



**Interuniversity Attraction Poles (IAP)
Phase VI**

2007 – 2011

IAP 6-14

**G PROTEIN-COUPLED RECEPTORS,
FROM STRUCTURE TO DISEASES**

Acronym : GPCRs

**Progress report
Year 2007**

Table of contents

Network composition.....	3
I Scientific report	4
1. Workpackage 1: Structural organisation of GPCRs.....	4
1.1. Structural models of GPCRs	4
1.2. Activation mechanisms	4
1.3. Ligand-receptor interactions	5
1.4. Design of agonists and antagonists	6
2. Workpackage 2: Dimerization of GPCRs	6
2.1. Glycoprotein hormone receptors.....	7
2.2. Chemokine receptors	7
2.3. ChemR23 and other receptors.....	7
2.4. Yeast sugar sensing receptor Gpr1	7
3. Workpackage 3: Signaling cascades activated by GPCRs.....	8
3.1. Proteolytic processing of I-TAC/CXCL11 by CD13/aminopeptidase N.....	8
3.2. Synergy between CC and CXC chemokines in monocyte chemotaxis	8
3.3. Signaling pathways regulated by ChemR23 in dendritic cells	9
3.4. Downstream signalling effects of insect GPCRs	9
3.5. Glucose-sensing receptor	9
4. Workpackage 4: Functional characterization of receptors in physiological processes.....	10
4.1. Human and mouse ChemR23	10
4.2 Human and mouse FPRL2	12
4.3. Glucocorticoid-induced receptor	13
4.4. Other knock out models	13
4.5. Purinergic receptors in inflammation and epithelial functions.....	14
4.6. Functional and molecular genetic analysis of insect G protein-coupled receptors.....	15
4.7. Glucose-sensing receptor in yeast and <i>Candida albicans</i>	16
4.8. Viral receptors.....	17
5. Workpackage 5: Role of GPCRs in human diseases and animal models	17
5.1. Glycoprotein hormone receptors.....	17
5.2. Chemokine receptors and chemokine variants in cancer and inflammatory diseases ..	18
5.3. Chemerin in inflammation and cancer.....	21
5.4. Mutations of the arginine-vasopressin receptor type 2	23
5.5. QTL analysis in animal models	23
6. Workpackage 6: Identification of novel receptors and their ligands.....	24
6.1. Chemoattractant and neuropeptide receptors.....	24
6.2. Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and IL-8/CXCL8.....	25
6.3. Glucose-sensing receptors	26
6.4. Studies on insect orphan GPCRs	27
6.5. Orphan LGRs.....	27
7. Workpackage 7: Olfactory receptors and evolution of gpcr families	28
7.1. Olfactory receptors.....	28
7.2. Bioinformatics and evolution of the GPCR gene family	29
II Network organisation and management	30
III Publications.....	33
III.1. Publications of each team	33
III.2. Co-publications.....	38
Annex 1: Program of the first plenary meeting	39

NETWORK COMPOSITION

BELGIAN PARTNERS

Coordinator : Partner 1 (P1)

Name : **Marc PARMENTIER**

Institution : **Université Libre de Bruxelles**

Institution's abbreviation : **ULB**

Partner 2 (P2)

Name : **Jozef VANDEN BROECK**

Institution : **Katholieke Universiteit Leuven**

Institution's abbreviation : **KUL**

Partner 3 (P3)

Name : **Johan THEVELEIN**

Institution : **Katholieke Universiteit Leuven**

Institution's abbreviation : **KUL**

Partner 4 (P4)

Name : **Jozef VAN DAMME**

Institution : **Katholieke Universiteit Leuven**

Institution's abbreviation : **KUL**

Partner 5 (P5)

Name : **Daniel DESMECHT**

Institution : **Université de Liège**

Institution's abbreviation : **ULG**

EUROPEAN PARTNER

EU-Partner 1 (EU1)

Name : **Leonardo PARDO**

Institution: **Universidad Autonoma de Barcelona**

Institution's abbreviation : **UAB**

Country : **Spain**

I. SCIENTIFIC REPORT

WORKPACKAGE 1. STRUCTURAL ORGANIZATION OF GPCRS

1.1. Structural models of GPCRs (EU1, P1)

1.1.1. Glycoprotein-hormone receptors (EU1, P1).

The crystal structures of the FSHR in complex with FSH and the TSHR in complex with a thyroid-stimulating autoantibody have recently been published. These structures have confirmed that the large N-terminal ectodomain (ECD) of the glycoprotein hormone receptor (GpHR) family belongs to the family of proteins with leucine-rich repeats (LRRs). The FSHR binds FSH through a concave inner surface, formed by ten LRRs, in a manner that resembles a hand-clasp. Homology models of GpHRs in complex with different hormones, using the structure of the FSHR-FSH complex as a template were constructed. These models and targeted mutagenesis, have helped in the identification of certain 'hot spot' positions both at the hormone and the concave inner surface of the receptor that play attractive and repulsive interactions for binding and specificity, respectively (Caltabiano et al. 2008).

1.1.2. Rhodopsin-like GPCRs (EU1, P1).

The crystal structure of the β_2 -adrenergic receptor bound to the partial inverse agonist carazolol has been published at the end of 2007. This structure together with the previously reported crystal structure of rhodopsin have been used in a combined manner to develop computational models of the transmembrane domain of GpHRs and chemokine receptors.

1.2. Activation mechanisms

1.2.1. Glycoprotein-hormone receptors (P1, EU1)

Identification of the first germline mutation in the extracellular domain of the follitropin receptor responsible for spontaneous ovarian hyperstimulation syndrome as an approach to the relation between binding and activation of glycoprotein hormone receptors. The receptors for follitropin (FSHR), thyrotropin (TSHR), and lutropin/chorionic gonadotropin (LHCGR) are the members of the glycoprotein hormone (GPH) receptors (GpHR) family. They present a bipartite structure with a large extracellular amino-terminal domain (ECD), responsible for high-affinity hormone binding, and a carboxyl-terminal serpentine region, implicated in transduction of the activation signal. Spontaneous ovarian hyperstimulation syndrome (sOHSS) is a rare genetic condition in which human chorionic gonadotropin (hCG) promiscuously stimulates the FSHR during the first trimester of pregnancy. Surprisingly, germline FSHR mutations responsible for the disease have so far been found only in the transmembrane helices of the serpentine region of the FSHR, outside the hormone binding domain. When tested functionally, all mutants were abnormally sensitive to both hCG and thyrotropin (TSH) while displaying constitutive activity. This loss of ligand specificity was attributed to the lowering of an intramolecular barrier of activation rather than to an increase of binding affinity. We have identified the first germline mutation responsible for sOHSS (C³⁸³A, Ser¹²⁸Tyr), located in the ECD of the FSHR. Contrary to the mutations described previously, the Ser¹²⁸Tyr FSHR mutant displayed increase in affinity and sensitivity toward hCG and did not show any constitutive activity, nor promiscuous activation by TSH. Thus, sOHSS can be achieved from different molecular mechanisms involving each functional domains of the FSHR. Based on the structure of the FSHR/FSH complex and site-directed mutagenesis studies, we have built robust molecular models for the GPH/GpHR complexes and we propose a molecular explanation to the binding characteristics of the Ser¹²⁸Tyr mutant (Akcurin et al. 2008).

1.2.2. CCR5 (P1, EU1)

In GPCRs, the interaction between the cytosolic ends of TM3 and TM6 was shown to play an important role in the transition from inactive to active states. The interaction between Arg at position 3.50 of the highly conserved (D/E)R(Y/W) motif in TM3 with its adjacent Asp/Glu residue at position 3.49 and an additional Asp/Glu at position 6.30 near the cytoplasmic end of TM6 is known as the ionic lock. Charge-neutralizing mutation of Asp/Glu^{3.49} in TM3 and Asp/Glu^{6.30} in TM6 results in increased constitutive activity in a number of structurally-related class A GPCRs. However, 30% of GPCRs, including all chemokine receptors, contain a positively charged residue at position 6.30 which does not support an interaction with R^{3.50}. We have shown that R^{6.30}D and R^{6.30}E substitutions, which allow an ionic interaction with R^{3.50}, resulted in an almost silent receptor devoid of constitutive activity and strongly impaired in its ability to bind chemokines but still able to internalize. These results indicate that the constitutive and ligand-promoted activity of CCR5 can be modified by modulating the interaction between the DRY motif in TM3 and residues in TM6 suggesting that the overall structure and activation mechanism are well conserved in GPCRs. However, the molecular interactions locking the inactive state must be different in receptors devoid of D/E6.30 (Springael et al. 2007).

