conditions used, L-norvaline did not affect smooth muscle contractility in the absence of IL-13 pretreatment. Gentle removal of the epithelium from tracheal rings prevented the development of IL-13-induced hyperresponsiveness. Immunoblot analysis indicated strong concentration-related increases in arginase I expression after 24 h exposure to IL-13 (10–100 ng/mL), with less expression seen at earlier time points. Immunohistochemical analysis of IL-13-treated tissues indicated arginase I expression in both epithelial and smooth muscle layers. IL-13-induced arginase I expression and hyperresponsiveness was abolished by the PI3K inhibitors wortmannin (100 nM) or LY294002 (10  $\mu$ M). Taken together, these observations suggest that arginase I may be involved in IL-13 induced-hyperresponsiveness through PI3K- and epithelial-dependent pathways. We acknowledge a fully-funded studentship to H.S.M.F. from the Egyptian Government.

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## S8.C002

### Regulation of inducible nitric oxide synthase expression by levo- and dextrosimendans

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Levosimendan is used to treat decompensated heart failure. It increases the contractility of the myocardium by sensitizing troponin C to calcium. In addition, levosimendan has been reported to have beneficial effects in experimental models of septic shock. Because heart failure and sepsis have been associated with excessive nitric oxide (NO) production through inducible nitric oxide synthase (iNOS) pathway, we investigated the effects of simendans on NO production and iNOS expression. In addition, effects on proinflammatory cytokines tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 6 (IL-6) were measured. Macrophages and fibroblasts were exposed to inflammatory stimuli to induce iNOS expression. Protein expression was detected by Western blot and mRNA expression was measured by quantitative reverse transcriptase/real time-PCR. iNOS promoter activity was investigated using luciferase reporter constructs. The activation of transcription factors was investigated by EMSA and luciferase reporter constructs. Cytokines were measured by ELISA. Levosimendan and its enantiomer dextrosimendan decreased iNOS expression and NO production in a dose-dependent manner. Simendans decreased iNOS mRNA expression and iNOS promoter activity, but did not affect iNOS mRNA decay. Simendans did not alter nuclear translocation or DNA binding of NF- $\kappa$ B or STAT1 (pivotal transcription factors for iNOS) but they inhibited NF- $\kappa$ B-mediated transcription as measured by reporter gene method. Simendans (at 10  $\mu$ M drug concentration) reduced slightly IL-6 production but they had practically no effect on TNF- $\alpha$  synthesis. The present study shows that levo- and dextrosimendan decrease iNOS expression and NO production by down-regulating iNOS promoter activity, and these cellular mechanisms may contribute to the beneficial clinical effects of levosimendan.

# Novel tricks for old enemies: anti-remodelling strategies for lung diseases (09.30–12.30)

## S9.1

### Modifying the Wnt system as a therapeutic approach for pulmonary fibrosis

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Despite recent improvements in diagnostic and therapeutic options, the pathogenesis of most major lung diseases including asthma, chronic obstructive lung disease, pulmonary fibrosis, or lung cancer remain incompletely understood and specific therapies are scarce.

Lung function is maintained by a functional interaction between epithelial and mesenchymal cell types, leading to tissue homeostasis of the bronchial, alveolar, vascular, and interstitial compartments. Key players in this delicate pulmonary microenvironment include epithelial cells and mesenchymal cells, such as interstitial and perivascular fibroblasts and myofibroblasts. These cell types are key regulators of the composition of the extracellular matrix (ECM), which in turn primes cell function within the lung.

The ECM is altered in all chronic lung diseases, such as asthma, chronic obstructive lung disease, pulmonary fibrosis and hypertension, or lung cancer, due to dysfunctional epithelial-mesenchymal crosstalk. Our understanding of soluble mediators involved in disturbed epithelial-mesenchymal interactions leading to altered ECM composition and lung structure in disease will thus enable novel therapeutic avenues. This seminar will demonstrate, using the example of Wnt signalling and the Wnt-induced signaling pathway protein (WISP) 1, a secreted growth factor with increased expression in pulmonary fibrosis, how implementation of cutting-edge molecular and cell biological technologies have led to elucidation of a novel disease-relevant target amenable to therapeutic intervention. Its effects on alveolar epithelial cells, as well as interstitial fibroblasts, *in vitro* and *in vivo*, will be detailed, and how therapeutic strategies of WISP1 neutralization led to attenuation of experimental lung fibrosis.

## S9.2

### Novel therapeutic targets in pulmonary arterial hypertension

N Morrell University of Cambridge School of Clinical Medicine, UK

Heterozygous germline mutations in the bone morphogenetic protein type II receptor (BMPR-II) are responsible for 80% of cases of familial pulmonary arterial hypertension (PAH). BMPR-II is a receptor the TGF-beta superfamily of receptors and transduces signals for a number of bone morphogenetic proteins (BMPs). The expression of BMPR-II is reduced in the pulmonary vasculature of patients with familial PAH, but also in patients with PAH in whom no mutation in the BMPR-II gene is detected. Studies in animal models have also confirmed the downregulation of BMPR-II in experimental PAH. Thus a critical reduction in BMPR-II expression and function may be a key feature of diverse forms of PAH. A reduction in BMPR-II expression leads to a reduction in signalling via the Smad1/5 proteins. Pulmonary artery smooth muscle cells derived from patients harbouring a mutation in BMPR-II are resistant to the growth suppressive effects of BMPs, which may contribute to the abnormal proliferation of vascular cells that characterise the pathology of PAH. In addition, growth factors such as platelet derived growth factor or serotonin can further inhibit BMP signalling and may contribute to the "second hit" required for disease manifestation. Some of the major targets of BMP signalling are transcription factors belonging to the inhibitors of DNA binding (Id) family. This key axis in pulmonary vascular cells involving BMPR-II/Smads/Id genes controls vascular cell proliferation and survival. Disruption of this axis leads to abnormal vascular function and proliferation and contributes centrally to the pathogenesis of PAH.

## S9.3

### The 'sweet and bitter' involvement of glycosaminoglycans in lung diseases

E Papakonstantinou Aristotle University of Thessaloniki, Greece

The extracellular matrix (ECM) plays a significant role in the structural integrity and functional behavior of the lung. The ECM is composed of various macromolecules, among which are the glycosaminoglycans (GAG). GAG are long, linear and highly charged heterogeneous polysaccharides that are composed of a variable number of repeating disaccharide units. There are two main types of GAG: sulphated GAG (heparan sulphate, heparin, chondroitin sulphate, dermatan sulphate, and keratan sulphate) and nonsulphated GAG (hyaluronic acid). With the exception of hyaluronic acid, GAG are usually covalently attached to a protein core, forming an overall structure that is referred to as proteoglycan. In the lungs, GAG are distributed in the interstitium, in the subepithelial tissue and bronchial walls, and in airway secretions. Their involvement in lung function includes the following: they regulate hydration and water homeostasis, provide structural stability, modulate the inflammatory response, and influence tissue repair and remodeling. Although many studies have described the role of proteoglycans in a wide range of pulmonary diseases, the contribution of GAG is much less well understood. There is evidence that depending on their structure and size GAG may either live up to their sweet taste and provide a protective effect against injury in various respiratory diseases, or in contrast, they may induce a 'bitter' effect, promoting inflammatory responses. The understanding of changes in GAG expression that occur in lung diseases may permit early diagnosis of ECM alterations and lung remodeling processes, and it may promote development of innovative and selective pharmacological therapeutic strategies.

## S9.4

### The role of the smooth muscle cell in airway remodelling in asthma

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The concept that the airway smooth muscle cell (ASMC) is a key player in the pathogenesis of asthma was postulated 90 years ago and was replaced by the hypothesis that asthma is due to a deregulated immune response of the lung to environmental stimuli. Recent studies revived the idea of deregulated ASMC as a major contributor to asthma. Histopathological studies showed a significant increase in number and size of ASMC in the upper airways of asthma patients. Isolated ASMC of asthma patients maintained several characteristics of inflammation which are the increase of proliferation, secretion of pro-inflammatory cytokines and growth factors, and deposition of extracellular matrix. They also show a different composition of the extracellular matrix compared to non-asthma control cells. Most asthma symptoms are well controlled by inhaled glucocorticoids, beta2-agonists and leukotriene antagonists. Glucocorticoids and leukotriene antagonists limit inflammation, while the beta2-agonists relax the airway. However, in regard to airway remodelling the effect of any of these classes of drugs is controversial. At least glucocorticoids seem to have a reducing effect on airway remodelling in long term studies. We reported recently that *in vitro* the activity of the glucocorticoid receptor in lung fibroblasts and ASMC is different when compared in active and inactive cells. Similarly, the deposition and composition of the extracellular matrix is affected by cell density and activity. In inactive cells glucocorticoids inhibit the deposition of extracellular matrix, while they stimulate it in active cells. In contrast beta2-agonists always inhibit extracellular matrix deposition.

## S9.5

### Role of actinins in lung pathophysiology

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Actinins, members of the TGF- $\beta$  superfamily, have been implicated in a diverse array of cellular processes including growth/differentiation, matrix production and inflammation. Activin-A has been considered, without being proven, a putative contributor in the pathogenesis of several respiratory disorders, including severe asthma, COPD and interstitial fibrosis. To validate functionally the capacity of Activin-A *in vivo*, we have used adenoviral gene transfer to transiently over-express Activin-A in the lungs of C57BL/6 mice and monitored changes in the structure and function of the lung tissue. Over-expression of human Activin A in the mouse airways resulted in the development of severe lung pathology that was characterized at the early stages by strong peri-vascular inflammation and gradual increase of peribronchial and alveolar inflammation, increased levels of neutrophils and macrophages in the airways, destruction of alveolar septa, and dramatic reduction in the lung compliance. The later stages were characterized by development of interstitial fibrosis and a further decline in compliance. These findings demonstrate that Activin-A can initiate a cascade of events that can damage the lung tissue and lead to the development of respiratory pathology. Thus, Activin-A is incriminated for the first time for the development of the respiratory and other diseases where increased Activin-A levels have been previously described.

## S9.C001

### A comparison between the incidences of respiratory distress syndrome in preterm infants who were born within 24 h of dexamethasone being administered to the mother vs. those mothers without

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The purpose of the present study is to compare between the incidences of respiratory distress syndrome in preterm infants who were born within 24 h of dexamethasone being first administered to the mother vs. those mothers without. A prospective study was performed on pregnant women with the complaint of preterm labor during 30–36 weeks of pregnancy and a probability of delivery within 24 h. In the dexamethasone group (100 cases), the drug was administered 5 mg every 12 h intra-muscularly. However, patients delivered within 24 h of the first dexamethasone administration and before finishing the course of treatment. The control group (70 patients), did not receive dexamethasone at all, and delivered within 24 h of admission. In the dexamethasone group the RDS were observed in 15% of neonates vs. 27.1% in the control group, which has a significant difference ( $P < 0.05$ ). Between 30–31.6 weeks of pregnancy, the rate of RDS in the neonates of the dexamethasone group were 18.9% vs. 35% in the control group ( $P = 0.001$ ). Between 32–33.6 weeks of pregnancy, the RDS were observed in 14.3% and 30.7% of neonates of the dexamethasone and control group respectively ( $P = 0.001$ ). Also between 34–36 weeks of pregnancy there were 10.7% RDS in the dexamethasone group vs. 16.7% in the control group ( $P = 0.004$ ), which for all gestational ages have statistically significant differences. RDS in male sex was more prevalent than female ( $P = 0.001$ ), and neonates who were born by cesarean section suffered from RDS more than vaginally born neonates ( $P = 0.005$ ). It is suggested to administer corticosteroids for the patients whose delivery is imminent, and it is thought that this occurred less than 24 h within the drug's administration.

**S9.C002**

**Human respiratory epithelial models for pulmonary drug delivery and toxicity studies**

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The respiratory tract is currently considered as an alternative to gastrointestinal or dermal drug delivery systems, due to the fast absorption and the absence of first pass metabolism characteristic of this tissue's physiology. For the drug development process, scarcity of good and reliable *in vitro* models (as an alternative for animal testing) remains a long standing problem. For this purpose Calu-3 culture conditions, such as culture medium, trypsinization, freezing/thawing and transport buffer selection were standardized in our laboratory. Effects of a popular solvent, dimethylsulfoxide (DMSO) were also analysed. We also performed preliminary transport experiments with the marker compounds: fluorescein, mannitol, rhoda-

mine123, digoxin and propranolol. In addition, compounds currently administered through the lung were analysed for their transport across the monolayer. The results obtained indicate that the Calu-3 cell line is a potential model for drug absorption studies, and high throughout screening of toxic compounds, drugs and pre-formulations in the upper respiratory tract. pAEPc (porcine Alveolar Epithelial primary Cells) have been previously isolated and characterized in our lab, and have also proven to be a good model. They consist of a mixture of type I and type II pneumocyte-like cells, and exhibit good barrier properties. Improvement of the model is currently taking place, regarding time of duration of the cell culture, cryopreservation or extent of cell differentiation, to set up a system that could be routinely used for drug transport studies. Financial support by EU Project-No.: MRTN-CT-2004-512229 'Pathogenesis of pulmonary disease' PULMONET is gratefully acknowledged.

# Myocardial receptor-mediated signalling in heart failure – new treatment modalities and individualised treatment (09.30–12.30)

## S10.1

### Receptor polymorphisms determine the kinetics of $\beta$ -adrenergic receptor conformational changes

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$\beta$ 1- and  $\beta$ 2-adrenergic receptors ( $\beta$ ARs) mediate crucial catecholamine effects in the body and thus play a key role in the sympathetic nervous system. Several frequently occurring polymorphisms have been identified whose clinical relevance is controversially discussed. The most important are Ser49Gly and Gly389Arg for the  $\beta$ 1AR, Arg16Gly and Gln27Glu for the  $\beta$ 2AR. In order to investigate the impact of these single amino acid changes on the receptor level we generated mutant  $\beta$ AR variants which contain the cyan- and yellow-emitting variant of the green fluorescent protein (CFP/Cer and YFP). By using a fluorescence resonance energy (FRET) approach we could determine the activation characteristics of the polymorphic receptors in real time and in living cells. Transfection of both receptor sensors in HEK293 cells showed proper expression at the cell membrane. Activation of the FRET receptors by norepinephrine lead to a symmetrical increase in CER emission and decrease in YFP emission and consequently to a decrease of the FRET ratio. Concentration response curves of  $\beta$ 1AR- and  $\beta$ 2AR-sensor activation for isoproterenol gave EC50 values which are in good agreement with radioligand binding studies performed with the native receptors. Furthermore they retained the functionality of the wild-type receptors with respect to cAMP production. We found, that receptor polymorphisms critically determine the kinetics of receptor activation and inactivation upon stimulation by agonists. These data suggest that naturally occurring receptor polymorphisms critically determine the kinetics of activation and deactivation of a G protein coupled receptor with likely consequences for its signalling properties.