1.3. Ligand-receptor interactions

1.3.1. ChemR23, characterization of two independent binding sites for chemerin domains (P1, EU1)

A ChemR23 model was constructed by partners EU1 and P1 on the basis of bovine rhodopsin. This model is being used to determine how chemerin or its C-terminal peptides bind and activate the receptor. Partner P1 has identified previously that, in the chemerin-9 nonapeptide (YFpGqFaFs-COOH), the four aromatic residues, the glycine and the terminal carboxyl group are essential for binding and activation of ChemR23. Preliminary experiments had also suggested the existence of a binding site for the cystatin domain of chemerin. This hypothesis has been tested further.

In order to investigate the binding site of the chemerin-9 nonapeptide, we have constructed a set of mutants affecting the amino acids potentially involved on the basis of the receptor model. These include residues N^{3.29}, L^{3.32}, I^{3.33}, and M^{3.36} in TM3, R^{5.42} and F^{5.43} in TM5, Y^{6.51}, H^{6.52} and N^{6.55} in TM6. All of these have been mutated to alanine, and the mutants were successfully expressed in CHO-K1 cells and tested by FACS. The cell lines were tested in functional assays, using chemerin and chemerin-derived peptides. Although the testing is still ongoing for some mutants, we identified three mutations affecting significantly the binding of chemerin-9 (R^{5.42}A, F^{5.43}A and Y^{6.51}A), and much less strongly that of full size chemerin. Mutation of L^{3.32} and M^{3.36} have only modest consequences.

From the model, Arg5.42 was identified as a candidate partner for the carboxyl group of the chemerin-9 nonapeptide. Indeed, position 5.42, in transmembrane helix 5, is involved in ligand binding in many G protein-coupled receptors. A strong decrease in the affinity and efficacy of the chemerin-9 nonapeptide was observed for the R^{5.42}A mutant of ChemR23, while recombinant full-size protein was only weakly affected. Additional mutants of this residue were made (R^{5.42}K, R^{5.42}L, R^{5.42}T, R^{5.42}E), and the consequences on the efficacy of the peptide was R > K > A > L > T > E, in agreement with an interaction with the COOH group of the peptide. Moreover, the neutralization of the COOH group in chemerin-9 by amidation decreased strongly its efficacy on WT ChemR23, but not on the R^{5.42} mutants, suggesting indeed a direct interaction. Using iodinated full-size chemerin as a tracer in a binding assay, it was shown that the nonapeptide does not compete efficiently for the binding of the tracer. A number of monoclonal antibodies directed at human ChemR23 have also been tested for their ability to block the functional response. Several mAbs blocked the activity of full-size chemerin, but not of the nonapeptide.

Altogether, these results demonstrate the existence of two independent binding sites, one for the cystatin-like domain of chemerin, the other for the C-terminal nonapeptide. Only this latter interaction is able to promote the activation of the receptor. Additional mutants will be constructed, in order to determine more precisely the nature of the binding site. Mutants of the receptor for investigating the binding site of the cystatin domain are still to be constructed as well.

The chemerin-9 peptide has been studied by infrared spectroscopy and nuclear magnetic resonance. Although the data support a poorly constrained structure of this peptide, they are compatible with the presence of a beta-hairpin structure, centered onto the Pro-Gly motif of the peptide.

1.3.2. Molecular pharmacology of insect tachykinin-like peptide receptors (P2, EU1)

Tachykinins are multifunctional neuropeptides that have been identified in vertebrates as well as invertebrates. The C-terminal FXGXRa-motif constitutes the consensus active core region of invertebrate tachykinins. In *Drosophila melanogaster*, two putative G protein-coupled tachykinin receptors have been cloned: DTKR and NKD. We recently focused on the functional characterization of DTKR, the *Drosophila* ortholog of the stable fly's tachykinin receptor (STKR). Tachykinins containing an alanine residue instead of the highly conserved glycine (FXAXRa) display partial agonism on STKR-mediated Ca²⁺-responses, but not on cyclic AMP-responses. STKR therefore seems to differentiate between a number of tachykinins. Gly- and Ala-containing tachykinins are both encoded in the *Drosophila* tachykinin precursor, thus raising the question of whether DTKR can also distinguish between these two tachykinin types. DTKR was activated by all *Drosophila* tachykinins and inhibited by tachykinin receptor antagonists. Ala-containing analogs did not produce the remarkable activation behavior previously observed with STKR, suggesting different mechanisms of discerning ligands and/or activating effector pathways for STKR and DTKR. (Poels *et al.*, 2007)

1.4. Design of agonists and antagonists (EU1, P1)

A three-dimensional model of CCR5 has been built to fulfill structural peculiarities of its alpha-helix bundle and to distinguish known CCR5 antagonists from randomly chosen drug-like decoys. In silico screening of a library of 1.6 million commercially available compounds against the CCR5 model by sequential filters (drug-likeness, 2-D pharmacophore, 3-D docking, scaffold clustering) yielded a hit list of 59 compounds, out of which 10 exhibited a detectable binding affinity to the CCR5 receptor. Unexpectedly, most binders tested in a functional assay were shown to be agonists of the CCR5 receptor. A follow-up database query based on similarity to the most potent binders identified three new CCR5 agonists. Despite a moderate affinity of all nonpeptide ligands for the CCR5 receptor, one of the agonists was shown to promote efficient receptor internalization, which is a process therapeutically favorable for protection against HIV-1 infection (Kellenberger *et al.* 2007).

WORKPACKAGE 2. DIMERIZATION OF GPCRS

G protein-coupled receptors (GPCRs) have long been considered to be monomeric membrane proteins. Although numerous recent studies have indicated that GPCRs can form multimeric complexes, the functional and pharmacological consequences of this phenomenon have remained elusive. With the discovery that the functional GABA_B receptor is an obligate heterodimer and with the use of energy transfer technologies, it is now accepted that GPCRs can form heteromultimers. In some cases, specific properties of such heteromers not shared by their respective homomers have been reported. Although in most cases these properties have only been observed in heterologous expression systems, there are a few reports describing data consistent with such heteromultimeric GPCR complexes also existing in native tissues. Together with a number of other groups active in the field, we have reviewed the well-documented examples of such native multimeric complexes, listed a number of recommendations for recognition and acceptance of such multimeric receptors, and gave recommendations for their nomenclature (Pin *et al.* 2007). As part of a consortium, we have also proposed the requirements for an information system that can manage the elements of information needed to describe comprehensively the phenomena of both homo- and hetero-oligomerization of GPCRs (Skrabanek *et al.* 2007).

2.1. Glycoprotein hormone receptors (P1)

We have shown that dimerization of glycoprotein hormone receptors is associated with an allosteric behavior, responsible for the negative binding cooperativity displayed, in particular by the TSH receptor (Urizar et al 2005). Using the large collection of TSH receptor mutants displaying constitutive activity that we have generated over the past years, we have explored the influence of constitutive activity on negative cooperativity. The results identify a robust inverse relation between constitutive activity and negative cooperativity, with the most constitutive receptors having completely lost their allosteric behavior. This phenomenon is not due to the reduced ability of constitutive mutants to dimerize, as a strong dimerization signal can still be observed with the mutants in BRET or FRET type of experiments. Our results lead to the concept that, contrary to wild type receptor dimers, constitutively active TSH receptor mutants keep a symmetrical conformation with both protomers displaying the same affinity for TSH binding.

2.2. Chemokine receptors (P1)

Chemokine receptors are presently used as targets for candidate drugs in the frame of inflammatory diseases and human immunodeficiency virus infection. They were shown to dimerize, but the functional relevance of dimerization in terms of drug action remains poorly understood. We reported previously the existence of negative binding cooperativity between the subunits of CCR2/CCR5 heterodimers. We have extended these observations to heterodimers formed by CCR2 and CXCR4, which are more distantly related. We also show that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heterodimerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. These data demonstrate that chemokine receptor antagonists regulate allosterically the functional properties of receptors on which they do not bind directly, with important implications on the effects of these potential therapeutic agents (Sohy et al. 2007).

Allosteric regulation of ligand binding is a well-established mechanism regulating the function of G protein-coupled receptors (GPCR). Allosteric modulators have been considered so far as molecules binding to an allosteric site, distinct from that of the reference ligand (orthosteric site), and able to modulate the binding affinity at the orthosteric site and/or the signaling properties resulting from orthosteric site occupancy. Given that most GPCR are known to form dimers or higher order oligomers, we explored whether allosteric interactions could also occur between protomers within oligomeric arrays, thereby influencing binding and signaling receptor properties. Two main conclusions emerged from such studies. First, allosteric modulators can affect one receptor by binding to another receptor within a dimeric or oligomeric complex. Second, allosteric modulators might act on a given receptor by targeting the "orthosteric site" in another receptor of the complex. Allosteric regulation within di(oligo)mers thus implies that the pharmacological properties of a given receptor subtype can be influenced by the array of dimerization partners coexpressed in each particular cell type. Ligands could thus act as agonists or antagonists on 1 receptor, while modulating allosterically the function of a variety of other receptors to which they do not bind directly. Allosteric regulation across GPCR oligomeric interfaces is expected to greatly influence the practice of pharmacology. It will likely affect the design of drug discovery programs, which rely mostly on the overexpression of the receptor of interest in a cell line, thereby focusing on homo-oligomers and ignoring the potential effects of other partners (Springael et al. 2007).

2.3. ChemR23 and other receptors (P1)

We have started to study the interaction of ChemR23 with chemokine receptors. The fusions with EGFP and luciferase have been constructed, and BRET was used to demonstrate transfer of energy between ChemR23 and several chemokine receptors when coexpressed (CCR2, CCR5, CCR7 and CXCR4). Cell lines stably co-expressing ChemR23 and one of these receptors are being established, in order to study the consequences of the dimerization on the pharmacological and functional properties of the receptors.

2.4. Yeast sugar sensing receptor Gpr1 (P3, P1)

As a collaboration between P3 and P1, we assayed the dimerization of Gpr1 *in vivo* using Bioluminescence Resonance Energy Transfer (BRET). We constructed Gpr1-luciferase (Gpr1-Rluc) and Gpr1-YFP fusion proteins and co-expressed them in yeast. We observed energy transfer between donor and acceptor molecules higher than the background and to similar levels as earlier reported for the Ste2 pheromone receptor in yeast (Gehret *et al.*, 2006), suggesting receptor oligomerization. Further investigations will include the identification of amino acid residues required for dimerization, the role of dimerization in sugar sensing and the effect of ligand addition on energy transfer within the Gpr1-Gpr1 dimer.

WORKPACKAGE 3. SIGNALING CASCADES ACTIVATED BY GPCRS

Besides the classical cascades activated by GPCRs, a number of G protein-independent signaling pathways have been delineated over the recent years. In addition, it became increasingly evident that the same receptor can stimulate different intracellular cascades according to the agonist which is used for stimulation. These properties are linked to the hypothesis following which different active states of a receptor may exist. An additional dimension to this complexity is due to the processing of ligands by proteases, particularly for chemokines, which can affect their receptor specificity, or the functional consequences of the interaction with their receptors. Some of these aspects are being studied for yeast, insect and mammalian receptors.

3.1. Proteolytic processing of I-TAC/CXCL11 by CD13/aminopeptidase N (P4, P1)

Leukocyte migration during normal (e.g. lymphocyte homing) and pathologic conditions (e.g. inflammation) is regulated by a number of protein families including adhesion molecules, cytokines, chemokines, and proteases. A complex network of interactions between these proteins allows for the fine-tuning of the directional migration of leukocyte subfamilies. Cytokines regulate chemokine and chemokine receptor expression, chemokines activate integrins, and the chemokine gradient determines the direction of leukocyte migration. Proteases are not only important for the degradation of the extracellular matrix but more and more evidence also points toward their crucial role in the regulation of chemokine activity and receptor specificity. The CXC chemokine ligand 11 (CXCL11) or interferon (IFN)-inducible T-cell α -chemoattractant (I-TAC) belongs to the CXC chemokine family characterized by the presence of 1 amino acid in between the 2 NH₂-terminal cysteines. I-TAC/ CXCL11 is produced by a variety of cells including leukocytes, fibroblasts, and endothelial cells upon stimulation with IFNs.

CXCR3 ligands were secreted by tissue fibroblasts and peripheral blood-derived mononuclear leukocytes in response to interferon- γ (IFN- γ) and Toll-like receptor (TLR) ligands. Subsequent purification and identification revealed the presence of truncated I-TAC/CXCL11 variants missing up to 6 amino acids. In combination with CD26/dipeptidyl peptidase IV, the metalloprotease aminopeptidase N (APN), identical to the myeloid cell marker CD13, rapidly processed I-TAC/CXCL11, but not CXCL8, to generate truncated I-TAC/CXCL11 forms. Truncated I-TAC/CXCL11 had reduced binding, signaling, and chemotactic properties for lymphocytes and CXCR3- or CXCR7-transfected cells. CD13/APN-truncated I-TAC/CXCL11 failed to induce an intracellular calcium increase but was still able to bind and desensitize CXCR3 for intact I-TAC/CXCL11 signaling. I-TAC/CXCL11 efficiently bound to CXCR7, but I-TAC/CXCL11 was not able to induce calcium signaling or ERK1/2 or Akt phosphorylation through CXCR7. CD26-truncated I-TAC/CXCL11 failed to attract lymphocytes but still inhibited microvascular endothelial cell (HMVEC) migration. However, further processing of I-TAC/CXCL11 by CD13 resulted in significant reduction of inhibition of HMVEC migration. Taken together, during inflammation or cancer, I-TAC/CXCL11 processing by CD13 may lead to a reduced number of tumor-infiltrating lymphocytes and in a more angiogenic environment (Proost *et al.*, 2007).

3.2. Synergy between CC and CXC chemokines in monocyte chemotaxis (P4, P1)

Tissue infiltration by leukocytes is an important phenomenon in a variety of normal as well as pathological processes including leukocyte homing, inflammation and cancer. This leukocyte recruitment is tightly

regulated by the interplay between endothelial cells and leukocytes, a process in which G protein-coupled receptor (GPCR) agonists, including complement factor 5a, bacterial-derived peptides (e.g. fMLP) as well as chemokines, play a central role. Chemokines have been detected during inflammation in many tissues, suggesting that most, if not all, cell types can secrete chemokines, after induction by appropriate stimuli. These co-induced chemokines may cooperate to attract leukocytes to the site of infection, thereby enhancing the outcome of an inflammatory response. Many different ways exist to enhance the cell influx mediated by chemokines. One possibility is the synergistic interaction between cytokines to induce chemokines followed by subsequent cooperation amongst co-induced chemokines to further increase leukocyte recruitment.