## S10.2

### Beta-1- and Beta-2-adrenoceptor polymorphisms and cardiovascular diseases

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Beta-1- and beta-2-adrenoceptors (AR) play a pivotal role in the regulation of cardiovascular function. Both betaAR subtypes are polymorphic: Two single nucleotide polymorphisms (SNPs) have been described for the beta1- (Ser49Gly/Arg389Gly) and four for the beta2AR (Arg-19Cys/Arg16Gly/Gln27Glu/Thr164Ile) that might be of functional relevance. In recombinant cell systems Gly49beta1AR is more susceptible to agonist-promoted downregulation and Arg389beta1AR is 3–4 times more responsive to agonist-evoked stimulation. With respect to beta2AR, Cys-19 is associated with greater beta2AR expression; Gly16 is more susceptible, whereas Glu27 is almost resistant to agonist-promoted downregulation and Ile164 is 3–4 times less responsive to agonist-evoked stimulation. Several studies addressed the possibility that these SNPs might have phenotypic consequences *in vivo*; influencing and/or contributing to the pathophysiology of cardiovascular/pulmonary diseases (hypertension, congestive heart failure, arrhythmia or asthma). However, at present it appears that these SNPs are very likely not disease-causing genes but predictive for the responsiveness to agonists/antagonists. Patients homozygous for the Arg389beta1AR are good responders to agonists as well as antagonists whereas patients for the Gly389beta1AR are poor or non-responders. Subjects heterozygous for the Thr164Ilebeta2AR variant exhibit blunted responses to beta2AR stimulation while asthma patients carrying the Arg16/Gln27/Thr164beta2AR haplotype and receiving regularly short- or longacting beta2AR agonists appear to be rather susceptible to agonist-induced desensitization and in consequence reduced bronchodilating/protective effects and/or increased asthma exacerbations. Nevertheless, the clinical relevance of such findings is still under discussion.

## S10.3

### Regulation of the failing cardiac ventricle through 5-HT2A and 5-HT4 receptors: mechanisms and possible therapeutic consequences

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In heart failure, cardiac responsiveness to neurohumoral stimulation is changed. We recently found functional 5-HT4 receptors coupled to a positive inotropic response in porcine and human cardiac ventricle, with increased 5-HT4 mRNA in heart failure. Whereas the normal rat cardiac ventricle does not respond to serotonin, functional 5-HT4 receptors are expressed after myocardial infarction and in chronic heart failure and mediate a positive inotropic response to serotonin. In acute heart failure, the rat cardiac ventricle expresses both 5-HT4 and 5-HT2A receptors, which mediate inotropic responses through different mechanisms. The 5-HT4 receptor mediates a positive inotropic as well as a lusitropic response dependent on cAMP, comparable to stimulation of beta-adrenoceptors. The 5-HT2A receptor mediates a positive inotropic response without a lusitropic response, comparable to stimulation of alpha-1-adrenoceptors. This inotropic response involves phosphorylation of myosin light chain, probably resulting in sensitisation of the myofilaments to Ca<sup>2+</sup>. Based on the similarity of 5-HT4- and beta-adrenoceptor-mediated effects in the failing cardiac ventricle and the beneficial effects of beta-adrenoceptor blockade in heart failure we examined the effects of treatment with a 5-HT4 receptor antagonist in rats with chronic post-infarction heart failure. We observed reduced cardiac remodelling, consistent with a beneficial effect of treatment with a 5-HT4 antagonist in heart failure. These results have been followed up by a randomised clinical trial, evaluating the safety and efficacy of treatment with the 5-HT4 receptor antagonist piboserod in patients with heart failure.

## S10.4

### Targeting myocardial alpha-1-adrenergic receptors to treat heart failure

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Heart failure (HF) is a major clinical problem, and new treatments are needed badly. Current HF therapy emphasizes beta-adrenergic receptor (AR) blockade to prevent

toxic cardiac effects of catecholamines from the sympathetic nervous system. However, catecholamines also activate cardiac alpha-1-ARs, which have been relatively neglected, because heart alpha-1-AR levels are much lower than beta-ARs, and alpha-1-ARs have been classically associated with smooth muscle contraction. Nevertheless, alpha-1-AR knockout and transgenic mice and human clinical trials with alpha-1-antagonists have all come together to suggest the novel idea that cardiac alpha-1-AR activation is adaptive and protective. Cardiac myocyte alpha-1-ARs stimulate adaptive hypertrophy, protect against hemodynamic stress, and prevent myocyte death from apoptosis and necrosis. Increased cardiac myocyte alpha-1-stimulation by endogenous catecholamines might in part explain the beneficial effects of beta-AR-blockers. Alpha-1-ARs exist as three molecular subtypes, named A, B, and D, with the A and B subtypes predominant in myocardium, and the D subtype in coronary arteries. Recent studies focus on the alpha-1B subtype in adaptive hypertrophy, and the alpha-1A subtype in cardio-protection. An alpha-1A-selective agonist can prevent cardiomyopathy in a mouse model, without changing blood pressure. In summary, a new model suggests that future HF treatment might include alpha-1-AR stimulation, as well as beta-AR blockade.

## S10.C001

### Dual critical role of angiotensin AT-1 and serotonin 5-HT<sub>2B</sub> receptors in non-cardiomyocytes for beta-adrenergic agonist induced pathological left-ventricular hypertrophy

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We previously demonstrated that pharmacological inhibition or genetic ablation of the 5-HT<sub>2B</sub> receptor (5-HT<sub>2BR</sub>) in global knockout mice abolished  $\beta$ -adrenergic agonist (isoproterenol, ISO)-induced pathological cardiac hypertrophy (CH) *in vivo*. However, cellular type expressing 5-HT<sub>2BR</sub> involved in this process is unknown. Thus, we generated new transgenic mice with a 5-HT<sub>2BR</sub> expression restricted to cardiomyocytes (CMs). We showed that mice with genetic ablation of serotonin 5-HT<sub>2BR</sub> exclusively in non-CM cells (Tg-5-HT2B<sup>-/-</sup>) were resistant to ISO (30  $\mu$ g/g/day, 7 days)-induced left ventricular mass:body weight ratio increase (4.4 mg/g  $\pm$  0.3 at day 7 vs. 4.2  $\pm$  0.2 at d0, n = 7) compared to mice with a global 5-HT<sub>2BR</sub> expression (tg-5HT2B<sup>+/+</sup>) (6.1 mg/g  $\pm$  0.4 at day 7 vs. 4.6  $\pm$  0.2 at day 0, P  $\leq$  0.01, n = 7). Importantly, only the Tg-5-HT2B<sup>-/-</sup> mice exhibited a fully conserved cardiac function after 7 days of ISO infusion as assessed by the measurement of the systolic ejection volume (27  $\mu$ L  $\pm$  2 at day 0 vs. 26  $\mu$ L  $\pm$  3 at day 7 and 29  $\mu$ L  $\pm$  3 at day 0 vs. 22  $\mu$ L  $\pm$  2 at day 7 respectively, P  $\leq$  0.05). In parallel, we also uncovered that angiotensinogen null-mice (Agt<sup>-/-</sup>) did not develop CH after ISO infusion (4.3  $\pm$  0.3 at day 7 vs. 4.5  $\pm$  0.2 at day 0, n = 5) compared to the wildtype mice (WT) (5.9  $\pm$  0.4 at day 7 vs. 4.4  $\pm$  0.1 at day 0, P < 0.01, n = 5). Moreover, ISO (10  $\mu$ M)-dependent hypertrophic cytokine release was abolished in Agt<sup>-/-</sup> CFs culture or was prevented by the AT-1R antagonist ZD7155 (100 nM) in WT CFs cultures. Finally, a significant correlation between 5-HT<sub>2B</sub> ventricular overexpression, plasma cytokine concentrations, and sympathetic activity was observed in patients with congestive heart failure. These results highlight that interactions between AT-1 and 5-HT<sub>2B</sub> receptors co-expressed by non-cardiomyocyte cells are key downstream events in adrenergic agonist induced—angiotensin-dependent—pathological CH, and suggest that 5-HT<sub>2B</sub> receptor antagonists may represent novel effective therapeutics to treat heart failure.

## S10.C002

### Phosphodiesterases regulating the negative inotropic response of C-type natriuretic peptide in heart failure

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The circulating levels of natriuretic peptides are increased in heart failure. C-type natriuretic peptide (CNP) and brain natriuretic peptide (BNP) activate NPR-B and NPR-A receptors, respectively. The role of CNP in heart failure is not fully understood. Earlier studies have shown that CNP elicits a negative inotropic response by activating the cGMP - protein kinase G (PKG) pathway. The phosphodiesterases (PDEs) potentially degrading cGMP in the heart are PDE1, 2, 3 and 5. The role of PDEs in the regulation of the negative inotropic response to CNP has not been investigated. In this study we address the role of PDE2 and PDE3 in the regulation of the negative inotropic response to CNP. We also investigated the negative inotropic response to BNP. Muscle contraction and total cGMP levels were measured in left ventricular muscle strips from male Wistar rats with congestive heart failure (CHF) 6 weeks after myocardial infarction. In a concentration-dependent manner, CNP increased cGMP levels and caused a negative inotropic response, which was reduced by the PKG blocker Rp-8-Br-PET-cGMP (1  $\mu$ M), demonstrating involvement of PKG. BNP also increased cGMP levels concentration-dependently, but elicited no negative inotropic response. The presence of the PDE2 inhibitor EHNA (10  $\mu$ M) or the PDE3 inhibitor cilostamide (1  $\mu$ M) did not enhance the cGMP increase to CNP beyond an additive effect, whereas the negative inotropic effect of CNP was increased by inhibition of PDE3, but not PDE2. Combined PDE2 and PDE3 inhibition showed an amplified cGMP increase by CNP with no further increase in negative inotropic response compared to the effect of CNP in the presence of PDE3 inhibitor alone. Despite comparable effects on cGMP levels, CNP and BNP stimulation do not cause the same negative inotropic response in CHF muscle strips, indicating compartmentation of the signal. This is supported by a mismatch between the effects of PDE inhibition on cGMP levels and negative inotropic response after CNP stimulation. We conclude that there is a strong compartmentation of the cGMP activating the PKG pathway and causing negative inotropic response in failing hearts.

# Symposia Presentations

## Wednesday 16 July

### Receptor heteromerization: new challenges for pharmacology (09.30–12.30)

#### S11.1

##### GPCR dimerisation: molecular basis and relevance for function and pharmacology

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It is now well established that monomers of G protein-coupled receptors can interact to form both homo- and hetero-dimers. Combinations of molecular modelling, chimeric constructs and interactions between fragments of receptors have generated information on key elements responsible for such interactions with transmembrane domains 4 and 5 frequently being implicated. One key role of dimerisation appears to be in receptor biogenesis and trafficking of the receptor complex to the membrane. Both mutated receptors associated with disease that inadvertently become trapped in the endoplasmic reticulum and deliberate modification of receptors to add endoplasmic reticulum trapping motifs result in such variants acting as dominant negatives that function to prevent cell surface delivery of co-expressed wild type forms of the same receptor and may be employed to explore the selectivity of G protein-coupled receptor hetero-dimerisation. Studies on the class C, metabotropic glutamate-like receptors have indicated that binding of a single molecule of agonist is sufficient to produce functional responses via G protein-coupled receptor dimers. Strategies based on co-expression of a wild type  $\beta_2$ -adrenoceptor and a variant of this receptor that is only able to bind and be activated by a synthetic small molecule agonist that does not interact with the wild type receptor indicate that a similar model is likely true for class A rhodopsin-like G protein-coupled receptor homo-dimers. Co-expressed pairs of class A G protein-coupled receptors that can form hetero-dimers often display pharmacological characteristics distinct from the corresponding homo-dimers. In a number of cases this is likely to reflect allosteric interactions across the interface of the hetero-dimer and suggest that such hetero-dimers may be potential targets for therapeutic intervention in a tissue-selective fashion.

#### S11.2

##### The two-state dimer model for G-protein-coupled receptors: new perspectives for academic research and for drug development

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Drugs targeting G-protein-coupled receptors are developed assuming that receptors are monomers but they are homo/heteromers. Drug discovery must take homo/heteromers into account to broaden therapeutic profiles and get fewer side-effects. G-protein-coupled receptors are targeted by a significant number of therapeutic drugs marketed to fight against a variety of diseases. Selection of novel lead compounds are based on pharmacological parameters obtained assuming that GPCR are monomers. However, many G-protein-coupled receptors (GPCRs) are expressed on the plasma membrane as dimers/oligomers. Therefore, drug development must consider GPCR as homo- and hetero-oligomers. A novel model, which considers dimers instead of monomers, has been developed that explains receptor operation: from ligand binding to signaling. This model and the strategy of considering receptor dimers would lead to broaden the therapeutical potential, identifying novel pharmacological profiles and decreasing the side effects of new drugs. Cooperativity and allostery will be presented as alternatives to current models assuming high- and low-affinity binding sites.

#### S11.3

##### Disruption of cell surface dopamine receptor oligomers, as visualized in living cells

TBC

G protein coupled receptors (GPCRs) form oligomers, however the details required for the formation of these oligomers, for hetero- or homo-oligomers, are unknown. There is little evidence regarding modulation of oligomer forms at the cell surface, and whether the constituents of homo- or hetero-oligomers remain together, following agonist treatment. We developed a method to study dopamine receptor oligomers, we inserted a nuclear localization sequence (NLS) into a GPCR, and showed oligomerization occurred when it co-translocated to the nucleus with a GPCR, not containing a NLS. Fluorophore-tagged D1 expressed with D1(NLS), or D2(NLS), translocated to the nucleus, and revealed D1/D1(NLS) and D1/D2(NLS) oligomers. Placement of the NLS in helix eight rendered these receptors conformationally sensitive. Antagonist and agonist such as (+)BTC or dopamine, retained D1(NLS) at the surface, and ligand removal allowed translocation of intact oligomers from the surface to the nucleus. In cells expressing D1 and D2 (NLS), treated with (+)BTC (which targets both D1 and D2) both receptors were retained at the cell surface. Removal of (+)BTC allowed D2(NLS) to traffic from the surface, and due to hetero-oligomerization, D1 also translocated. Using this method we examined the dynamics of receptor oligomers, and visualized whether the size of oligomers were functionally regulated. We demonstrated that D1-D2 hetero-oligomers were separated by agonist treatments, and also that these oligomers reformed following agonist removal.

#### S11.4

##### Cross talk between receptor tyrosine kinase and G-protein coupled receptors

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G-protein coupled receptors (GPCRs) and receptor tyrosine kinase (Trk) have distinct structure and transducing mechanisms; therefore, cross talk among them was unexpected. First hints appeared while reporting that at nerve-muscle cultures cAMP gated the synaptic action of BDNF, an endogenous Trk ligand (Booher and Poo, 1999). A2A adenosine receptors are positively coupled to cAMP and devoted to interact with other modulators (Sebastião and Ribeiro, 2000). Whether A2A receptors could influence Trk receptor mediated actions was therefore evaluated.

Adenosine A2A receptor antagonists prevent BDNF facilitatory actions on transmission (Diógenes *et al.*, 2004) and LTP (Fontinha *et al.*, 2008) at hippocampal synapses, and at the neuromuscular junction (Pousinha *et al.*, 2006); this A2A triggering of BDNF actions is PKA-dependent. The age-related changes of the ability of BDNF to influence hippocampal synaptic transmission could be related to changes of adenosine A2A receptor mediated actions (Diógenes *et al.*, 2007). A2A receptors also trigger synaptic actions of other neurotrophic factors at other brain areas, such as GDNF at dopaminergic striatal nerve endings (Gomes *et al.*, 2006). Therefore, tonic adenosine A2A receptor activity is a required step to allow synaptic actions of neurotrophic factors. Supported by FCT and Regeneron.

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#### S11.C001

##### The GABA<sub>B</sub> receptor agonist, baclofen, and the positive allosteric modulator, CGP7930, inhibit visceral pain-related responses to colorectal distension in rats

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Baclofen, acting on GABA<sub>B</sub> receptors, produces somatic analgesia. We evaluated the effects of baclofen and the GABA<sub>B</sub> positive allosteric modulator, CGP7930, on pseudo-affective responses to colorectal distension (CRD) in conscious rats. Sprague-Dawley rats were subjected to repeated, noxious CRD (12 × 80 mmHg). The visceromotor (VMR) and cardiovascular responses (arterial blood pressure and heart rate) to CRD were monitored in telemetrized animals as markers for visceral pain. The pressure-volume relationship during CRD (0–20 mmHg) was used as a measure of colonic compliance. Oral baclofen (1, 3 or 10 μm/kg; n = 12 each) reduced the VMR to CRD by 15%, 13% and 35% (P < 0.05 vs. vehicle), respectively. In telemetrized rats, baclofen (10 μmol/kg, po, n = 6) simultaneously reduced the VMR (37% inhibition) and the hypertensive (52% inhibition) and tachycardic responses (56% inhibition) to CRD (all P < 0.05 vs. vehicle). Likewise, intravenous baclofen (0.3, 1 or 3 μm/kg; n = 8 each) inhibited the VMR to CRD and, in telemetrized rats, prevented the rise in blood pressure and inhibited the tachycardic response to CRD. The GABA<sub>B</sub> positive allosteric modulator, CGP7930, (3, 10 or 30 μm/kg, i.v.) reduced the VMR to CRD by 3%, 29% (P < 0.05 vs. vehicle) and 31% (P < 0.05 vs. vehicle), respectively. At 30 μm/kg, CGP7930 attenuated the rise in blood pressure (18%) and inhibited by 24% (P < 0.05 vs. vehicle) the tachycardic response to CRD. Neither baclofen (3 μm/kg, i.v.) nor CGP7930 (30 μm/kg, i.v.) affected pressure-volume relationships during CRD. Activation of GABA<sub>B</sub> receptors, either by full agonism or by positive modulation, produces anti-nociceptive effects in a rat model of visceral pain. Targeting GABA<sub>B</sub> receptors may represent a valid approach in the treatment of visceral pain conditions.

#### S11.C002

##### TNF $\alpha$ regulation of proteinase activated receptor-2 and -4 heterodimerisation

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G-protein coupled receptors (GPCRs), including members of the proteinase-activated receptor (PAR) family, can form homo- and heterodimers (Leger *et al.*, 2006 and Kaneider *et al.*, 2007). Previous work in our laboratory has demonstrated dual upregulation of PAR-2 and PAR-4 in HUVECs following treatment with TNF $\alpha$  (Ritchie *et al.*, 2007) and PAR-2/PAR-4 heterodimerisation in HEK293 cells (Cunningham *et al.*, 2007). This study investigates the potential for TNF $\alpha$  to regulate PAR-2/PAR-4 heterodimerisation. Receptor interaction was detected from single cells using wide field FRET microscopy. FRET images were corrected and ratiometric FRET (RFRET) values quantified using Youvan's algorithm (Youvan *et al.*, 1997), with an RFRET value >1 indicating FRET occurrence. In HEK293 cells FRET was detected between PAR-4 CFP and PAR-2 YFP (RFRET value 1.88 ± 0.035, P < 0.01, n = 24). Surprisingly, when expressed in NCTC2544 cells FRET was negligible (RFRET value 1.11 ± 0.023), despite notable translocation of intracellular PAR-4 to the membrane when co-expressed with PAR-2. When treated with TNF $\alpha$  (10 ng/mL) PAR-2 YFP and PAR-4 CFP were co-internalised and FRET detected (RFRET value 1.41 ± 0.043). These findings suggest that PAR-2 may be required to drive PAR-4 expression to the plasma membrane. In addition, this study also implicates an important regulatory role for TNF $\alpha$  in PAR-2/PAR-4 heterodimerisation and internalisation of the complex.

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# Calcium sensing receptors: pharmacology, function, and medication (09.30–12.30)

## S12.1

### Calcium, allosteric modulators and calcium sensing receptor activity

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The  $\text{Ca}^{2+}$  sensing receptor (CaR) senses minor changes in ionized  $\text{Ca}^{2+}$  in extracellular fluids and represents the major molecular determinant of  $\text{Ca}^{2+}$  homeostasis. The CaR belongs to the class III G-protein coupled receptors (GPCRs), which includes receptors for pheromones, sweeteners, the neurotransmitters glutamate and  $\gamma$ -aminobutyric acid and for basic amino acids (GPCR6A). These receptors are characterized by a long extracellular aminoterminal domain called a Venus flytrap module (VFTM) containing the ligand binding pocket. Naturally occurring activating and inactivating CaR mutations are responsible of genetic diseases linked to disturbance of  $\text{Ca}^{2+}$  homeostasis and have been helpful for understanding the CaR activation process. Interestingly, functional analysis of CaR mutants developed from its VFTM model indicates that  $\text{Ca}^{2+}$  does not interact with amino acids homologous to those delineating the glutamate binding cavity in mGluRs, but with a site adjacent to this cavity. Calcimimetics (NPS R-568, Calindol) and calcilytics (NPS 2143, Calhex 231) have been recently developed and proposed to be of therapeutic interest for diseases linked to perturbation of  $\text{Ca}^{2+}$  homeostasis such as hyperparathyroidism and osteoporosis. These allosteric modulators interact at the level of the transmembrane domains (TMs) of the CaR. The ligand binding pockets of these molecules are overlapping but not identical. The close homology of the CaR and the GPCR6A TMs and pharmacological experiments suggest that calcimimetics may also modulate GPCR6A. Altogether, these data further highlight the molecular mechanisms involved in class III GPCR activation and its regulation by allosteric modulators that represent potentially important molecules in therapeutic.

## S12.2

### Calcium-sensing receptors in the vasculature: pharmacology and function

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The role of the extracellular calcium-sensing receptor (CaR) in calcium homeostasis is now well established. Although initial studies focussed on the parathyroid gland and the kidneys, recent investigations have highlighted that the mammalian vasculature possesses two distinct  $\text{Ca}^{2+}$ -sensing receptors. One of these, the classical CaR, is largely restricted to the vascular endothelium whereas the related G protein-coupled receptor, designated GPCR6A, is present in both the endothelial cells and myocytes of blood vessels. In the vascular endothelium, activation of both the CaR and GPCR6A results in the selective opening of TRAM34-sensitive, intermediate conductance,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (IKCa) with subsequent myocyte hyperpolarization and relaxation. The inability of the CaR to activate the small conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels that are also found in vascular endothelial cells is consistent with the co-localisation of the CaR and IKCa proteins in non-caveolin fractions of endothelial cells. This contrasts with the SKCa channels that are found in the caveolin-rich components of these cells. The positive allosteric modulator, calindol, activates both the CaR and GPCR6A while L-ornithine is a diagnostic activator of GPCR6A. The circulatory function of the CaR and GPCR6A is still unclear but a role in blood pressure control is likely. Consistent with isolated vessel studies, the positive allosteric modulator NPS R568 is hypotensive in SHR rats and the CaR protein is down-regulated in a rat model of Type 2 diabetes, a condition that is associated with hypertension in man.

## S12.3

### Characterization of GPCR6A – a 7TM receptor activated by L-alpha-amino acids and positively modulated by divalent cations

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We recently reported the cloning and analysis of expression of a novel human family C 7TM receptor, termed hGPCR6A. To identify agonists at this orphan receptor we faced the challenges of achieving surface expression in mammalian cell lines and establishing an appropriate functional assay. Generating a chimeric receptor construct, h6A/5.24, containing the ligand binding amino-terminal domain (ATD) of hGPCR6A with the 7TM domain of the homologous goldfish 5.24 receptor allowed us to overcome these obstacles. Homology modeling of the hGPCR6A ATD based on the crystal structure of the mGluR1 predicted interaction with alpha-amino acids, and was employed to rationally select potential ligands. Measurement of calcium-dependent chloride currents in *Xenopus laevis* oocytes facilitated the deorphanization of h6A/5.24 and identification of L-alpha-amino acids as agonists. The most active agonists were basic L-alpha-amino acids, L-Arg, L-Lys and L-ornithine, suggesting that these may function as endogenous signaling molecules. Mutation of two specific residues of the hGPCR6A ligand binding pocket completely abolishes activity of the chimera, supporting the proposed model of hGPCR6A. Cloning, cell surface expression and deorphanization of the mouse and rat orthologues further reinforces the assignment of the agonist preferences of hGPCR6A. Most recently, we have developed efficient assays based on measurement of intracellular calcium and inositol phosphate levels in mammalian cells, which have been used to characterize a range of commercially available L-Arg and L-Lys analogs and allosteric modulation by divalent cations.

## S12.4

### Calcimimetics for hyperparathyroidism in patients receiving haemodialysis

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The clinical introduction of calcimimetics has led to a change in the treatment of secondary hyperparathyroidism in chronic haemodialysis patients. The long-term administration of the calcimimetic cinacalcet proved to be superior to « optimal » standard therapy, in that it induced a decrease in both plasma iPTH and the  $\text{Ca} \times \text{P}$  product, in contrast to the effects of active vitamin D sterols which often increase the  $\text{Ca} \times \text{P}$  product. The effect of cinacalcet is long-lasting. It generally is given together with either calcium-containing or non-calcium containing phosphate binders and/or with active vitamin D derivatives, under the condition that serum Ca and phosphorus remain well controlled. Cinacalcet may become the first treatment option for uraemic secondary hyperparathyroidism in association with elevated plasma calcium and phosphorus levels, and possibly even in patients with normal serum calcium levels. Gastrointestinal side effects such as nausea, vomiting, and diarrhea may occur. They disappear in the majority of patients with time. Attention needs to be paid to avoid hypocalcaemia. The introduction of low cinacalcet doses and frequent serum calcium monitoring generally allows one to avoid symptomatic hypocalcaemia. Of note, a cinacalcet trial in patients with chronic kidney disease stages 3–4 was interrupted because of frequent hypocalcaemic episodes and a trend towards hyperphosphataemia. An important issue is that of cinacalcet's effects on patient outcomes. Two randomised controlled trials are in progress to answer this question. In conclusion, the introduction of cinacalcet allows a better control of hyperparathyroidism in dialysis patients.

## S12.C001

### Amino acids and the rat mesenteric arterial calcium-sensing receptor

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The presence and functional activity of the extracellular calcium-sensing receptor (CaR) in the rat mesenteric artery has been demonstrated (Weston *et al.*, 2005, Geraghty *et al.*, 2007). Conigrave *et al.* (2000) found that an L-amino acid mixture of composition similar to that of fasting human plasma mobilises intracellular  $\text{Ca}^{2+}$  in HEK293 cells that over-express the CaR (CaR-HEK293). In the present study we investigated whether a similar amino acid mix could activate the CaR in rat mesenteric arteries. Mesenteric artery branches (200–300  $\mu\text{m}$ ) were dissected from male Wistar rats (250–300 g) following  $\text{CO}_2$  asphyxiation and cervical dislocation. Vessels were either fixed and stained for CaR immunofluorescence or used for pressure myography. All values are given as mean  $\pm$  SEM. A two-way repeated measures ANOVA with post hoc Bonferroni test was used;  $P < 0.05$  was considered significant. Immunohistochemical studies confirmed the presence of the CaR in the rat mesenteric artery. In pressure myograph experiments, increasing extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o$ ; 1–10 mM) induced vasorelaxation of pre-constricted vessels ( $4 \pm 9.2$  mm U46619, a  $\text{TXA}_2$  agonist,  $n = 4$ ). Calhex 231, a negative allosteric modulator of the CaR, significantly inhibited this relaxation [ $4$ – $10$  mM ( $\text{Ca}^{2+}$ );  $n = 4$ ] demonstrating the functional activity of the CaR in these arteries. Increasing concentrations of the ten most potent L-amino acids (Conigrave *et al.*, 2000) in the ('fasting rat') mixture had no effect on the vascular tone of pre-constricted arteries ( $4 \pm 2$  mm U46619;  $n = 4$ ). A 'post-prandial' amino acid mixture (Agli *et al.*, 1998) had no effect on a U46619 (0.1–300 nM) concentration-response curve ( $P > 0.05$ ;  $n = 4$ ). Although amino acid mixtures activate the CaR in CaR-HEK293 cells (Conigrave *et al.*, 2000), they had no effect on vascular tone in the present study. However, local  $[\text{Ca}^{2+}]_o$  could be  $> 1$  mM in contracted vessels (Hofer, 2005) and further experiments in the presence of higher  $\text{Ca}^{2+}$  concentrations are planned.

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## S12.C002

### Insights into Class III GPCR function from studies of the T1R taste receptor family

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Mammals use Class III G protein-coupled receptors (GPCRs) to detect and transduce sweet and umami taste stimuli. Both native receptors are heterodimers containing one shared (T1R3) and one unique GPCR: T1R1 for umami and T1R2 for sweet. Both subunits of each receptor are required for normal stimulus sensitivity and selectivity. The T1R1:T1R3 umami receptor detects stimuli such as monosodium glutamate (MSG) and other L-amino acids and is highly selective for L- over D-amino acids. The T1R2:T1R3 sweet receptor binds a wide variety of structurally distinct ligands, including sugars, high-potency sweeteners and sweet proteins. The use of human rodent chimeric T1Rs has helped clarify the roles of each subunit in the detection and transduction of some sweet stimuli that display species specificity (e.g., aspartame, cyclamate, brazzein). However, the specific contributions of T1R2 and T1R3 to the detection of sweet stimuli preferred by both humans and rodents (e.g., sugars) is less well understood. We developed an *in vitro* system for quantifying ligand interactions with T1R proteins and found that sugars bind to the extracellular N-terminal domains of both T1R2 and T1R3, and do so at physiologically-relevant concentrations. Furthermore, each T1R subunit exhibits distinct changes in protein structure upon binding of sugar ligands. We conclude that both T1R2 and T1R3 function to bind sugars, with each subunit contributing unique structural properties that permit the T1R2:T1R3 heteromeric receptor to efficiently transduce natural sugar stimuli.

# Novel approaches to the treatment of Parkinson's disease (09.30–12.30)

## S13.1

### Present understanding in mechanism of neurodegeneration in Parkinson's Disease

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## S13.2

### Non-human primate models of motor and non-motor symptoms of Parkinson's disease

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Parkinsonian patients present a myriad of motor and non-motor symptoms, both receiving now particular attention from the clinicians and the industry to meet the needs. In this context, we need to appraise the relevance and validity of the experimental models. Here, I will review the pros and cons of the different non-human primate species currently in use as well as the main regimens of neurotoxin administration, referring to the specific symptoms they generate or enable to study. Of particular interest is the fact that experimental parkinsonism in non-human primates reproduces the complexity of Parkinson's disease symptoms and offers a venue for validating therapeutic interventions.