CC and CXC chemokines co-induced in fibroblasts and leukocytes by cytokines and microbial agents determine the number of phagocytes infiltrating into inflamed tissues. Interleukin-8 (IL-8/CXCL8) and stromal cell-derived factor-1 (SDF-1/CXCL12) significantly and dose-dependently increased migration of monocytic cells, expressing the corresponding CXC chemokine receptors CXCR2 and CXCR4, towards suboptimal concentrations of the monocyte chemoattractant protein-1 (MCP-1/CCL-2) or MCP-3/CCL7. These findings were confirmed in monocytic THP-1 cells using different chemotaxis assays. In contrast, the combination of two CC chemokines (MCP-1/CCL2 plus MCP-3/CCL7) or two CXC chemokines (IL-8/CXCL8 plus SDF-1/CXCL12) did not provide synergy in monocyte chemotaxis. These data show that chemokines competing for related receptors and using similar signaling pathways do not synergize. Receptor heterodimerization is probably not essential for chemokine synergy as shown in CCR2/CXCR4 co-transfectants. Interestingly, MCP-1/CCL2 mediated extracellular signal-regulated kinase (ERK)1/2 phosphorylation was significantly enhanced by IL-8/CXCL8 in monocytes, indicating cooperative downstream signaling pathways during enhanced chemotaxis. Moreover, in contrast to intact SDF-1/CXCL12, truncated SDF-1/CXCL12(3-68), which has impaired receptor signaling capacity but can still desensitize CXCR4, was not able to synergize with MCP-1/CCL2 in monocytic cell migration. These data indicate that for synergistic activity between chemokines binding and signaling of the two chemokines to their proper receptor is necessary (Gouwy *et al.*, submitted).

3.3. Signaling pathways regulated by ChemR23 in dendritic cells (P1)

The transcriptional program regulated by ChemR23 activation in dendritic cells was investigated by using microarrays. Monocyte-derived DCs were stimulated for 1, 4 and 16 hours with 50 nM human recombinant chemerin, and the transcriptional program was compared to that of unstimulated cells. A relatively small set of genes was found to be upregulated, a subset of which being involved in the interferon gamma signaling pathway. These genes included TRP2 (Tetratricopeptide repeat protein 2, a chaperone) and TRP4, STAT1 and IRF4 (interferon regulatory factor 4). The upregulation of these genes was verified by quantitative RT-PCR, confirming upregulation of the genes from 2 fold (IRF4) to over 10 fold (TRP2 and TRP4) at 16h of stimulation by chemerin. The significance of this stimulation in the frame of the overall function of chemerin needs to be determined.

3.4. Downstream signalling effects of insect GPCRs (P2)

Microarray technology is applied to identify molecules and pathways that act downstream of GPCRs and that regulate important physiological and developmental processes in insects. This technique allows us to analyze differential gene expression patterns on a genome-wide scale resulting from defective GPCR signaling. We have set up microarray hybridization experiments thereby using mutant *Drosophila* strains, such as *ricketts* (*i.e.* expressing non-functional DLGR2 receptors) and *pupal* (*i.e.* with defective bursicon production), to examine molecular events that underlie the processes of cuticle sclerotization, melanization and wing spreading behaviour in insects. These interrelated processes have previously been shown to be regulated by the neurohormone bursicon that acts on DLGR2. At present, we have already identified several transcripts which code for proteins (*i.e.* structural proteins, enzymes...) that likely participate in these processes. In the future, we also aim to use a similar approach to unravel downstream signaling events initiated by other insect GPCR/ligand couples.

3.5. Glucose-sensing receptor (P3)

The cAMP-protein kinase A (PKA) pathway in yeast consists of an extracellular ligand-sensitive G-protein coupled receptor, a G-protein signal transmitter and the effector, adenylate cyclase, of which the product, cAMP, acts as an intracellular second messenger. The latter activates PKA by dissociating the regulatory from the catalytic subunits. Yeast cells (*Saccharomyces cerevisiae*) contain a glucose/sucrose sensitive 7-transmembrane domain receptor, Gpr1, that was proposed to activate adenylate cyclase through the $G\alpha$ protein Gpa2. Consistently, we show here that adenylate cyclase only binds to active, GTP-bound Gpa2. Two related kelch-repeat proteins, Krh1/Gpb2 and Krh2/Gpb1, are associated with Gpa2 and were suggested to act as $G\beta$ mimics for Gpa2, based on their predicted 7-bladed β -propeller structure. However, we find that although Krh1 associates with both GDP and GTP-bound Gpa2, it displays a preference for GTP-Gpa2. The strong downregulation of PKA targets by Krh1 and Krh2 does not require Gpa2, but is strictly dependent on both the catalytic and the regulatory subunits of PKA. Krh1 directly interacts with PKA via the catalytic subunits, and Krh1/2 stimulate the association between the catalytic and the regulatory subunits *in vivo*. Indeed, both a constitutively active *GPA2* allele, and deletion of *KRH1/2*, lower the cAMP requirement of PKA for growth. We propose that active Gpa2 relieves the inhibition imposed by the kelch-repeat proteins on PKA, thereby bypassing adenylate cyclase for direct regulation of PKA. Importantly, we show that Krh1 also downregulates mouse PKA, suggesting Krh-control of PKA has been evolutionarily conserved.

One major class of G proteins typically functions as heterotrimeric complexes consisting of $G\alpha$, $G\beta$ and $G\gamma$ subunits. However, recent work in yeast has identified an atypical $G\alpha$ protein, Gpa2p, which functions without cognate $G\beta\gamma$ subunits. Two novel kelch repeat protein binding partners of Gpa2p, Krh1p and Krh2p, do not function as alternative $G\beta$ subunits, as initially thought, but rather as Gpa2p effectors. They directly link Gpa2p to protein kinase A, thus forming an adenylyl cyclase bypass pathway that enables inputs other than cellular cAMP concentration to affect protein kinase A (PKA) activity. Because mammalian PKA expressed in yeast is also subject to control by the same bypass pathway, it is exciting to postulate that a functionally similar mechanism might exist in mammalian cells and that other $G\alpha$ proteins could exhibit similar characteristics to Gpa2p (Peeters et al. 2006, 2007).

WORKPACKAGE 4. FUNCTIONAL CHARACTERIZATION OF RECEPTORS IN PHYSIOLOGICAL PROCESSES

A number of specific receptors, among which several were identified by the partners over the previous years, have been studied in details in order to determine their role in physiological processes. Using both *in vitro* studies and *in vivo* models of genetically modified organisms, we have studied chemoattractant receptors and a set of neuromodulatory receptors in human and mouse, neuropeptide receptors in insects, and the glucose/sucrose sensing GPCR system in yeast and *Candida albicans*.

4.1. Human and mouse ChemR23 (P1, P4 and P5)

Chemerin was identified by partner P1 as the natural ligand of ChemR23 over the previous IAP program. Partner P1 has, in collaboration with partners of the network and other groups, pursued the characterization of this new chemoattractant system acting on myeloid and plasmacytoid dendritic cells.

4.1.1. Distribution studies.

In collaboration with the group of Silvano Sozzani (Brescia), we have shown that blood $CD56^{\text{low}}CD16^+$ natural killer (NK) cells selectively express functional ChemR23 and that this receptor is coexpressed with CXCR1, the CXCL8 receptor, and the KIR receptors. *In vitro* culturing of NK cells with IL-2 or IL-15 induced a delayed and time-dependent down-regulation of ChemR23 that was associated with the inhibition of NK cell migration to chemerin. Biopsies obtained from patients with oral lichen planus presented an infiltration of $CD94^+CD3^-CD56^+$ NK cells that coexpressed ChemR23. The same biopsies were infiltrated by myeloid, DC-SIGN⁺ and plasmacytoid, $CD123^+BDCA2^+$, ChemR23⁺ dendritic cells that were occasionally

associated with NK cells. In the same histologic sections, chemerin was expressed by inflamed dermal endothelium. These findings propose a role for the ChemR23/chemerin axis in the recruitment of blood NK cells and strongly implicate chemerin as a key factor for the colocalization of NK cells and DC subsets in pathologic peripheral tissues (Parolini et al. 2007).

We demonstrated also that chemerin influences adipose cell function. Chemerin and ChemR23 mRNAs were found to be highly expressed in adipose tissues, and their expression levels were up-regulated in mice fed a high-fat diet. Both chemerin and ChemR23 mRNA expression dramatically increased during the differentiation of 3T3-L1 cells and human preadipocytes into adipocytes. Furthermore, recombinant chemerin induced the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK 1/2) and lipolysis in differentiated 3T3-L1 adipocytes. Thus, chemerin might behave as an adipokine regulating adipocyte function by autocrine/paracrine mechanisms (Roh et al. 2007).