## S13.3

### Cell models of genetic Parkinson's disease for testing neuroprotective therapeutic agents

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While most Parkinson's disease in the clinic is apparently sporadic, rare genetic forms have greatly clarified pathogenesis, and are fostering the development of experimental therapeutics. Two of the major genes whose mutations can cause PD are alpha-synuclein and LRRK2. Alpha-synuclein aggregates are present in Lewy bodies in both sporadic and familial disease. We have developed cell models inducibly expressing mutant alpha-synuclein (A30P, A53T, or E46K), and studied mechanisms of cell death. Cell death mechanisms include mitochondrial impairment, proteasome inhibition, ER stress activation, and increases in reactive oxygen species. Baicalein has previously been reported to inhibit aggregation of alpha-synuclein. We have confirmed these effects using *in vitro* preparations of E46K alpha-synuclein. Baicalein strikingly inhibits formation of aggregates containing beta structure and inhibits formation of fibrils. We have now found that baicalein strikingly inhibits cell death in the inducible PC12 cell model. This may make baicalein or related compounds interesting candidates to test in mouse models. We have also been studying mutant LRRK2 in cell models. Mutant LRRK2, but not wild type LRRK2, causes striking cell toxicity. Cell toxicity is at least in part dependent on kinase and GTPase activity of LRRK2, and mediated by interaction with other proteins within the cell. Furthermore, in a *Drosophila* model, mutant LRRK2 causes striking and relatively selective degeneration of dopaminergic neurons. Thus LRRK2 kinase activity, GTPase activity or other protein interactions may represent interesting therapeutic targets. Cell models can help elucidate pathogenesis of PD, and contribute to development of experimental therapeutics.

## S13.4

### Exogenous modulation of adult neurogenesis in preclinical models of Parkinson's disease

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Neurogenesis persists in the adult subventricular zone (SVZ)/olfactory bulb (OB) and in the hippocampus. A decreased proliferation of neural precursor cells was described in the SVZ of Parkinson disease (PD) patients as well as in PD animal models. The aim was to decipher the potential of oral application of the D2/D3 receptor agonist pramipexole (PPX) to modulate adult neurogenesis in a PD model. 6-Hydroxydopamine lesioned adult rats received either PPX (1.0 mg/kg) or PBS orally and bromodeoxyuridine for 10 days and were perfused after treatment or 4 weeks after PPX withdrawal. Analysis revealed a significant augmentation in SVZ proliferation by PPX. Consecutively, enhanced neuronal differentiation and more newly generated neurons were present in the OB 4 weeks after PPX withdrawal. Dopaminergic neurogenesis was increased in the OB. Oral PPX treatment selectively increases adult neurogenesis in the SVZ/OB system. Deciphering the neurogenic potential of D2/D3 mediated pathways may lead to new avenues to induce neural repair. One of the most exciting ideas for endogenous repair is: i) to direct newborn cells to diseased regions and ii) to locally differentiate into the specific neuronal phenotype that succumb due to the disease. We show that application of the growth factors EGF and FGF-2 increases neurogenesis in the OB, promote migration of newly-generated DCX-positive neuroblasts from the SVZ into the adjacent striatum,

however fail to obtain a dopaminergic phenotype. Both studies indicate that exogenous applied compounds are capable of inducing cellular repair mechanisms.

## S13.C001

### Behavioural tests to reveal long-term functional benefit of potentially neuroprotective compounds in cerebral ischemia in mouse and stroke

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All compounds showed neuroprotective in models of ischemia on the basis of short-term reduction in infarct volume have failed in clinical trials. Therefore a better evaluation of therapeutic strategies including long term behavioral outcome is needed. In this context, the present study aimed to select sensorimotor tests that show long lasting deficits after cerebral ischemia in mice. Male Swiss mice (27–30 g) anesthetised with ketamine and xylazine hydrochloride (50 mg/kg and 6 mg/kg ip respectively) underwent intraluminal occlusion of the left middle cerebral artery ( $n = 19$ ). Sham-operated mice ( $n = 11$ ) and non operated mice ( $n = 7$ ) were also included in the study. Behavioral tests were assessed at day 2 after ischemia and once a week until day 56 after the surgery. The neurological score evaluates sensorimotor reflexes. Beam walking tests assess the capacity of the mouse to walk on beams of different wide (1, 2 and 3 cm) and beam balance tests the ability to balance on a rectangular or a cylindrical beam. The pole test values the capacity of the mouse to go down a vertical wooden pole. For the chimney test, the mouse has to back up a vertical glass tube. The grip test measures the time during which the mouse hangs on a horizontal string and the string test evaluates the way the mouse grips and moves on the string. Adhesive removal test measures the time to remove adhesives put on each forepaw. The circle test values the ability and rapidity of the mouse to exit concentric circles of different diameters. Whatever the time after surgery, the grip test and the beam balance test on the rectangular beam did not reveal any deficit after ischemia. In all the other tests, animals exhibited a deficit on day 2 after ischemia. An early functional recovery (before day 9) was observed for the chimney test, the string test and the balance beam test on the cylindrical beam. The neurological score, the beam walking tests, pole test, adhesive removal test and circle test revealed a deficit up to 56 days. In conclusion, among our battery, five tests are able to reveal long-term sensorimotor deficits. These tests appear of particular interest to screen strategies relevant for the treatment of clinical stroke.

## S13.C002

### Effect of L-carnitine on mitochondrial antioxidants and nucleic acids in the substantia nigra of 1-methyl-4-phenyl-1,3,4,6-tetrahydropyridine induced neurotoxicity in aged albino rats

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Reactive oxygen species (ROS) have been hypothesized to play an important role in aging and aging related neurodegenerative diseases such as Parkinsonism. In aging there exists an imbalance between the production of free radicals and antioxidant defense mechanisms in the substantia nigra, which may lead to cell death. The present study was designed to determine whether administration of L-Carnitine (LC) ( $\beta$ -hydroxy- $\gamma$ -trimethylamino butyrate) when administered (300 mg/kg body weight/day, i.p. started 3 days prior to 1-Methyl-4-Phenyl-1,3,4,6-Tetrahydropyridine (MPTP) (20 mg/kg body wt/i.p twice in 1 h interval) administration, six animals in each group for 21 days would prevent age-related mitochondrial oxidative stress itself as well as the neurotoxin MPTP induced changes in the mitochondrial antioxidant defence systems, lipid peroxidation (LPO), protein carbonyl content (PCC), DNA and RNA contents and xanthine oxidase (XO) activity in the substantia nigra of aged rat brain. Aged control rats elicited a statistically significant decline by students *t*-test ( $P < 0.05$ ) in DNA and RNA contents, antioxidants such as reduced glutathione (GSH) content and Mn-superoxide dismutase (Mn-SOD) activity and a significant increase ( $P < 0.05$ ) in LPO, PCC and XO as compared to LC-treated aged rats. A highly significant increase in LPO, PCC and XO activity and a highly significant decrease in DNA, RNA and GSH contents as well as Mn-SOD activity were observed in the mitochondria of the neurotoxin MPTP-challenged aged rats when compared to aged control rats. Administration of LC to the MPTP-challenged group of aged rats was effective in reducing the mitochondrial LPO, PCC and XO activity and exerted a significant increase in GSH, DNA and RNA contents and Mn-SOD activity. Thus, LC by acting as a potent antioxidant and nitrogenomic molecular medicine exerted significant neuroprotective effect and proved efficacious in amelioration of MPTP induced neurotoxicity and protecting brain against aging related oxidative neurodegeneration itself and aging related neurological disorders such as Parkinsonism.

# Endothelium-derived contracting factors: from bench to bedside (09.30–12.30)

## S14.1

### Uridine adenosine tetraphosphate: a novel endothelial-derived vasoconstrictive mediator

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Uridine-(5')-adenosine-(5')-tetraphosphate (Up4A) gained increasing interest as a potent vasoconstrictor in recent years. (Gui *et al.*, 2008). The Up4A was isolated from the supernatant of stimulated human endothelium and was identified by mass-spectrometry. Up4A most likely exerts vasoconstriction via purinoceptors. Up4A is the first dinucleoside polyphosphate containing both purine and pyrimidine moieties isolated from living organisms. Mean total plasma Up4A concentrations are significantly increased in juvenile hypertensives compared with juvenile normotensives (Jankowski *et al.*, 2005). Plasma Up4A concentrations correlates with left ventricular mass and intima media wall thickness in hypertensives. Moreover, Up4A was identified in renal tissue (Jankowski *et al.*, 2007). Up4A acts as a strong vasoconstrictive mediator on afferent arterioles, but has no significant effect on the tone of efferent arterioles, suggesting a functional role of Up4A as an autocrine hormone for glomerular perfusion. The release of Up4A from renal tubular cells may be an additional mechanism whereby tubular cells could affect renal perfusion. In addition, Up4A has an effect on contractility of isolated rat pulmonary artery (Jankowski *et al.*, 2008). Up4A stimulated contraction in a concentration-dependent manner. Up4A is a potent vasoconstrictor, but not a vasodilator, of the rat pulmonary artery. Up4A likely acts through a suramin-sensitive P2Y receptor. The contractile effect of Up4A involves the entry of extracellular  $Ca^{2+}$  and release of  $Ca^{2+}$  from intracellular stores, but not  $Ca^{2+}$  sensitization via the RhoA/Rho kinase pathway. Up4A, therefore, plays an important role in the regulation of pulmonary vascular tone.

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## S14.2

### Endothelium-dependent contractions and the prostanoid TP and IP receptors

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In spontaneously hypertensive rats (SHR), the endothelial dysfunction is due to the release of endothelium-derived contracting factors (EDCF) that counterbalance the effect of NO. In the SHR, the endothelium-dependent contractions involve the production of reactive oxygen species, the activation of cyclooxygenase-1, the diffusion of EDCF and the subsequent stimulation of TP-receptors on vascular smooth muscle. The EDCFs released by acetylcholine have been identified as PGH2 and paradoxically prostacyclin. Prostacyclin, by stimulating its receptor (IP-receptors), generally produces vascular relaxation and inhibits platelet aggregation. In the aorta of SHR, prostacyclin is the principal metabolite of arachidonic acid released from the endothelial cells by acetylcholine. However, in SHR aortae prostacyclin activates the smooth muscle TP-receptor and produces contraction. Indeed, the IP-receptor is not functional in the SHR aorta as early as at 12 weeks of age, but its activity is not reduced in platelets. This dysfunction of the IP-receptor is explained neither by a general dysfunction involving the adenylate-cyclase pathway nor by a reduction in the expression of the IP receptor. Therefore, prostacyclin is a Janus face prostaglandin, in the rule it protects the vascular wall, but in the SHR it can contribute to endothelial dysfunction. Whether prostacyclin plays a detrimental role as an EDCF in other animal models and in human remains to be determined. Nevertheless, since EDCFs converge to activate TP-receptors, selective antagonists of this receptor, by preventing endothelium-dependent contractions, curtail the endothelial dysfunction in hypertension and diabetes.

## S14.3

### Endothelium-dependent contractions and endothelial dysfunction in human essential hypertension

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Vascular endothelium plays a primary role in the control of vascular function and structure, principally through the production of nitric oxide (NO) and other endothelium-derived relaxing factors (EDRFs) including prostacyclin and different endothelium-derived hyperpolarizing factors, which represent mainly a compensatory mechanism under conditions of reduced NO availability. However, endothelial cells, under given conditions, can induce contraction (constriction) of the underlying vascular smooth muscle cells. Such endothelium-dependent increase in contractile tone can be the consequence of the reduced production of nitric oxide or its increased breakdown by reactive oxygen radicals, but also to the production of vasoconstricting peptides (angiotensin II, endothelin-1) and/or the release of vasoconstricting metabolites of arachidonic acid. These latter, in particular, have been defined as 'endothelium-derived contracting factor' (EDCF) as they contribute to the real-time changes of the underlying vascular smooth muscle cells contraction status. Indeed EDCFs diffuse from the endothelial cells and induce contraction through the activation of the TP-receptors on the smooth muscle cell. Scientific evidence suggest that both in human and animals EDCF-mediated responses are exacerbated by cardiovascular risk factors, such as aging, essential hypertension and diabetes mellitus and that they conceivably contribute to the impairment of endothelium-dependent vasodilatations in aged subjects and essential hypertensive patients. Accordingly it was shown that the blockade of the TP-receptors prevents endothelium-dependent contractions, and improves endothelial-dependent vasodilation in hypertension and diabetes, suggesting an

important pathophysiological interplay between EDRFs and EDCFs in genesis of endothelial dysfunction.

## S14.4

### Endothelin receptor antagonists and cardiovascular diseases

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Endothelin-1 (ET-1) is a powerful vasoconstricting, mitogenic, pro-inflammatory and pro-fibrotic peptide generated by vascular endothelial cells and acting on target tissues (including blood vessels, heart and kidney) through ETA and ETB receptors. Whereas ETA receptors have a clear pathological role, ETB receptors cause endothelium-dependent vasodilatation, natriuresis and ET-1 clearance. Several ET receptor antagonists (ETRAs) are licensed for the treatment of pulmonary artery hypertension, but other indications for ETRAs appear promising, including hypertension, chronic kidney disease and cancer. ETRAs reduce blood pressure (BP) in essential hypertension and may have clinical potential in resistant cases. Hypertension in chronic kidney disease, including diabetic nephropathy, is currently one of the most promising targets for ETRAs. ET-1 generation is enhanced in renal disease, and the kidney is a complex and important target for ET-1, having high densities of ETA and ETB receptors. Recent experimental studies support use of ETRAs to reduce BP and prevent or even reverse renal impairment. Recent clinical studies also show that ET-1 plays an important pathogenic role in the genesis of hypertension, atherosclerosis, arterial stiffness, renal dysfunction, and proteinuria in patients with chronic renal disease, primarily through actions on the ETA receptor, and additional to those seen with standard ACE inhibitor therapy. Indeed, ETB receptor may play a protective role in this situation. Taken together, the evidence suggests that ETRAs may be powerful new therapeutic agents for the treatment of hypertension in chronic renal disease. Long-term studies on BP, proteinuria and progression of renal impairment are now needed.

## S14.C001

### Modulation of sphingolipid metabolism orchestrates vascular contractility in isolated carotid arteries from spontaneously hypertensive rats

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Besides their role in growth regulation, the sphingomyelin metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) have vasoactive properties. Since hypertension is associated with pronounced alterations in vessel growth and contractile properties, we investigated whether modulation of sphingolipid metabolism induces altered contractile responses in isolated arteries during hypertension. Therefore, the vasoactive effects of modulation of sphingolipid metabolism in isolated carotid arteries from 6 months old male, spontaneously hypertensive rats (SHR) were compared to these from normotensive Wistar Kyoto rats (WKY) in a wire myograph. Inhibition of sphingosine kinase by means of dimethylsphingosine (DMS, 10  $\mu$ M) induced a transient contraction in arteries obtained from SHR but not WKY with maximal effects of  $1.49 \pm 0.14$  mN/mm ( $n = 6$ ). Furthermore, the exogenous application of sphingomyelinase (SMase, 0.1 U/ml) induced a pronounced constriction in arteries from SHR ( $1.73 \pm 0.13$  mN/mm,  $n = 7$ ), whereas it only induced minor constriction in vessel segments from WKY ( $0.56 \pm 0.07$  mN/mm,  $n = 6$ ). Concomitant administration of SMase and DMS had, only in SHR, greater effects than either agent alone ( $2.49 \pm 0.21$  mN/mm,  $n = 5$ ). Both, DMS- and SMase-induced constrictions in SHR were completely prevented by the cyclooxygenase inhibitor indomethacin (10 M) ( $0.01 \pm 0.01$  and  $0.01 \pm 0.01$  mN/mm respectively,  $n = 5$ ). Moreover, removal of the endothelium prior to the addition of DMS or SMase, completely abolished vasoconstriction ( $0.03 \pm 0.02$  and  $0.05 \pm 0.02$  mN/mm respectively,  $n = 5$ ). From these experiments we conclude that modulation of sphingolipid metabolism by inhibition of sphingosine kinase or application of SMase, induces pronounced contractions in carotid artery segments from SHR but not from normotensive WKY rats. These contractile responses are mediated by endothelium-derived cyclooxygenase products. Hypertension thus seems to be associated with alterations in sphingolipid metabolism that influence vascular tone, which may play an important role in the aetiology and pathophysiology of hypertension. This research was performed within the framework of project T2-108 of the Dutch Top Institute Pharma.