In order to compare to what is known in human, we have evaluated the distribution of chemerin and ChemR23 in the mouse system by using monoclonal antibodies. These antibodies have been validated by FACS and IHC on CHO cell lines expressing chemerin or ChemR23 (also named Dez in mouse) and tested on isolated primary cells, such as mouse bone marrow-derived dendritic cells (BMDCs) or DCs purified from mouse spleens. CD11c and ChemR23 double fluorescence staining on mouse CD11c⁺ dendritic cells clearly demonstrated expression of the receptor on these antigen presenting cells. FACS analysis of myeloid DCs (derived from bone marrow, BMDCs) showed significant expression of ChemR23 on ~40% of the immature population (CD11c⁺MHCII^{low}) whereas only 5% of mature myeloid DCs (CD11c⁺MHCII^{high}) expressed the receptor. As observed in human, most plasmacytoid DCs (pDCs, CD11c⁺mPDCA⁺) purified from mouse spleen expressed ChemR23 (90%). No staining for ChemR23 was observed neither in the CD11c negative cell population nor in BMDCs obtained from ChemR23 KO mice. At the mRNA level, these observations were confirmed by semi quantitative RTPCR analysis, where ChemR23 expression was found in the CD11c⁺ dendritic cell population, whereas no expression could be found in the CD11c-negative population. At the tissue level, ChemR23 expression was found in the stroma of surface epithelial tissues (skin, lung, and colon), and in lymphoid organs (thymus, spleen, mesenteric lymph nodes), in agreement with the expression pattern of the human receptor.

Chemerin expression was investigated in several mouse tissues by immunohistochemistry (IHC) to determine which cell type could produce the ligand. A positive staining was detected in the epithelial cell structure of most tissues investigated (enterocytes, crypt cells, ciliated respiratory epithelium, tracheal submucosal glands, lung respiratory bronchioles, oviduct). Prochemerin transcripts were also found in most tested mouse tissues (skin, lung, liver, ileum, colon, ovary, uterus, and testis) and lymphoid organs (thymus, spleen, mesenteric lymph nodes, Peyer's patches). However, no detectable prochemerin transcripts were found in leukocyte populations purified from the spleen, neither in dendritic cell populations or in other mononuclear cells present in the CD11c-negative cell population. The expression pattern of chemerin in epithelial cells that constitute the first barrier against pathogens, as well as in High Endothelial Veinules which allow immune cell recirculation may suggest a central role of chemerin in the initiation and/or the regulation of the immune response by recruiting ChemR23 positive DCs at the inflammatory sites and present the antigen to immune cells in the draining lymph nodes.

4.1.2. (Pro)chemerin processing.

Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active on ChemR23. However, prochemerin can be rapidly converted into a full ChemR23 agonist by proteolytic removal of a carboxy terminal peptide. This maturation step is mediated by the neutrophil-derived serine proteases elastase and cathepsin G. We have now investigated proteolytic events that control negatively chemerin activity. We demonstrated that neutrophil-derived proteinase 3 (PR3) and mast cell chymase are involved in the generation of specific and stable inactive chemerin variants. Mass spectrometry analysis showed that PR3 specifically converts prochemerin into a chemerin form lacking the last eight carboxy-terminal amino-acids, and which is inactive on ChemR23. Whereas PR3 had no effect on bioactive chemerin, mast cell chymase

was shown to abolish chemerin activity by the removal of three additional amino acids from its C-terminus. This effect was shown to be specific to bioactive chemerin, as mast cell chymase does not use prochemerin as a substrate. These mechanisms leading to the production of inactive variants of chemerin, starting either from the precursor or the active variants highlight the complex interplay of proteases regulating the bioactivity of this novel mediator during early innate immune responses (Guillabert et al. in preparation).

4.1.3. Biological actions *in vitro* and *in vivo*.

The mouse ChemR23 receptor was expressed in CHO-K1 cells, and mouse chemerin expressed in CHO-cells and purified. Synthetic peptides corresponding to prochemerin and chemerin C-terminus were synthesized. The mouse system was found to function very similarly to the human one. The mouse ligand cross-reacted with the human receptor and vice-versa, with almost identical affinities and efficacies. Mouse prochemerin required the proteolytic removal of 6 or 7 amino acids, as in human, in order to become an agonist of ChemR23. The C-terminal nonapeptide of mouse chemerin was found to act on ChemR23 with an EC₅₀ of about 5 nM (Guillabert, Wittamer et al. unpublished), only ten-fold lower than that of full size chemerin (0.5 nM). Provided the similar distribution, mouse appears as a valid model for evaluating ChemR23 function.

4.1.4. Knock out model.

Expression of ChemR23 is restricted to macrophages and dendritic cells and its agonist chemerin can be found at high level in human inflammatory fluids. We postulate therefore that chemerin is involved in the recruitment of APCs and regulates the inflammatory process and the development of adaptive immune responses. In order to determine this role in *in vivo* situations, we have obtained a knock out model for ChemR23, and have started to characterize the phenotype associated with ChemR23 deficiency. They were generated by the insertion of a Neo cassette in the coding exon 3, and the deletion of the sequence encoding transmembrane domains 1 and 2 of the receptor. The mice have been bred on a C57black background for 12 generations so far. The animals are fertile and do not exhibit obvious defects in SPF conditions. No obvious differences were observed in terms of histology of lymphoid organs, leukocyte populations in blood or lymphoid organs, as well as in a first set of inflammatory challenges (LPS-induced shock, shigella infection). The absence of functional expression of ChemR23 at the cell surface was confirmed by calcium release and chemotaxis assays performed on WT and ChemR23 KO BMDCs. Indeed, intracellular Ca²⁺ levels increased in immature dendritic cells in response to mouse chemerin (30 nM), with a reactivity comparable to that observed with the chemokine CCL5 (30 nM) or ATP (1 μM). This specific intracellular Ca²⁺ mobilization through chemerin activation of ChemR23 was demonstrated by the absence of activation of DCs prepared from ChemR23 KO mice. Chemerin acts as a chemotactic factor for dendritic cells in human. Similar observations were made for mouse chemerin which promoted a dose-dependent migration of mouse immature dendritic cells (BMDCs) whereas DCs deficient in ChemR23 receptor could not migrate toward chemerin, confirming that chemerin promotes DCs chemotaxis through specific activation of ChemR23. Other aspects of these studies are described in WP5.

4.2 Human and mouse FPRL2 (P1)

4.2.1. Characterization of the mouse functional ortholog of FPRL2.

F2L (formylpeptide receptor (FPR)-like (FPRL)-2 ligand), a highly conserved acetylated peptide derived from the amino-terminal cleavage of heme-binding protein, is a potent chemoattractant for human monocytes and dendritic cells, and inhibits LPS-induced human dendritic cell maturation. We recently reported that F2L is able to activate the human receptors FPRL-1 and FPRL2, two members of the FPR family, with highest selectivity and affinity for FPRL2. To facilitate delineation of mechanisms of F2L action *in vivo*, we have attempted to define its mouse receptors. This is complicated by the nonequivalence of the human and mouse FPR gene families (three vs at least eight members, respectively). When cell lines were transfected with plasmids encoding the eight mouse receptors, only the one expressing the receptor Fpr2 responded to F2L (EC₅₀ approximately 400 nM for both human and mouse F2L in both calcium flux and cAMP inhibition

assays). This value is similar to F2L potency at human FPRL1. Consistent with this, mouse neutrophils, which like macrophages and dendritic cells express Fpr2, responded to human and mouse F2L in both calcium flux and chemotaxis assays with EC₅₀ values similar to those found for Fpr2-expressing cell lines (approximately 500 nM). Moreover, neutrophils from mice genetically deficient in Fpr2 failed to respond to F2L. Thus, Fpr2 is a mouse receptor for F2L, and can be targeted for the study of F2L action in mouse models (Gao et al. 2007).