## S14.C002

### Long-term treatment with ouabain induces endothelial dysfunction that increases the vasoconstriction to noradrenaline of mesenteric resistance arteries

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We demonstrated that 5 weeks of ouabain (OUA) treatment induces arterial hypertension (HA) and did not change the noradrenaline (NA) vasoconstrictor response in mesenteric resistance arteries (MRA). However, a hypotrophic inward remodeling was observed in this artery. In the present study we analyze whether a long-term OUA treatment during 20 weeks induces a severe HA and vascular dysfunction in MRA of rats. Arterial blood pressure (BP) levels were measured by tail-cuff method during 20 weeks in OUA- (8.0  $\mu$ g/day, sc,  $n = 21$ ) or Vehicle (VHE,  $n = 24$ )-treated male Wistar rats. 3rd branches of mesenteric bed were mounted in a wire small-vessel myography. The constrictor response to KCl (120 mM) and endothelial-dependent relaxation to acetylcholine (ACh, 0.01 mM–30  $\mu$ M) were performed. Constrictor-response curves to noradrenaline (NA, 10 mM–30  $\mu$ M) were performed in absence and presence of endothelium, L-NAME (LN,

100  $\mu\text{M}$ ); indometacin (INDO, 10  $\mu\text{M}$ ); ridogrel (RIDO, 1  $\mu\text{M}$ ); and Cu/Zn superoxide dismutase (SOD, 150 U/mL). Western Blot technique was used to evaluate the protein expression of cyclooxygenase-2 (COX-2). 20 weeks of OUA-treatment increased both BP (+20%) and the NA-sensitivity (VHE  $6.06 \pm 0.04$  vs. OUA  $6.36 \pm 0.05$   $P < 0.05$ ,  $t$ -test). The difference of area under the curve (dAUC %) for NA indicated that mechanical endothelial removal or LN incubation significantly increased NA-response only in VHE (VHE  $43.2 \pm 7.9$  vs. OUA  $6.3 \pm 3.2$  %  $P < 0.05$ ,  $t$ -test), and INDO reduced it in a larger manner in the OUA as compared to VHE (VHE  $-10.4 \pm 1.9$  vs. OUA  $31.9 \pm 2.3$  %  $P < 0.05$ ,  $t$ -test). Furthermore, RIDO (VHE  $6.4 \pm 2.8$  vs. OUA  $38.7 \pm 3.2$  %  $P < 0.05$ ,  $t$ -test) and SOD (VHE

$9.8 \pm 5.1$  vs. OUA  $29.8.3 \pm 4.5$  %  $P < 0.05$ ,  $t$ -test) induces a significantly reduction in NA-response only in the OUA group. KCl- or ACh-induced response did not change in OUA group. OUA-treatment increased protein expression of COX-2 on MRA (+77%). Our data suggest that endothelial dysfunction after 20 weeks of OUA-treatment could be a mechanism that contributes to the maintenance of HA. This vascular dysfunction is associated with a reduction in nitregic modulation associated with an increment in superoxide anion levels and an increase in the synthesis of thromboxane  $A_2$  and/or prostaglandin  $H_2$  probably by COX-2 protein activation. We were supported by FAPESP/ CNPq. Supported by FAPESP and CNPq.

# Immunopharmacology (09.30–12.30)

## S15.1

### Immune regulation by histamine

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Histamine and 4 different histamine receptors constitute a multifaceted system with distinct functions of receptor types due to their differential expression, which changes according to the stage of cell differentiation and influences of the microenvironment. Differences in cellular expression and affinities of these receptors for histamine is highly decisive for the biological effects of histamine and drugs that target histamine receptors. Histamine possesses all the properties of a classical leukocyte chemoattractant, including: agonist-induced actin polymerization, mobilization of intracellular calcium, alteration in cell shape, and up-regulation of adhesion molecule expression. Histamine induces the CC chemokines, monocyte chemoattractant protein 1 and 3, RANTES, and eotaxin in explant cultures of human nasal mucosa via H<sub>1</sub>R, suggesting a prolonged inflammatory cycle in allergic rhinitis between the cells that release histamine and their enhanced migration to nasal mucosa. It has been demonstrated that differential patterns of histamine receptor expression on Th1 and Th2 cells determine reciprocal T cell responses following histamine stimulation. Th1 cells show predominant, but not exclusive expression of H<sub>1</sub>R, while Th2 cells show increased expression of H<sub>2</sub>R. Histamine enhances Th1-type responses by triggering the H<sub>1</sub>R, whereas both Th1- and Th2-type responses are negatively regulated by H<sub>2</sub>R. In addition, histamine actively participates in functions and activity of dendritic cell precursors as well as their immature and mature forms. In conclusion, immune regulatory functions of histamine are exciting and important and new data are emerging.

## S15.2

### Intolerance to tolerability: data intensive early phase drug studies in man-the key to translational development

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Administration to human subjects completes the translational process. When this is done successfully perhaps no step in the process reduces uncertainty more. However the studies in man have to be sufficiently informative to be able to make translational links to what happened in animal models. There is a common belief that the primary aim of first-in-man studies is limited to safety and tolerability only, and still many studies are designed with this in mind. There is no regulatory guidance that states this and in fact the safety/tolerability objective cannot normally be accomplished in the traditional first in man study due to its size. This is due to the fact that safety and tolerability issues generally arise around events that are serious, but unlikely to occur in all subjects. This is the reason that many drugs that were taken off the market for safety reasons have early development studies that explicitly state that the drug is 'safe and well tolerated'. The key to good translational development is that the dose of the drug is chosen in a manner that proof of pharmacological principle can occur in the first studies in man. Unfortunately many studies still employ dosages that are determined by NOAEL dose levels in toxicology studies, in which generally it is not known if the expected pharmacological effect is, or at which concentration. This can easily be resolved by more integrated preclinical development, and by developing the right biomarkers. Translational development requires dense pharmacological information.

## S15.3

### Cytokine blockade: a major improvement in translational medical research

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My colleagues and I identified TNF as a useful target for therapy, chiefly using studies of human disease tissue. In rheumatoid synovial cultures, TNF blockade was very effective at down-regulating other aspects of pathology, including cytokines and destructive enzymes. Subsequent clinical trials have validated TNF as a therapeutic target for not only rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis and its arthritis, ankylosing spondylitis etc. What can be learnt from this? Is studying human disease tissue generally useful? An exploration of atherosclerosis tissue, and potential targets will be summarized. Is TNF unique among cytokines in being a therapeutic target? As cytokines are involved in controlling all biological processes, it is possible, even likely, that all diseases, involving multiple biological processes, are regulated in a critical way by cytokines. So are there cytokine targets for all unmet medical needs? This will be discussed.

## S15.4

### Screening for the cytokine-stimulatory potential of drugs - why and how

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Cytokines play multiple roles in human health and disease, and both cytokine and anti-cytokine immunotherapies thus have achieved ever increasing utility in clinics. Seeking immunomodulatory compounds that would positively or negatively influence the cytokine network is a great challenge for pharmacological research. There is a cross-talk between cytokines and drugs: while many drugs can change the patterns of cytokine production in human recipients, cytokines may alter their pharmacokinetic and pharmacodynamic profile. It is suggested therefore that it is very important to characterize the immunobiological potential of drugs and newly developed compounds. Here we focus on the methodological aspects of screening for drug interference with cytokine production. We have developed a test platform

based on an *in vitro* nitric oxide (NO) assay in mouse peritoneal macrophages. The ability of compounds to up-regulate the production of NO, triggered primarily by interferon- $\gamma$ , is tightly correlated with their intrinsic cytokine-stimulatory activity. The data can be extrapolated with high fidelity to the effects in human cells. The compounds that have been found to produce cytokines in mouse cells also produce cytokines in human peripheral blood mononuclear cells. From the point of view of pivotal immunopharmacological screening, it is important to underline that the ability of compounds to up-regulate NO production in mouse macrophages can be used as a reliable indicator of their cytokine-stimulatory effects in human cells.

## S15.C001

### Lymphocyte trafficking at resolution is mediated by PGD<sub>2</sub>, via iNOS nitrosylation of COX-2

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Inducible nitric oxide synthase (iNOS) is classically described as a pro-inflammatory inducible enzyme producing large amounts of nitric oxide at the onset of inflammation. Here we show that iNOS also has pro-resolution properties. Similarly cyclooxygenase-2 (COX-2) originally shown to have a role at the onset of inflammation, has recently been shown to have pro-resolution properties via the production of prostaglandin D<sub>2</sub> (PGD<sub>2</sub>). Previous work by our group has investigated the mechanisms of resolution and shown the importance of lymphocytes at resolution by protecting against secondary infection. We have now brought these two together and show here that iNOS binds and nitrosylates COX-2, producing PGD<sub>2</sub>, which acts as a chemoattractant for lymphocytes. Male wild type C57/blk6 (25–30 g) (WT) and male iNOS knockout (25–30 g) (KO) mice were given a resolving zymosan peritonitis: cell populations were analysed by FACS, cytokines and PGD<sub>2</sub> were measured in inflammatory exudate. iNOS immunoprecipitation and biotin switch assays for nitrosylated proteins were performed on *ex vivo* macrophage cultures. High expression of iNOS was found at the resolution phase of zymosan peritonitis, but did not coincide with high levels of nitric oxide. Interestingly we found COX-2 bound to iNOS at the resolution phase; further investigation showed that iNOS nitrosylates COX-2 in wild types, but in iNOS KO's this is substantially reduced. FACS analysis of peritoneal washouts showed that there was no difference between cell numbers in WT vs. iNOS KO's. Bioplex analysis of the inflammatory exudate showed no difference in cytokines and chemokines. Further FACS analysis showed a reduction in the number of lymphocytes in iNOS KO's; this data coincided with a reduction of PGD<sub>2</sub> at the resolution phase. Our data shows that PGD<sub>2</sub> acts as a chemoattractant for lymphocytes at resolution which is mediated by nitrosylated COX-2 via pro-resolution iNOS.

## S15.C002

### A critical role for the anti-inflammatory protein annexin A1 in microglial phagocytosis

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The primary cause of neurodegeneration in Parkinson's disease (PD) is unclear, but, in accord with the view that the local inflammatory response contributes to the progression of neurodegenerative disease, multiple lines of evidence implicate persistent microglial activation in the substantia nigra, the principal area affected in PD (Whitton, 2007). The protein annexin A1 (ANXA1) is a key promoter of resolution in peripheral inflammation, in part acting to stimulate macrophage phagocytosis of inflammatory cells (Scannell *et al.*, 2007). Whilst its actions in the CNS remain relatively unexplored, post mortem examination of PD patients has identified upregulated ANXA1 expression in microglia surrounding degenerating dopaminergic neurones (Knott *et al.*, 2000) and, in rodents, exogenous ANXA1 can suppress production of inflammatory mediators by microglia (Minghetti *et al.*, 1999). We hypothesise that ANXA1 plays an important role in the resolution of neuroinflammation, acting to enhance microglial phagocytosis of damaged dopaminergic neurones, and hence removing a potent pro-inflammatory stimulus. Using an *in vitro* approach, we established that the BV2 microglial cell line could phagocytose neurone-like cells of the PC12 line, and that this was significantly enhanced after the PC12 cells were damaged by the dopaminergic neurotoxin 6-hydroxydopamine. Using FACS analysis, western blot and electron microscopy, we demonstrated that ANXA1 was not expressed in PC12 cells, either before or after 6-hydroxydopamine treatment; however, ANXA1 was located in the cytoplasm, but not on the cell surface, of BV2 cells. When BV2 cells were mixed with PC12 cells after exposure to 6-hydroxydopamine, FACS analysis revealed a significant cell surface expression of ANXA1, indicating a translocation of the protein from the cytoplasm of the BV2 cells. The significance of this effect was demonstrated by the ability of a neutralising anti-ANXA1 antibody, but not the immunoglobulin isotype control, to block the phagocytosis of PC12 cells by BV2 cells. These data support a critical role for ANXA1 in the efficient phagocytosis of apoptotic neurone-like cells by microglia. As the presence of apoptotic cells can be a major pro-inflammatory stimulus, their removal is central to the efficient resolution of inflammation with minimal tissue damage. Whilst further work is necessary to translate these findings to the *in vivo* situation in the brain, these data suggest that ANXA1 may play an endogenous homeostatic role to limit inflammation, a function of particular importance in the sensitive CNS tissue. This work was funded by the Wellcome trust and Imperial College London.

#### References:

- Knott *et al.* Mol Cell Neurosci. 2000; 16: 724–739.
- Minghetti *et al.* Br J Pharmacol. 1999; 126: 1307–1314.
- Scannell *et al.* J Immunol. 2007; 178: 4595–4605.
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# Symposia Presentations

## Thursday 17 July

### Recent advances in drug transporters and their polymorphisms (09.30–12.30)

#### S16.1

##### The serotonin transporter in pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is associated with elevated pulmonary pressures and pulmonary vascular remodeling. Increased expression and activity of the serotonin transporter (SERT) has been associated with PAH. This phenotype has also been associated with exaggerated PAH in patients with COPD and with an increased risk of developing PAH at high altitudes. We have demonstrated that mice over-expressing SERT develop increased pulmonary pressures and are more susceptible to hypoxia-induced and dexfenfluramine-induced PAH (MacLean *et al.*, 2004). We have recently shown that this effect is only observed in female mice. Increased SERT expression can result in proliferation of pulmonary artery smooth muscle cells (PASMCs). The 5-HT<sub>1B</sub>-receptor mediates both PASMC proliferation and pulmonary vasoconstriction and inhibition of this receptor ablates the development of PAH in the hypoxic rodent model (Keegan *et al.*, 2001). We and others have shown that there is co-operativity between the 5-HT<sub>1B</sub>-receptor and SERT in both serotonin-induced vasoconstriction and proliferation (Lawrie *et al.*, 2005, Morecroft *et al.*, 2005). Here we will discuss further the synergistic interactions of serotonin, SERT, the 5-HT<sub>1B</sub> receptor, gender and other factors in the development of experimental PAH.

##### References:

Keegan *et al.* *Circulation Res.* 2001; 89: 1231–1239.  
Lawrie *et al.* *Circulation Res.* 2005; 97: 227–235.  
MacLean *et al.* *Circulation.* 2004; 109: 2150–2155.  
Morecroft *et al.* *J Pharm Exp Ther.* 2005; 313: 539–548.

#### S16.2

##### Transporters in the cardiovascular system: physiological and pharmacological functions

HK Kroemer *Ernst Moritz Arndt University, Germany*

#### S16.3

##### Impact of OATP transporters on drug disposition

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Organic anion transporting polypeptides (OATP) constitute a superfamily of influx transporters. Human OATP family consists of 11 members, of which OATP1A2, OATP1B1, OATP1B3 and OATP2B1 likely play significant roles in drug disposition. OATP1B1 is expressed on the sinusoidal membrane of hepatocytes and transports a wide variety of endogenous and exogenous compounds. The 521T>C (Val174Ala) single nucleotide polymorphism of the *SLCO1B1* gene has been associated with reduced hepatic uptake and increased plasma concentrations of diverse drugs. For example, the AUC of plasma simvastatin acid, atorvastatin, pravastatin and rosuvastatin have been 221%, 144%, 91% and 65% larger, respectively, in individuals with the c.521CC genotype than in those with the c.521TT genotype (Niemi *et al.*, 2006, Pasanen *et al.*, 2006, Pasanen *et al.*, 2007). The *SLCO1B1* 521T>C polymorphism shows a distinct geographical distribution with high variant allele frequencies occurring in the north (15–25% in Europe, 7–15% in Asia) and low frequencies near the equator (1–5% in Sub-Saharan Africa) (Pasanen *et al.*, 2008). OATP1B3 and OATP2B1 are expressed on the sinusoidal membrane of hepatocytes. Moreover, OATP2B1 and OATP1A2 are expressed on the luminal membrane of enterocytes and may facilitate drug absorption. OATP1A2 is also expressed in cholangiocytes and the endothelial cells forming the blood-brain barrier. More studies are required to establish the role of OATP1A2, OATP1B3 and OATP2B1 in drug disposition.

##### References:

Niemi *et al.* *Clin Pharmacol Ther.* 2006; 80: 356–366.  
Pasanen *et al.* *Pharmacogenet Genomics.* 2006; 16: 873–879.  
Pasanen *et al.* *Clin Pharmacol Ther.* 2007; 82: 726–733.  
Pasanen *et al.* *Pharmacogenomics.* 2008; 9: 19–33.

#### S16.4

##### Drug transporters in the human blood-placental barrier

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Transporters in placenta may either increase or decrease fetal exposure depending on their function and localization (Myllynen *et al.*, 2007). Main components of the human term placental barrier are a tight layer of multinucleated syncytiotrophoblasts on maternal side and endothelial cell layer facing fetal blood. The brushborder of syncytiotrophoblasts facing maternal blood contains high levels of

p-glycoprotein/MDR1/ABCB1, BCRP/ABCG2 and MRP2/ABCC2 which are efflux transporters. ABCB1 and ABCG2 in transgenic mice decrease fetal exposure to xenobiotics (Leslie *et al.*, 2005). Some MDR1 and BCRP gene polymorphisms have been shown to associate with lower protein levels in placenta and may thus have functional significance for fetal exposure (Shiverick *et al.*, 2007). For complete placental pharmacokinetics, we use human placental perfusion model that retains full structure and function of human term placenta better than other experimental models (DiSanto *et al.*, 2003). We found that a specific inhibitor of BCRP/ABCG2 increases the transplacental transfer of a heterocyclic amine, PhIP a known substrate for BCRP/ABCG2. Also, the fetal to maternal concentration ratio of PhIP correlated with the expression of BCRP/ABCG2 protein in placental tissue. In BeWo chorioncarcinoma cells PhIP increased/rescued the viability of cells after the inhibition of BCRP/ABCG2.

##### References:

DiSanto S *et al.* *Placenta.* 2003; 24: 882–894.  
Leslie EM *et al.* *Toxicol Appl Pharmacol.* 2005; 204: 216–237.  
Myllynen P *et al.* *Expert Opin Drug Metab Toxicol.* 2007; 3: 331–346.  
Shiverick K *et al.* *Placenta. Trophoblast Res.* 2007; 21: S125–S128.

#### S16.C001

##### The effects of gender on the development of pulmonary arterial hypertension in mice over-expressing the serotonin transporter

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Pulmonary arterial hypertension (PAH) is a progressive, often fatal disease, associated with both constriction and remodelling of the pulmonary vasculature. In its idiopathic and familial forms, it occurs more often in women than men (>2:1). A polymorphism causing increased activity/expression of the serotonin transporter (SERT) has been associated with the development of familial PAH (fPAH). We have previously reported that female mice over-expressing SERT (SERT+ mice) develop increased pulmonary arterial pressures. We now investigate the effects of gender on development of PAH in SERT+ mice under both normoxic and hypoxic (10% O<sub>2</sub> for 2 weeks) conditions. Mice were anaesthetised using 3% (induction) and 1–1.5% (maintenance) isoflurane. Right ventricular pressure (RVP) was obtained in anaesthetised mice (C57/BL6, 25–45 g) via a 25-gauge needle inserted directly into the right ventricle using a transdiaphragmatic approach. Systolic RVP (sRVP) was increased in both male (24.0 ± 0.8 mmHg, n = 11) and female (20.3 ± 0.8 mmHg, n = 12, P < 0.05) and female (32.0 ± 3.7 mmHg, n = 9) of 18.5 ± 0.4 mmHg, n = 5, P < 0.05) SERT+ mice compared to wildtype controls. Female SERT+ mice, however, displayed greater sRVP than male SERT+ mice (P < 0.05). Hypoxia increased sRVP in both male (40.6 ± 3.1 mmHg, n = 4, P < 0.001) and female (31.0 ± 3.7 mmHg, n = 11, P < 0.05) wildtype mice. Hypoxic female SERT+ mice displayed elevated sRVP (46.8 ± 5.2, n = 11, P < 0.05) in comparison to their wildtype controls, while hypoxic male SERT+ mice did not (39.2 ± 1.5, n = 8). In conclusion, female mice over-expressing SERT develop increased pulmonary arterial pressures compared to their male counterparts. As the reason for the prevalence of fPAH in female humans is still unclear, SERT+ mice make an attractive model with which to study the effects of gender on the development of fPAH.

#### S16.C002

##### The effect of *SLCO1B1* polymorphism on repaglinide pharmacokinetics is independent of repaglinide dose

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Hepatic uptake by organic anion transporting polypeptide 1B1 (OATP1B1) is an important step preceding the metabolism of repaglinide in the liver. This study aimed to establish whether the effect of *SLCO1B1* (encoding OATP1B1) c.521T>C (p.Val174Ala) polymorphism on the pharmacokinetics of repaglinide is dose-dependent. Twelve healthy volunteers with the *SLCO1B1* c.521TT genotype (controls) and eight with the c.521CC genotype ingested a single 0.25 mg, 0.5 mg, 1 mg, or 2 mg dose of repaglinide in a dose-escalation study with a washout period of at least 1 week. The mean area under the plasma concentration-time curve from time 0 to infinity (AUC<sub>0-∞</sub>) of 0.25 mg, 0.5 mg, 1 mg, or 2 mg repaglinide was 82% (95% confidence interval 47%, 125%), 72% (24%, 138%), 56% (24%, 95%), or 108% (59%, 171%) (P ≤ 0.001) larger in participants with the *SLCO1B1* c.521CC genotype than in those with the c.521TT genotype, respectively. Repaglinide peak plasma concentration and AUC<sub>0-∞</sub> increased linearly along with repaglinide dose in both genotype groups (r > 0.88, P < 0.001). There was a tendency towards lower blood glucose concentrations after repaglinide administration in the participants with the c.521CC genotype than in those with the c.521TT genotype. In conclusion, the effect of *SLCO1B1* c.521T>C polymorphism on the pharmacokinetics of repaglinide is independent of repaglinide dose throughout the clinically relevant dose range.

# Epac: a novel signalling factor in the pulmonary and cardiovascular system

(09.30–12.30)

## S17.1

### The regulation and function of Epac proteins

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Epac1 and Epac2 are guanine nucleotide exchange factors for Rap proteins directly activated by cAMP, which function among others in the control of integrin-mediated cell adhesion, cell-cell junction formation and secretion. Previously, we have determined the crystal structure of Epac2 in the inactive conformation, revealing the auto-inhibited conformation of the protein. We have now determined the active conformation in the presence of cAMP and Rap1, showing in atomic detail the mechanism of activation. In addition, we found that Epac1, but not Epac2, has an additional level of regulation by cAMP, which is its translocation from the cytosol to the plasma membrane. This is most likely driven by diffusion and requires the DEP domain in the open, cAMP-bound, conformation of the protein. The translocation is essential for proper activation of Rap1 at the plasma membrane by Epac1 and for downstream biological effects including integrin-mediated cell adhesion. Independently, signalling through the small GTPase Rho induces a similar translocation of Epac1, although to a more polarized plasma membrane localization. To study the function of Epac1 and its downstream effects in more detail we have focused on endothelial cells. Activation of endogenous Epac1 with an Epac-selective cAMP analog results in increased barrier function as measured by transendothelial electrical resistance. We are currently studying the involvement of various Rap effectors in this process.

## S17.2

### Compartmentalized cAMP signalling in cardiac myocytes

M Zaccolo *University of Glasgow, UK*

In the heart, cAMP generated upon beta-adrenergic receptor (beta-AR) stimulation controls strength, duration and frequency of contraction. Our long term goal is to understand the molecular mechanisms that underpin the involvement of cAMP signalling in heart disease and to explore the possibility to exploit such mechanisms for the development of novel therapeutic strategies. In the heart, several G-protein coupled receptors (GPCRs) signal through cAMP yet lead to clearly distinct effects. A key question thus is how the stimulation of different receptors that act via the same second messenger can elicit the appropriate and specific functional response. Our working hypothesis is that disruption of spatial control of cAMP signalling may lead to inappropriate activation of downstream targets and result in disease. By using a genetically encoded, FRET-based cAMP sensor, we were able to show that beta-AR stimulation generates multiple microdomains with increased concentration of cAMP in cardiac myocytes. Free diffusion of cAMP is limited by the activity of PDEs, the enzyme that degrade cAMP. In particular PDE4 and PDE2, despite its very low expression level, play a key role in this. Data in the literature report that the inotropic effect of catecholamines is associated to the activation of the particulate isoform of PKA (PKA type II). On the contrary, PGE1 stimulation of cardiac myocytes activates the soluble fraction of PKA (PKA type I) and does not increase inotropy. We are currently investigating whether such a difference in the functional effects of PKA isoforms is dependent on selective PKA subset activation by individual pools of cAMP generated by individual GPCR stimulation.

## S17.3

### Regulation of anti-inflammatory signalling by Epac in vascular endothelial cells

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The ubiquitous second messenger cyclic AMP is a key regulator of immune and inflammatory responses. For example, it has been determined that the ability of cyclic AMP to inhibit pro-inflammatory, interleukin (IL)-6 responses in human umbilical vein endothelial cells (HUVECs) is due to induction of 'suppressor of cytokine signalling-3' (SOCS-3), a negative regulator of IL-6 receptor signalling (Sands *et al.*, 2006). SOCS-3 induction by cyclic AMP occurs independently of cyclic AMP-dependent protein kinase (PKA), instead requiring activation of the cyclic AMP sensor 'exchange protein activated by cyclic AMP 1' (EPAC1). Our current investigations are geared towards understanding the mechanisms by which activation of EPAC1 leads to changes in SOCS-3 gene expression. Accordingly, we have identified the C/EBP family of transcription factors as a critical link between EPAC1 activation and SOCS-3 induction (Yarwood *et al.*, 2008). We found that selective activation of EPAC in HUVECs increases C/EBP DNA binding activity and recruitment of C/EBPbeta to the SOCS-3 promoter. In addition, knockdown of C/EBPbeta and delta isoforms abolishes both SOCS-3 induction and inhibition of IL-6 signalling in response to cyclic AMP. Finally, overexpression of C/EBPalpha, beta or delta potentiates EPAC-mediated accumulation of SOCS-3 in HUVECs. These effects do not appear to be restricted to HUVECs, as we have observed similar phenomena in COS1 cells and in murine embryonic fibroblasts. In summary, our findings constitute the first description of an EPAC-C/EBP pathway that can control cyclic AMP-mediated changes in gene expression independently of PKA. Here we present evidence of intermediary signalling mechanisms linking EPAC activation to the regulation of C/EBP-dependent SOCS-3 transcription.

#### References:

Sands WA *et al.* *Mol Cell Biol.* 2006; 26: 6333-6346.  
Yarwood SJ *et al.* *J Biol Chem.* 2008; (in press).

## S17.4

### Epac: effectors and biological functions in the heart and lung

M Schmidt, SS Roscioni, CRS Elzinga *University of Groningen, the Netherlands*

Since their discovery about ten years ago, it is now accepted that Epac proteins act as cyclic AMP-activated guanine nucleotide exchange factors for Ras-like GTPases. Epac1 (also known as cAMP-GEF-I) and Epac2 (also known as cAMP-GEF-II) signal to a plethora of diverse effectors, including Ras and Rho GTPases, phospholipase

C- $\epsilon$ , phospholipase D, mitogen-activated protein kinases, protein kinase B/Akt, ion channels, secretory-granule associated proteins and regulators of the actin-microtubule network, the latter probably involved in the spatiotemporal dynamics of Epac-related signaling. Importantly, it is now accepted that Epac proteins are novel cAMP sensors that regulate several pivotal cellular processes, including calcium handling, cell proliferation, cell survival, cell differentiation, cell polarization, cell-cell adhesion events, gene transcription, secretion, ion transport, and neuronal signaling. Epac proteins might even play a role in the regulation of inflammation, the development of cardiac hypertrophy, airway development and lung functioning. Upon highlighting multi-faceted effectors and diverse biological functions driven by Epac proteins, certain controversial signaling properties of cAMP in the pulmonary and cardiovascular systems will be discussed.

## S17.C001

### Lipid raft-mediated transmembrane signalling associated with lysosomal targeting and trafficking of acid sphingomyelinase in endothelial cell membrane

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Lipid rafts (LRs) have been demonstrated to mediate transmembrane signalling through their clustering, which is recently considered as a possible missing link between many receptors and intracellular signalling machinery. However, the molecular mechanism mediating LR clustering to form signalling platforms on the cell membrane remains unknown. The present study tested a hypothesis that the formation of LR platforms and consequent transmembrane signalling in coronary arterial endothelial cells (CAECs) are attributed to lysosome trafficking and fusion to the cell membrane, whereby acid sphingomyelinase (ASMase) is activated and LR clustering occurs in the membrane of these cells. By measurement of fluorescent resonance energy transfer (FRET) between FITC-labelled anti-lamp1 (a lysosome marker protein), Rac1, or Fas and TRITC-conjugated cholera toxin subunit B (CTXB) which binds to GM1 gangliosides (GM1), a LR marker, it was found that in FasL-stimulated CAECs, membrane lamp1, Rac1, or Fas and GM1 were trafficking toward the cell membrane in that RRET was produced between TRITC-CTXB and each of FITC-labelled molecules. Confocal microscopy also showed colocalization of Cy3-conjugated ceramide antibody and CTXB, suggesting that ceramide molecules are enriched in the LR platforms, which were colocalized with ASMase, a ceramide producing enzyme. All these FasL-induced changes in FRET and colocalization of ASMase or ceramide with LR platforms could be abolished by lysosome function inhibitors, bafilomycin (Baf) or glycy-L-phenylalanine- $\beta$ -naphthylamide (GPN). Further pursuit of lysosomal ASMase resources demonstrated that the siRNA of sortilin, an intracellular transporter for targeting of ASMase to lysosomes blocked FasL-induced formation of LR platforms in CAECs. Functionally, Electron Spin Resonance analysis showed that blockade of lysosomal trafficking and ASMase targeting substantially blocked FasL-induced O<sub>2</sub><sup>-</sup> production via LR platforms. It is concluded that LR-mediated transmembrane signalling relies on the lysosomal trafficking and fusion to cell membrane and consequent presentation of ASMase, which may represent a novel function of lysosomes in CAECs. Besides classical and secretory lysosomes, a new type of signalling lysosomes could exist to mediate transmembrane signalling via LR platforms in these cells.