4.2.2. Distribution of cells expressing FPRL2 and HBP.

We have studied the detailed distribution of FPRL2 in leukocytes by Q-PCR and flow cytometry with anti-FPRL2 antibodies, resulting in the identification of new cellular targets of the FPRL2 ligand (hF2L), macrophages, plasmacytoid DCs and eosinophils. These 3 leukocyte populations are involved in innate immunity and in the modulation of adaptive immunity. Using tissue microarrays (TMA), staining with antibodies against FPRL2 and CD68, we have demonstrated the restricted expression of FPRL2 to specific macrophage populations found in lung, gut, and skin, three organs chronically exposed to pathogens and exogenous antigens. In the lung, FPRL2 is expressed by alveolar and interstitial macrophages. In the skin, FPRL2 is also expressed by dermal DCs. No FPRL2 expression was observed on other specialized macrophages, such as Kupffer cells, or macrophages from lymph nodes or spleen. Macrophages from lung, gut and skin share similar properties and characteristics in terms of CDs, released cytokines and cytotoxic activity. Involved in cellular immunity and antigen capture, they differ from other macrophage populations involved in cell debris disposal and the establishment of humoral immunity. It is accepted that the functional properties of macrophages are strongly influenced by their micro-environment in tissues, particularly in tumors, in which Tumor Associated Macrophages (TAMs) can, according to their polarization, display anti- or pro-tumoral properties. We will therefore investigate the modulation of FPRL2 expression in human diseases, particularly in various types of cancer.

The expression of FPRL2 on eosinophils was observed by semi-quantitative RT-PCR and flow cytometry. We have shown the chemotactic activity of hF2L on eosinophils in the physiological nanomolar concentration range, comparable to that promoted by the positive control, eotaxin. However, no degranulation of eosinophils by hF2L was observed. These data suggest a specific chemoattractant activity of hF2L on eosinophils with no activation of these cells. As eosinophils are prominent effectors in allergic responses, the F2L-FPRL2 system might be involved in the regulation of lung inflammatory and allergic diseases, such as asthma. In this context, understanding the pathway leading to the proteolytic cleavage of the precursor HEBP1 and the release of hF2L will certainly facilitate the understanding of the processes in which FPRL2 is involved.

4.3. Glucocorticoid-induced receptor (P1)

Glucocorticoid-induced receptor (GIR) is an orphan GPCR with predominant expression in brain and thymus. High levels of GIR expression have been described in limbic forebrain and hypothalamic regions of the brain of mouse, rat and human, suggesting a role for GIR in memory, cognition, stress, reward or the control of emotion. We have generated a knock-out model for GIR in which part of the coding region is replaced by a tau-LacZ reporter gene. Using this reporter gene, we confirmed the high expression of GIR in scattered large striatal neurons coexpressing choline acetyl-transferase, a specific marker for cholinergic neurons. Strong labelling was also observed in neurons of the olfactory bulb, the olfactory tubercle, the thalamus, and less abundantly in the piriform cortex and hippocampus. The knockout mice were tested in a number of behavioural settings. The mice displayed hyperlocomotion in the open field and were prone to anxiety. Motor coordination was affected, particularly in old mice, as shown in the rotarod and strength grip tests. In situ hybridization has shown reduced expression of the proenkephalin gene and overexpression of substance P and prodynorphin genes, suggesting increased activity of dopamine D₁-expressing neurons and reduced activity of D₂-expressing neurons, while the number of D₁ and D₂ binding sites in the striatum appeared unchanged. Microarray analysis of the striatum has revealed overexpression in knockout mice of a number of genes also upregulated following acute or chronic cocaine treatment (c-fos, egr1, egr2, PP-1, Na/K

ATPase). Cocaine administration resulted in stimulated locomotion in both genotypes, but more efficiently in KO animals. The treatment with D₁ and D₂ agonists or antagonists also affected the KO and wild-type mice differently. The mechanisms underlying the apparent hyperactivity of the dopaminergic system in the striatum of GIR knockout mice are still being investigated (Laurent et al. in preparation).

4.4. Other knock out models (P1)

4.4.1. A_{2a} receptor

Adenosine is released from metabolically active cells or generated extracellularly. It is a potent biological mediator that contributes to the protection of cells and tissues during stress conditions such as ischaemia. We had previously generated a knockout model for the A_{2a} receptor (Ledent et al. Nature 388: 674-678, 1997). Additional experiments were made in collaboration with various groups, in order to delineate further the role of adenosine receptors in various aspects of physiology.

Mice lacking the adenosine A_{2A} receptor are hypoalgesic, and previous studies have suggested a role for the A_{2A} receptor in sensitizing afferent fibres projecting to the spinal cord. To test this hypothesis, formalin was injected into the paw and nociceptive responses were measured in wildtype and A_{2A} receptor knockout mice. There was a significant reduction in nociception associated with sensory nerve activation in the knockout mice. In addition, the selective adenosine A_{2A} antagonist SCH58261 also antagonised the formalin test. These results support a key role for the adenosine A_{2A} receptor in peripheral nociceptive pathways (Hussey et al. 2007).

Endogenous adenosine is an important ligand trigger for the cardioprotective effects of postconditioning. To evaluate the role of the A_{2A} receptor in this process, global ischemia-reperfusion was performed with and without postconditioning in isolated hearts wild-type and A_{2A} knockout mice. In WT hearts, postconditioning improved recovery of postischemic developed pressure in early and late reperfusion. These beneficial effects of postconditioning were blocked by treatment with the selective A_{2A} antagonist ZM-241385 during reperfusion. Also, in A_{2A}KO hearts, postconditioning did not improve functional recovery. Thus the protective effects of postconditioning are attenuated by both selective A_{2A} receptor antagonism and targeted deletion of the gene encoding A_{2A} adenosine receptors, supporting the conclusion that endogenous activation of A_{2A} receptors is an essential trigger leading to the protective effects of postconditioning in isolated murine hearts (Morrison et al. 2007).

4.4.2. CB₁ receptor

We had previously generated a knockout model for the CB₁ receptor, the central receptor for the active compounds of *Cannabis*, and for the endogenous cannabinoid anandamide (Ledent et al. Science 285 : 401-404, 1999). This model was further tested in collaboration with a number of groups.

We showed that the endocannabinoid system regulates neuronal circuits critical for long-lasting effects of cocaine, presumably by acting on CB₁ receptors located on terminals of striatal medium spiny neurons (Corbillé et al. 2007). Investigating the neuroadaptations induced by chronic alcohol exposure on both NMDA and GABA_A receptors have suggested that the endocannabinoid/CB₁ receptor system is involved in alcohol dependence (Warnault et al. 2007), while the cognitive effects of nicotine and physostigmine were shown to be dependent on CB₁ receptors (Bura et al. 2007). Also, the cannabinoid system within the CNS was shown to play a critical role in regulating autoimmune inflammation, with the CNS directly suppressing T-cell effector function via the CB₂ receptor (Maresz et al. 2007). The analysis of the CB₁ KO mice also suggested the existence of a SR141716A-sensitive site that is different from the classical CB₁ receptor (Thiemann et al. 2007).