## S17.C002

### Lipoxin A4 levels in asthma: relation with disease severity and aspirin sensitivity

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Lipoxin A<sub>4</sub>, an endogenous anti-inflammatory eicosanoid (Serhan, 2005), has been found to be low in patients with severe asthma (Bonnans *et al.*, 2002). However, few studies also suggested more diminished lipoxin A<sub>4</sub> levels in Aspirin Exacerbated Respiratory Disease when compared to Aspirin Tolerant Asthma (Sanak *et al.*, 2000). It is, therefore, currently not clear whether the asthma severity or the presence of Aspirin Exacerbated Respiratory Disease has a primary role on the disturbed lipoxin metabolism. The aim of this study is to detect lipoxin A<sub>4</sub> and 15-epi-lipoxin A<sub>4</sub> levels in asthma patients with and without Aspirin Exacerbated Respiratory Disease of comparable severity. The study groups consisted of 22 subjects with Aspirin Exacerbated Respiratory Disease, 22 subjects with Aspirin Tolerant Asthma and 10 volunteers without asthma and aspirin sensitivity. This study was approved by the local ethics committee of Ankara University School of Medicine (approval date and number, October, 4, 2004, 58-1431), and each subject gave written informed consent. Whole blood samples were stimulated with calcium ionophore, A23187 (5 × 10<sup>-5</sup> M), and A23187 (5 × 10<sup>-5</sup> M) + aspirin (10<sup>-4</sup> M), lipoxin A<sub>4</sub>, and 15-epi-lipoxin A<sub>4</sub> levels were analyzed by enzyme immune assay method. Severe asthma patients in both Aspirin Exacerbated Respiratory Disease 0.5 to 0.8 ng/ml and Aspirin Tolerant Asthma 0.5 to 0.45 ng/ml groups showed diminished generation for Lipoxin A<sub>4</sub> to stimulation with A23187 in comparison to other severity degrees in their groups (P = 0.02 and P = 0.046, respectively). Lipoxin A<sub>4</sub> generation in both severe groups was comparable to each other (P > 0.05). Although severe cases with Aspirin Exacerbated Respiratory Disease showed a diminished capacity to generate 15-epi-lipoxin A<sub>4</sub>, this did not reach to a statistical significance. This study indicated that diminished lipoxin A<sub>4</sub> generation was unique to severe asthma phenotype regardless of co-morbid aspirin sensitivity. Lower lipoxin A<sub>4</sub> levels in severe asthma would suggest a possibility for lipoxin analogs as future treatment options in these patients.

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# Imaging technology in drug research and development (09.30–12.30)

## S18.1

### Molecular imaging of gene expression in the living subject: pre-clinical applications in biotherapeutics

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## S18.2

### Positron emission tomography: the conceptual idea using a multidisciplinary approach

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Positron Emission Tomography (PET) is a method for measuring biochemical and physiological processes *in vivo* in a quantitative way by using radiopharmaceuticals labeled with positron emitting radionuclides as  $^{11}\text{C}$ ,  $^{13}\text{N}$ ,  $^{15}\text{O}$  and  $^{18}\text{F}$  and by measuring the annihilation radiation using a coincidence technique. This includes also the measurement of the pharmacokinetics of labeled drugs and the measurement of the effects of drugs on metabolism. Also deviations of normal metabolism can be measured and insight in biological processes responsible for diseases can be obtained. The idea of *in vivo* measurement of biological and/or biochemical processes was already envisaged in the 1930's when the first artificially produced radionuclides of the biological important elements carbon, nitrogen and oxygen, which decay under emission of externally detectable radiation, were discovered with help of the then recently developed cyclotron. These radionuclides decay by pure positron emission and the annihilation of positron and electron results in two 511 keV gamma quanta under a relative angle of  $180^\circ$  which are measured in coincidence. This idea of PET could only be realized when the inorganic scintillation detectors for the detection of gamma radiation, the electronics for coincidence measurements and the computer capacity for data acquisition and image reconstruction became available. For this reason the technical development of Positron Emission Tomography as a functional *in vivo* imaging discipline started approximately 35 years ago.

## S18.3

### Investigating ligand affinity and selectivity using PET

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Positron emission tomography (PET) involves administration of a radiopharmaceutical and measuring its subsequent distribution over the body with a 3D PET-camera. Whatever the radiopharmaceutical, its distribution over the various tissues of an organism is invariably complicated in both the spatial and the temporal domain. Distribution depends on many factors: biochemical properties of the tissue (concentration of proteins such as receptors and pumps, concentration of low molecular compounds etc.), physiological and physical properties of the tissue, properties and dose of the radiopharmaceutical, presence of other pharmaceuticals and haemodynamic parameters. For pharmaceutical purposes, it is highly desirable to extract only the factors of interest, while removing all other factors that influence distribution. Usually, the factors of interest are 1. concentration of a specific receptor and 2. its affinity and selectivity for a specific ligand. Fortunately, many classical pharmacological models and methods can be adapted to characterize distribution in PET, at the mere expense of computational power.

Methods will be presented and exemplified that can provide the following in 3D maps of:

1. receptor expression
2. affinity of a known receptor for reversibly and irreversibly binding radiopharmaceuticals.
3. affinity of a known receptor for reversibly and irreversibly binding non-labeled ligands
4. neurotransmitter release.

Examples in the talk will be based on studies of macromolecules involved in the brain's reward system (opioid and dopamine receptors).

However, there are no fundamental obstacles to apply the methods to other target organs, macromolecules and drugs.

## S18.4

### Translatability of non-clinical and clinical imaging data to clinical efficacy

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Increasingly, pharmaceutical companies use neuroimaging techniques to support drug development programs for novel central nervous system (CNS) therapeutics that should have a sufficient therapeutic margin. Specifically, receptor occupancy information as measured by positron emission tomography (PET) is often used as a surrogate marker for clinical effect. To support the translation of a receptor occupancy level into an expected clinical effect, pre-clinically a series of pharmacokinetic (PK) – pharmacodynamic (PD) relationships is established to summarize information from multiple sources. Dopamine D2 receptors have long been

recognized as a drug target for antipsychotics. Pre-clinically, well-established behavioural models exist to characterize the effect of potential antipsychotics on behaviour. In addition, pre-clinical imaging of dopamine D2 receptors in rodent and primate brain supports the development of PK/PD models that link behavioural effects to levels of dopamine D2 receptor occupancy. Clinically, reference information is available from various marketed antipsychotic drugs. Nevertheless, significant uncertainty remains as will be discussed in this presentation. In a development program of a novel dopamine D2 antagonist, we observed that, despite the data richness of the field, species differences in the PK/PD relationship of dopamine D2 receptor occupancy and differences in efficacy estimates across different behavioural models contribute to uncertainty in estimating the required level of dopamine D2 occupancy for clinical efficacy. In addition, clinically, differences in PK/PD relationships following acute and chronic dose administration and an incomplete understanding of the required behaviour of a drug in time contribute to uncertainty.

## S18.C001

### The serotonin-1B receptor: a novel target for the pathophysiology of depression

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The serotonergic (5-HT) system has been widely implicated in the pathophysiology of Major Depressive Disorder (MDD). Although the 5-HT system is a popular target for drug therapy in MDD the role that serotonin plays in MDD is not clearly understood. An abundance of research suggests that several 5-HT receptor subtypes may be dysfunctional in patients with MDD including the 5-HT<sub>1B</sub> receptor. Methods: A comprehensive literature search on PubMed for the role of the 5-HT<sub>1B</sub> receptor in the pathophysiology of MDD. Evidence implicating 5-HT<sub>1B</sub> receptors in the pathophysiology of depression comes from a number of converging lines of evidence from genetic, pharmacological, neuroendocrine challenge, behavioral and postmortem studies in animals and humans. Two common genetic polymorphisms of 5-HT<sub>1B</sub> receptors, G861C and C129T, have been associated with fewer 5-HT<sub>1B</sub> binding sites and have been implicated in affective disorders. In an mRNA regulation and stress susceptibility study rats predisposed to learned helplessness (an animal model of depression) were found to have decreased 5-HT<sub>1B</sub> receptor mRNA in dorsal raphe nucleus compared with stress-resilient animals. In neuroendocrine challenge studies activation of 5-HT<sub>1B/D</sub> receptors via zolmitriptan produced a smaller increase in the growth hormone (GH) levels of melancholic depressed patients than in healthy controls. Results similar to those obtained for the depressed group were observed in a group of healthy men whose zolmitriptan induced GH level increase was attenuated by administration of ketanserin, a 5-HT<sub>1B/D</sub> antagonist. Additionally, pharmacological studies have demonstrated an increase in extracellular levels of 5-HT and augmentation of anti-depressant effects following administration of SSRIs in the absence or blockade of 5-HT<sub>1B</sub> receptors. 5-HT<sub>1B</sub> receptor agonists, administered alone or with anti-depressants, have been shown to be effective in preclinical models of depression. Recent interest has focused on p11, an s100 EF-hand protein family protein which colocalizes with 5-HT<sub>1B</sub> receptors. p11 plays a central role in the modulation of 5-HT<sub>1B</sub> receptor function which is dysregulated in preclinical models of depression and postmortem samples of MDD patients. A review of the 5-HT<sub>1B</sub> receptor literature provides strong evidence that 5-HT<sub>1B</sub> receptors and related factors such as p11 protein are involved in the pathophysiology of depression. Future research should examine the use of the 5-HT<sub>1B</sub> receptor as a target for pharmacotherapy as well as a biomarker for vulnerability to MDD.

## S18.C002

### Re-establishment of normal blood flow is mandatory to restore intramuscular high energy phosphate levels after transient ischemia

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Normalization of blood flow is required to salvage ischemic tissues, but might paradoxically cause reperfusion injury. The aim of this study was to determine whether restoration of skeletal muscle high energy phosphates after ischemia is affected by post-ischemic vessel stenosis. Leg ischemia was induced by a cuff on one thigh for 20 min and muscle high energy phosphates depleted by lower leg exercise ( $n = 5$  healthy male subjects). After calf ischemia, the cuff was either deflated or air pressure maintained at 20 mmHg below systolic pressure for 5 min (stenosis). Measurements of high-energy phosphates in gastrocnemius muscle were performed with a 3T spectrometer.  $^{31}\text{P}$  and  $^1\text{H}$  spectra were acquired as an estimate of myocellular concentrations of phosphocreatine (PCr) and inorganic phosphate (iP). PCr concentrations decreased to 31 % ( $\pm 16\%$ ) during ischemic exercise ( $P < 0.001$ ) and iP levels increased in parallel (not shown). While PCr re-established within 2 min after cuff deflation, no recovery was detectable when blood flow was impaired in the reperfusion phase. Muscle phosphate recovery depends on normalization of blood flow. These data indicate that intensified strategies to re-establish flow conditions are required for residual myocellular function after transient ischemia.

# Pharmacology and functional roles of KCNQ potassium channels (09.30–12.30)

## S19.1

### Deafness resulting from impaired inner ear K transport

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Potassium is of particular importance for hearing as the mechanosensitive channels in the apical membranes of sensory hair cells mediate a depolarizing K<sup>+</sup> influx. This requires the positive potential and high K<sup>+</sup> concentration of the scala media, which are created by the transepithelial transport of the stria vascularis in the lateral wall of the cochlea. KCNQ1/KCNE1 is the apical exit pathway for K<sup>+</sup> in marginal cells of the stria vascularis, but secretion also requires a basolateral Na, K-ATPase, the NaK2Cl cotransporter NKCC1, as well as ClC-K/barttin channels to recycle chloride. The exit from sensory outer hair cells (OHCs) requires the KCNQ4 potassium channel, which is exclusively expressed in the basal pole of these cells. We have shown that dominant negative mutations in KCNQ4 underlie the DFNA2 form of progressive hearing loss in humans, and have generated KCNQ4 mouse models. While the complete KO leads to deafness within a few weeks after birth, knock-in mice heterozygous for a dominant negative mutant identified in humans develop hearing loss over several months. The hearing loss is largely due to a selective degeneration of OHCs. Patch-clamping before degeneration shows that these cells have lost their M-type K current and are depolarized, which may cause their degeneration. Once K<sup>+</sup> has left OHCs via KCNQ4, it must be taken up by supporting Deiter's cells. We have shown that this probably occurs through the K-Cl cotransporter KCC4: when disrupted, OHCs degenerate with a similar time course.

## S19.2

### KCNQ channels in smooth muscle

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The KCNQ gene family (KCNQ1-5) encode for voltage-gated potassium channels (Kv7) that are crucial determinants of neuronal resting membrane potential and cardiac action potential. KCNQ1-encoded channels have a prominent role in defining cardiac action potentials and in epithelia, whereas KCNQ2-5 are predominantly expressed in neurons with KCNQ4 restricted to the cochlear and auditory nerves. Recently, we have used quantitative PCR and immunocytochemistry, combined with single cell electrophysiology and isometric tension measurements, to investigate the expression and functional impact of KCNQ genes in smooth muscle. These investigations revealed that KCNQ genes are expressed in vascular and non-vascular smooth muscle cells with KCNQ1 and KCNQ4 being the most abundant. KCNQ5 was also expressed in a number of tissues. Smooth muscles also expressed various KCNE gene family members although the abundant isoform was very tissue specific. In functional studies application of the pan-Kv7 channel blocker XE991 caused smooth muscle contractility to increase. This was associated with depolarisation of the resting membrane potential and inhibition of a non-inactivating potassium current. These effects were not mirrored by the Kv7.1-selective blocker chromanol 293B. These data suggested that Kv7 channels comprised of Kv7.4 and 7.5 were crucial determinants of smooth muscle activity. This postulate was supported by the finding that retigabine, which activates Kv7.2–7.5 but not Kv7.1, relaxed smooth muscle. Overall, our data provide compelling evidence that Kv7 channels are key regulators of cellular activity in most smooth muscle cells.

## S19.3

### Neural KCNQ channels

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Neural KCNQ channels comprise mainly KCNQ2 and KCNQ3 (Kv7.2, Kv7.3). These make up M-channels, which regulate excitability. They are closed by receptors coupled to Gq such as M1 and M3 muscarinic receptors. Closure results from PIP2 hydrolysis – either from consequent depletion of PIP2 or from IP3 production and release of Ca, or a combination of both, with ancillary sensitization to closure by DAG activation of PKC (attached to channel-associated AKAP) and Kv7 phosphorylation (Hoshi *et al.*, 2003; Delmas and Brown, 2005; Hughes *et al.*, 2007). Some functional effects of M-channel closure, determined from transmitter action, blocking drugs and KCNQ2 gene disruption or manipulation, are as follows: i) Sympathetic neurons facilitation of repetitive discharges and conversion from phasic to tonic firing, ii) Sensory nociceptive systems: facilitation of A-delta peripheral sensory fibre responses to noxious heat (Passmore *et al.*, 2007) and iii) Hippocampal pyramidal neurons: facilitation of repetitive discharges, enhanced after-depolarization and burst-firing, and induction of spontaneous firing through a reduction of action potential threshold at the axon initial segment (pro-epileptic effect) (Shah *et al.*, 2007). The neural KCNQ enhancer, retigabine, produces the opposite effects and additionally reduces the responses of both A-delta and C-fibres to peripheral noxious heat.