4.5. Purinergic receptors in inflammation and epithelial functions (P1)

Extracellular ATP and prostaglandin E₂ (PGE₂) are two cAMP-elevating agents inducing semimaturation of human monocyte-derived dendritic cells. We have compared the gene expression profiles induced by ATP_γS and PGE₂ in human DCs using microarray technology. At 6 h of stimulation, ATP_γS initiated an

impressive expression profile compared with that of PGE2 (1125 genes compared with 133 genes, respectively) but after 24 h, the number of genes regulated by ATP γ S or PGE2 was more comparable. Many target genes involved in inflammation have been identified and validated by quantitative RT-PCR experiments. We have focused on novel ATP γ S and PGE2 target genes in DCs, including CSF-1, the chemokine MCP-4/CCL13, vascular endothelial growth factor-A, and neuropilin-1. ATP γ S strongly down-regulated CSF-1 receptor mRNA and CSF-1 secretion, which are involved in monocyte and DC differentiation. Additionally, ATP γ S down-regulated several chemokines involved in monocyte and DC migration, including CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL8/MCP-2, and CCL13/MCP-4. Interestingly, vascular endothelial growth factor A, a major angiogenic factor displaying immunosuppressive properties, was secreted by DCs in response to ATP γ S, ATP, or PGE2, alone or in synergy with LPS. Finally, flow cytometry experiments have demonstrated that ATP γ S, ATP, and PGE2 down-regulate neuropilin-1, a receptor playing inter alia an important role in the activation of T lymphocytes by DCs. Our data give an extensive overview of the genes regulated by ATP γ S and PGE2 in DCs and an important insight into the therapeutic potential of ATP- and PGE2-treated human DCs (Bles et al. 2007).

Extracellular nucleotides regulate ion transport, mucociliary clearance as well as inflammatory properties of the airway epithelium by acting on P2 receptors. Cyclooxygenase-2 (COX-2) is a key enzyme involved in the synthesis of prostaglandins during inflammation. Using calcium imaging, DNA microarrays, real-time RT-PCR and PGE2 measurement, we have shown that ATP, UTP and INS365 (P2Y₂ receptor agonists) up-regulate COX-2 expression by approximately 3-fold and enhance the release of PGE2 in human A549 airway epithelial cells. Our data suggest that P2Y receptors may represent putative targets in airway inflammatory diseases (Marcet et al. 2007b).

Luminal P2 receptors are ubiquitously expressed in transporting epithelia. In steroid-sensitive epithelia (e.g., lung, distal nephron) epithelial Na⁺ channel (ENaC)-mediated Na⁺ absorption is inhibited via luminal P2 receptors. In distal mouse colon, we have identified that both a luminal P2Y₂ and a luminal P2Y₄ receptor stimulate K⁺ secretion. We have investigated further the effect of luminal ATP and UTP on electrogenic Na⁺ absorption in distal colonic mucosa of mice treated on a low Na⁺ diet for more than 2 weeks. Transepithelial electrical parameters were recorded in an Ussing chamber. Luminal ATP and UTP induced a persistent inhibition of the amiloride-sensitive current, with IC₅₀ values of 10 and 3 μ M respectively. In P2Y₂ KO mice, the effect of luminal UTP on amiloride-sensitive Na⁺ absorption was absent, while the effect of UTP was kept in P2Y₄ KO mice. Semiquantitative polymerase chain reaction did not indicate regulation of the P2Y receptors under low Na⁺ diet, but revealed a pronounced axial expression of both receptors with highest abundance in surface epithelia. Thus, luminal P2Y₂ and P2Y₄ receptors and ENaC channels co-localize in surface epithelium, but only the stimulation of the P2Y₂ receptor mediates inhibition of electrogenic Na⁺ absorption (Matos et al. 2007).

Extracellular nucleotides regulate ion transport and mucociliary clearance in human airway epithelial cells (HAECs) via the activation of P2 receptors, especially P2Y₂. Therefore, P2Y₂ receptor agonists represent potential pharmacotherapeutic agents to treat cystic fibrosis. Nucleotides also modulate inflammatory properties of immune cells like DCs, which play an important role in mucosal immunity. Using DNA-microarrays, quantitative RT-PCR and cytokine measurements, we showed that UTP up-regulated approximately 2- to 3-fold the expression and release of the antimicrobial chemokine CCL20 in primary HAECs cultured on permeable supports at an air-liquid interface. Both P2Y₂ (ATP γ S, UTP, INS365) and P2Y₆ (UDP, INS48823) agonists increased CCL20 release. UTP-induced CCL20 release was insensitive to NF- κ B pathway inhibitors but sensitive to inhibitors of ERK1/2 and p38/MAPK pathways. Furthermore, UTP had no effect on IL-8 release, but reduced the release of both CCL20 and IL-8 induced by TNF- α and LPS. Accordingly, UTP reduced the capacity of basolateral supernatants of HAECs treated with TNF- α or LPS to induce the chemoattraction of both CD4⁺ T lymphocytes and neutrophils. In addition, we showed that, in monocyte-derived DCs, ATP γ S, and UDP but not UTP or INS365 stimulated CCL20 release. Likewise, UDP but not ATP γ S was also able to increase CCL20 release from monocytes. Pharmacological experiments

suggested an involvement of P2Y₁₁ or P2Y₆ receptors through NF- κ B, ERK1/2, and p38/MAPK pathways. Altogether, our data demonstrate that nucleotides may modulate chemokine release and leukocyte recruitment in inflamed airways by acting on both epithelial and immune cells. Our results could be relevant for further clinical investigations in cystic fibrosis (Marcet et al. 2007a).

4.6. Functional and molecular genetic analysis of insect G protein-coupled receptors (P2)

We are currently studying a novel *Drosophila* G protein-coupled receptor (CG12290) that is distantly related to biogenic amine receptors and belongs to the rhodopsin class of GPCRs. Interestingly, CG12290 possesses a Asp-Thr-Trp (DTW) sequence where most GPCRs from this group own the Asp/Glu-Arg-Tyr (D/ERY) motif that plays a pivotal role in regulating GPCR conformational states. Expressing this receptor in several mammalian and insect cell lines indicates that this GPCR constitutively boosts intracellular cyclic AMP production. Via site-directed mutagenesis, we obtained 2 mutant receptors containing either a DRW or a complete DRY motif. These mutants display a drastically decreased constitutive activity, whereby the DRY mutant does not produce any basal cyclic AMP elevation. Knocking out CG12290 in *Drosophila* by means of imprecise P-element excisions results in lethality. This indicates that the *wt* (DTW) receptor is probably functional and plays an indispensable role during development. We will determine whether this lethality can be rescued by the *wt* receptor or also by the DRY mutant. Orthologs of this receptor have been identified in other insect genomes and also contain this DTW sequence, suggesting that the constitutive effect of this receptor type might be evolutionary conserved. RT-PCR analysis has indicated that CG12290 expression is developmentally regulated, which could form a potential means of controlling the constitutive activity. We have also localized the receptor transcript in the central nervous system by *in situ* hybridization.

For the study of *Drosophila* LGRs (leucine-rich repeats containing GPCRs), we are applying quantitative real-time PCR and *in situ* hybridization analyses to identify the tissues in which these receptors are expressed. Furthermore, we also plan to verify our hypotheses regarding the possible *in vivo* role(s) of these receptors by analysing several mutant fly strains and by creating functional knock down flies using RNA interference.

4.7. Glucose-sensing receptor in yeast and *Candida albicans* (P3)

Under specific environmental conditions, the yeast *Saccharomyces cerevisiae* can undergo a morphological switch to a pseudohyphal growth pattern. Pseudohyphal differentiation is generally studied upon induction by nitrogen limitation in the presence of glucose. It is known to be controlled by several signaling pathways, including MAP kinase, cAMP-PKA and Snf1 kinase pathways. We show that the alpha-glucoside sugars maltose and maltotriose and especially sucrose are more potent inducers of filamentation than glucose. Sucrose even induces filamentation in nitrogen-rich media and in the *mep2 Δ /mep2 Δ* ammonium permease mutant on ammonium-limiting medium. We demonstrate that glucose also inhibits filamentation by means of a pathway parallel to the cAMP-PKA pathway. Deletion of *HXK2* shifted the pseudohyphal growth pattern on glucose to that of sucrose, while deletion of *SNF4* abrogated filamentation on both sugars, indicating a negative role of glucose repression and a positive role for Snf1 activity in the control of filamentation. In all strains and in all media, sucrose induction of filamentation is greatly diminished by deletion of the sucrose/glucose sensing GPCR Gpr1 whereas this has no effect on induction by maltose and maltotriose. The competence of alpha-glucoside sugars to induce filamentation is reflected in the increased expression of the cell surface flocculin *FLO11*. In addition, sucrose is the only alpha-glucoside sugar capable of rapidly inducing *FLO11* expression in a Gpr1-dependent manner, reflecting the sensitivity of Gpr1 for this sugar and its involvement in rapid sucrose signaling. Our study identifies sucrose as the most potent nutrient inducer of pseudohyphal growth and shows that glucose inactivation of Snf1 kinase signaling is responsible for the lower potency of glucose. (Van de Velde and Thevelein 2008).