#### References:

- Delmas and Brown. *Nat Revs Neurosci.* 2005; 6: 850–862.  
Hoshi *et al.* *Nat Neurosci.* 2003; 6: 564–571.  
Hughes *et al.* *Pflug Arch.* 2007; 455: 115–124.  
Passmore *et al.* *Soc Neurosci Abstr.* 2007; 681: 8.  
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## S19.4

### Physiological and pharmacological regulation of Kv7 channels

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The activity of Kv7 channels is regulated by a numerous pathways. We have studied their targeting, regulation via inactivation and pharmacology, and find that the five subtypes of Kv7 channels are differentially regulated. The targeting of the Kv7 channel subtypes was studied by immunohistochemistry, confocal microscopy, and mutagenesis. The inactivation properties and the pharmacology were studied

by two-electrode voltage clamp on human cloned Kv7 channels expressed into oocytes. We find that in hippocampal neurons Kv7.2 and Kv7.3 are selectively targeted to the axon initial segment via binding to ankyrin-G just like Nav channels are targeted to this region. The five Kv7 channels display variable degrees of inactivation when expressed in the cell membrane. Kv7.4 and Kv7.5 show >30% inactivation at physiological potentials, Kv7.1 shows less inactivation and Kv7.2 none. The time-constants for inactivation and recovery from inactivation of Kv7.4 and 7.5 are in the range of several seconds. The channel inactivation is differently influenced by two activators of neuronal Kv7 channels: Retigabine does not influence the inactivation whereas BMS204342 abolishes the inactivation properties of Kv7.4. The BMS acrylamide S-1 also shows differential effects on the Kv7 channel subtypes. The compound blocks Kv7.1 and Kv7.1/KCNE1, whereas it increases conductance of Kv7.4 and Kv7.5. Further, the compound shifts the activation curve in the hyperpolarizing direction of all the neuronal Kv7.2, Kv7.2/3, Kv7.4 and Kv7.5 channels. The effects of S-1, BMS204352 and retigabine on Kv7.5 depends crucially on tryptophan residue 242. In conclusion, the activity of the five Kv7 channel subtypes is differentially regulated both physiologically and pharmacologically.

## S19.C001

### A role for KCNQ channels in hypoxia-induced pulmonary vasoconstriction and hypertension

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Voltage-dependent K<sup>+</sup> channels play an important role in regulating the resting membrane potential of pulmonary artery smooth muscle cells (PASMCs). They have also been implicated in hypoxic pulmonary vasoconstriction (HPV) and in pulmonary hypertension (PH), which result from acute and chronic exposure to hypoxia, respectively (Osipenko *et al.*, 1998). We recently suggested that KCNQ channels may be key regulators of the background K<sup>+</sup> conductance and resting membrane potential of PASMCs. Here we demonstrate a role for KCNQ channels in HPV and PH. Isolated, male Wistar rat lungs were prepared and perfused with physiological saline as described before (Herget *et al.*, 1987). The pulmonary arterial perfusion pressure, corresponding to pulmonary vascular resistance, was continuously measured. Acute challenges with hypoxia caused a significant increase in pulmonary arterial pressure. The amplitude of this response was significantly decreased in the presence of the KCNQ channel blocker, linopirdine, suggesting that KCNQ channels are involved in mediating this effect. To investigate a role in PH, pulmonary arterial compliance was studied in lungs from animals housed in normoxic or hypoxic conditions for 3 days. Pulmonary arterial pressure was recorded at different perfusion flow rates. Pulmonary vascular resistance (PVR), determined from the slope of the pressure versus flow plot, was significantly higher in rat lungs exposed chronically to hypoxia compared with control. This effect of chronic hypoxia was reproduced by linopirdine in control rat lungs. In line with this, there linopirdine had no significant effect on PVR in the lungs from chronic hypoxic rats. Reverse-transcriptase polymerase chain reaction showed reduced KCNQ4 mRNA in pulmonary arteries from rats chronically exposed to hypoxia. This study provides evidence that KCNQ4 channels play an important role in mediating pulmonary artery responses to hypoxia. The KCNQ4 channel might therefore be a potential therapeutic target for treating hypoxic pulmonary vascular disease.

#### References:

- Herget J *et al.* *Am J Physiol Heart Circ Physiol.* 1987; 253: H574–H581.  
Osipenko ON *et al.* *Br J Pharmacol.* 1998; 124: 1335–1337.

## S19.C002

### Functional effects of ether-á-go-go-related channel activators on murine vascular smooth muscle

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We have previously characterised the expression and functional properties of ether-á-go-go-related gene (ERG) channels in murine portal vein using molecular, electrophysiological and whole tissue techniques (mPV; Ohya *et al.*, 2002; Yeung and Greenwood, 2007). The aim of the present study was to expand our knowledge of vascular ERG channels by determining the expression of ERG genes in the murine thoracic aorta and assess the effects of ERG channel blockers (dofetilide) and activators [PD-118057 (PD) and R595047 (R595)] on the contractile nature of this tissue. 6–8 week old BALB/c mice were sacrificed by cervical dislocation. The thoracic aorta were excised and immediately placed into RNA Later and total RNA extracted. Quantitative PCR was employed to determine the relative expression of ERG genes. Further aortae were removed and 2 mm aortic segments were suspended for isometric tension recordings in a wire myograph. All tissues were bathed in Krebs solution maintained at 37°C and aerated by 95%O<sub>2</sub>/5%CO<sub>2</sub>. All data are mean ± SEM, with *n* segments from *N* animals. 60 mM KCl was applied to all tissues to assess viability. Quantitative PCR showed that the ERG1 gene message was abundant but ERG2 or ERG3 expression was negligible. Dofetilide (1 μM) produced a contraction of 1.4 ± 0.5 mN, equivalent to about 40% of the 60 mM KCl-induced spasm in 11 from 15 vessels. Aortic segments were precontracted with 1 μM phenylephrine (PE), 10 μM XE991 (Kv7 channel blocker) or 60 mM KCl and an ERG activator was applied to assess any change in vascular tone. 10 μM PD relaxed aortae precontracted with 1 μM PE or 10 μM XE991 by approximately 50% in both cases (*n* = 3). PD however had no relaxant effect on KCl-induced contractions. In contrast, 10 μM R595 relaxed KCl-induced contractions (66 ± 6%) and almost completely relaxed aortae precontracted with 10 μM XE991 (96 ± 3% relaxation). These data provide novel and further evidence that ERG channels are important regulators of vascular reactivity.

#### References:

- Ohya S *et al.* *Am J Physiol (Cell Physiol).* 2002; 283: 866–877.  
Yeung SYM and Greenwood IA. *Am J Physiol* 2007; 292: 468–476.

# Issues and challenges of receptor allosterism in drug discovery (09.30–12.30)

## S20.1

### Challenges of allosterism in drug discovery

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G protein-coupled receptors (GPCRs) account for approx. 2% of the human genome and represent the major targets for around 30% of all medicines on the market. Although classic small-molecule GPCR drug discovery has focused on ligands interacting with the binding site for the endogenous agonist (i.e., the orthosteric site), it is now known that GPCRs possess allosteric binding sites, and that ligands can utilise these sites to modulate receptor activity through conformational changes transmitted from the allosteric to the orthosteric site and/or to effector coupling sites. Thus, allosteric modulators offer enormous potential for expanding the chemical space associated with GPCR-targeting small molecules. However, in order to capitalise on the promise of GPCR allosteric modulators, a number of practical challenges need to be addressed. One such challenge is the need to appreciate and capture as many allosteric behaviours as possible when screening for such ligands. This is because allosteric modulators can have divergent effects on orthosteric ligand affinity vs. efficacy; other allosteric ligands can possess intrinsic efficacy in their own right. Another challenge is the need to develop quantitative models of allosteric drug action to facilitate modulator structure-activity studies. Finally, perhaps one of the most interesting emergent challenges in this field relates to the ability of some allosteric agonists and modulators to differentially traffic receptor stimulus, promising a great potential for further sculpting cellular signalling, but requiring a broad range of screening assays as a consequence.

## S20.2

### Mechanistic insights into allosteric modulation of NMDA receptors

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NMDA receptors (NMDARs) are glutamate-gated ion channels that are widely expressed in the central nervous system and that play key roles in synaptic plasticity. NMDARs are also implicated in numerous brain disorders. In particular, it is well established that over-activation of NMDA receptors, as occurring during cerebral ischemia or neurodegenerative diseases, can lead to neuronal death (excitotoxicity). On the other hand, pharmacological, genetic and clinical studies have also revealed that a deficit of glutamate neurotransmission, and in particular a hypo-activation of NMDARs, is central in the pathophysiology of human psychoses including schizophrenia. Accordingly, discovering small molecules capable of modulating NMDA receptors either by enhancing (positive allosteric modulator) or inhibiting (negative allosteric modulator) their activity holds therapeutic promise. Moreover, because NMDARs occur *in vivo* as multiple subtypes which differ in their functional properties, there is a growing interest in exploiting this pharmacological heterogeneity for the development of subtype-selective compounds with improved tolerability. In the recent years, we and others have shown that the large extracellular N-terminal domain (NTD) that precedes the agonist-binding domain forms in NR2A and NR2B subunits a bilobate modulatory domain binding subunit-selective allosteric inhibitors (zinc, ifenprodil). We have also characterized the molecular mechanisms by which the NTD communicates with the gating machinery to promote receptor inhibition. We propose that, by the way it operates ('Venus-flytrap' mechanism), the NTD could bind compounds acting as subunit-selective allosteric potentiators. We will thus discuss how a single domain could serve as the target of both positive and negative allosteric modulators.

## S20.3

### Developing allosteric modulators of GPCRs

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## S20.4

### Analysis of auto-sterism: how and when to look for positive modulation

C. Langmead *GlaxoSmithKline, UK*

The advent of functional high-throughput screening assays in the past ten years has greatly increased the diversity of novel compound pharmacologies that are being identified, as functional assays permit the identification of both orthosteric and allosteric ligands. Allosteric sites on a 7TM receptor are topographically distinct from the orthosteric-binding site, such that the receptor can accommodate both ligands simultaneously. A commonly used model for analysing allosteric modulation is the ternary complex model (ATCM), which takes into account modulation of orthosteric ligand affinity, and which is normally applied to data derived from a Schild analysis type experimental design. However, screening for positive allosteric modulators is routinely carried out using a low concentration of orthosteric agonist (~EC<sub>20</sub>) in the presence of a range of concentrations of modulator. This talk will discuss the benefits and drawbacks of analysing such data

according to the ATCM. More recent data suggests that allosteric ligands can robustly alter the efficacy of the orthosteric ligand and even activate the receptor in their own right, in addition to any effects on affinity. To accommodate emerging pharmacologies, the ATCM has been extended to incorporate the operational model of agonism which accounts for allosteric effects on agonist affinity, efficacy and also allosteric agonism. The manifestation of these effects and hence the correct analytical approach depend on the chosen assay system and may require parallel radioligand binding and/or further functional studies to fully define compound mechanism of action.

## S20.C001

### The endocannabinoids anandamide and 2-arachidonylglycerol are negative allosteric modulators of ligand binding at the human A<sub>3</sub> adenosine receptor

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Studies of the endogenous cannabinoid signalling system, exemplified by agonists such as anandamide and 2-arachidonylglycerol (2-AG) have revealed the potential of these ligands to exert modulatory actions on other receptor systems in addition to their ability to activate cannabinoid receptors. This study investigated the effect of cannabinoid ligands on the human adenosine A<sub>3</sub> (hA<sub>3</sub>) receptor expressed in Chinese Hamster Ovary (CHO) cells. Various cannabinoid ligands, including anandamide and 2-AG, were able to fully inhibit both antagonist ([<sup>3</sup>H] 8-Ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one - [<sup>3</sup>H] PSB-11) and agonist ([<sup>125</sup>I] N6-(4-amino-3-iodobenzyl) adenosine-5'- (N-methyluronamide) - [<sup>125</sup>I] AB MECA) binding at the hA<sub>3</sub> receptor. This inhibition occurred over a narrow (low micromolar) range of ligand concentration and was characterised by Hill coefficients significantly greater than unity. However, no inhibition of radioligand binding was observed at the hA<sub>1</sub> or hA<sub>2A</sub> adenosine receptors. Furthermore, the cannabinoid ligands did not affect the potency of the agonist 5-N-Ethylcarboxamidoadenosine (NECA) to inhibit both [<sup>3</sup>H] PSB-11 or [<sup>125</sup>I] AB MECA binding. The above findings suggest that the interaction of the cannabinoid ligands at the hA<sub>3</sub> receptor is non-competitive in nature. Accordingly, we investigated the influence of cannabinoid ligands on the rate of dissociation of [<sup>125</sup>I] AB MECA. In the presence of 2-AG and anandamide, the rate of dissociation was increased, consistent with their role as negative allosteric modulators of agonist binding. Non-eicosanoid cannabinoids did not affect the rate of dissociation. Since the hA<sub>3</sub> receptor has been shown to be expressed in astrocytes and microglia, these findings may be particularly relevant in certain pathological states such as ischaemia where levels of 2-AG and anandamide are raised.

## S20.C002

### Regulation of the M<sub>1</sub> muscarinic acetylcholine receptor by orthosteric and allosteric ligands

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4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride (AC-42) and 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone (77-LH-28-1) are selective M<sub>1</sub> muscarinic (mACh) receptor agonists with an allosteric mechanism of action. The effect of allosteric activation of the M<sub>1</sub> mACh receptor on receptor redistribution is unknown. In this study we have investigated the actions of AC-42, 77-LH-28-1 and three orthosteric agonists [oxotremorine-M (oxo-M), arecoline and pilocarpine] at the human M<sub>1</sub> mACh receptor, stably expressed in Chinese hamster ovary cells, in order to determine whether these compounds differentially affect the regulation of the M<sub>1</sub> mACh receptor at the level of internalization and down-regulation. In functional studies, oxo-M (EC<sub>50</sub> 0.8 μM) caused the greatest increase in [<sup>35</sup>S]-GTPγS-Gα<sub>q/11</sub> binding in membranes from Chinese hamster ovary cells stably expressing the M<sub>1</sub> muscarinic receptor. AC-42 (EC<sub>50</sub> 1.6 μM) and pilocarpine (EC<sub>50</sub> 2.4 μM) stimulated similar responses that were approx. 30% of the maximal oxo-M response. 77-LH-28-1 (EC<sub>50</sub> 0.5 μM) and arecoline (EC<sub>50</sub> 2.9 μM) were more efficacious and stimulated approx. 60% of the maximal oxo-M response. Using intact cell [<sup>3</sup>H]-NMS and [<sup>3</sup>H]-QNB binding assays to measure changes in cell-surface and total cellular expression, respectively, we have shown that after 24 h treatment, oxo-M (100 μM) caused 68 ± 3% internalization and 42 ± 3% down-regulation of the M<sub>1</sub> mACh receptor. Over a similar time-course, pilocarpine (1 mM) caused 50 ± 4% internalization and 36 ± 6% down-regulation. In contrast, AC-42 did not cause significant receptor internalization or down-regulation. Arecoline was able to induce both receptor internalization and down-regulation (42 ± 1% and 29 ± 5%, respectively), while 77-LH-28-1 caused 33 ± 5% internalization, but no significant down-regulation of the receptor. These data suggest that while AC-42 and 77-LH-28-1 are able selectively to activate G<sub>q/11</sub>-dependent signalling through the M<sub>1</sub> mACh receptor, they do not cause the same receptor redistribution as equi-efficacious orthosteric partial agonists, at least over the time-period studied here.