Based on sequence homology, the *C. albicans* cAMP-PKA pathway and its upstream sensing system seems to be very similar to that of *S. cerevisiae*. Previously we have also shown that the *C. albicans* Gpr1 and Gpa2 homologues function upstream of the PKA-pathway (Maidan et al., Molecular Biology of the Cell, 16, 1971-

1986, 2005). The ligand of CaGpr1, however, does not seem to be glucose or sucrose as CaGpr1 is dispensable for the glucose-induced cAMP increase. This effect seems to be completely mediated by the Cdc25-Ras1 branch of the pathway. Further analysis of this pathway has now clearly shown that CaGpr1 and CaGpa2 are not working in a linear pathway as in *S. cerevisiae*. A large number of phenotypes are completely different between *Cagpr1* and *Cagpa2* mutants. These include e.g. sensitivity to antifungals and oxidative stress tolerance. We have now also found genetic interactions between CaGpr1 and Hgt12, which has recently been described as a glucose transporter. We think, however, that this Hgt12 is a transceptor. This will be investigated in the near future. We are also investigating the possibility that CaGpr1 may be a sensor for the induction of sugar transporter genes, similar to Snf3 in *S. cerevisiae*.

We have also tried to complement the yeast *gpr1* and *gpa2* mutants with the *C. albicans* homologues. For this purpose we have optimized the codons for proper expression in yeast. The CaGpa2 protein can complement the yeast mutant for glucose-induced cAMP signaling. However, expression of the *CaGPR1* gene in a yeast *gpr1* mutant does not complement. Instead it seems that this protein causes a constitutively high PKA phenotype in the *gpr1* mutant, not upon expression in the wild type strain. How this works is a question we want to investigate in the near future.

4.8. Viral receptors (P1)

Many gammaherpesviruses encode G-protein-coupled receptors (GPCRs). Several in vivo studies have revealed that gammaherpesvirus GPCRs are important for viral replication and for virus-induced pathogenesis. The gammaherpesvirus alcelaphine herpesvirus 1 (AIHV-1) is carried asymptotically by wildebeest, but causes malignant catarrhal fever (MCF) following cross-species transmission to a variety of susceptible species. The A5 ORF of the AIHV-1 genome encodes a putative GPCR. In the present study, we investigated whether A5 encodes a functional GPCR and addressed its role in viral replication and in the pathogenesis of MCF. In silico analysis supported the hypothesis that A5 could encode a functional GPCR as its expression product contained several hallmark features of GPCRs. Expression of A5 as tagged proteins in various cell lines revealed that A5 localizes in cell membranes, including the plasma membrane. Using [35S]GTPgammaS and reporter gene assays, we found that A5 is able to constitutively couple to alpha i-type G-proteins in transfected cells, and that this interaction is able to inhibit forskolin-triggered cAMP response element-binding protein (CREB) activation. Finally, using an AIHV-1 BAC clone, we produced a strain deleted for A5 and a revertant strain. Interestingly, the strain deleted for A5 replicated comparably to the wild-type parental strain and induced MCF in rabbits that was indistinguishable from that of the parental strain. The present study is the first to investigate the role of an individual gene of AIHV-1 in MCF pathogenesis (Boudry et al. 2007).

WORKPACKAGE 5. ROLE OF GPCRS IN HUMAN DISEASES AND ANIMAL MODELS

The functional characterization of receptors initiated in WP4 is being pursued for human receptors of potential clinical relevance in human diseases and animal (essentially mouse) models of human diseases.

5.1. Glycoprotein hormone receptors (P1, EU1)

5.1.1. Mutations of the FSH receptor as a cause of the spontaneous ovarian hyperstimulation syndrome

Spontaneous ovarian hyperstimulation syndrome (sOHSS) is a rare genetic condition in which human chorionic gonadotropin (hCG) promiscuously stimulates the FSHR during the first trimester of pregnancy. Surprisingly, germline FSHR mutations responsible for the disease have so far been found only in the transmembrane helices of the serpentine region of the FSHR, outside the hormone binding domain. When tested functionally, all mutants were abnormally sensitive to both hCG and thyrotropin (TSH) while displaying constitutive activity. We have reported the first germline mutation responsible for sOHSS (c.383C4A, p.Ser128Tyr), located in the ECD of the FSHR. Contrary to the mutations described previously, the p.Ser128Tyr FSHR mutant displayed increase in affinity and sensitivity toward hCG and did not show

any constitutive activity, nor promiscuous activation by TSH. Thus, sOHSS can be achieved from different molecular mechanisms involving each functional domains of the FSHR. Based on the structure of the FSHR/FSH complex and site-directed mutagenesis studies, we provide robust molecular models for the GPH/GPHR complexes (see 1.1.1) and we propose a molecular explanation to the binding characteristics of the p.Ser128Tyr mutant (De Leener et al. 2008).

5.1.2. A novel TSH receptor activating germline mutation in hyperthyroidism

Autosomal dominant nonautoimmune hyperthyroidism (ADNAH) is caused by gain of function mutations in the TSH receptor (TSHr) gene and characterized by toxic thyroid hyperplasia with a variable age of onset in the absence of thyroid antibodies and clinical symptoms of autoimmune thyroid disease in at least two generations. We have studied a Turkish family with a novel TSHr gene mutation with distinct features all consistent with ADNAH. Thyroid function tests of the proband were as follows: free T3: 13.1 pg/ml (N: 1.8-4.6); free T4: 5.1 ng/dl (N: 0.9-1.7); TSH: 0.01 mIU/ml (N: 0.2-4.2); and TSH receptor antibody: 2 IU/ml (N: 0-10). A heterozygous missense mutation in exon 10 of the TSHr gene (c.1454C>T) resulting in the substitution of valine for alanine at codon 485 (p.Ala485Val) was found in the father and his son and daughter. This mutation had arisen de novo in the father. Functional studies of the novel TSHr germline mutation demonstrated a higher constitutive activation of adenylyl cyclase than wild type without any effect on phospholipase C activity. These results indicate that gain of function germline mutations in the TSHr gene should be investigated in families with members suffering from thyrotoxicosis and progressive growth of goiter, but without clinical and biochemical evidence of autoimmune thyroid disease. In addition, this family illustrates the fact that patients harboring the same mutation of the TSHr gene may show wide phenotypic variability with respect to the age at onset, and severity of hyperthyroidism and thyroid growth (Akcurin et al. 2008).

5.1.3. Causes of abnormal skeletal development in hypothyroidism.

By proposing TSH as a key negative regulator of bone turnover, recent studies in TSH receptor (TSHR) null mice challenged the established view that skeletal responses to disruption of the hypothalamic-pituitary-thyroid axis result from altered thyroid hormone (T_3) action in bone. Importantly, this hypothesis does not explain the increased risk of osteoporosis in Graves' disease patients, in which circulating TSHR-stimulating antibodies are pathognomonic. To determine the relative importance of T_3 and TSH in bone, a consortium of four laboratories was put together (S.Refetoff, Chicago; G.Williams London UK; J.Samarut, Lyon France; IRIBHM, Brussels) to compare the skeletal phenotypes of two mouse models of congenital hypothyroidism in which the normal reciprocal relationship between thyroid hormones and TSH was intact or disrupted. Pax8 null (Pax8^{-/-}) mice have a 1900-fold increase in TSH and a normal TSHR, whereas hyt/hyt mice have a 2300-fold elevation of TSH but a nonfunctional TSHR. We reasoned these mice must display opposing skeletal phenotypes if TSH has a major role in bone, whereas they would be similar if thyroid hormone actions predominate. Pax8^{-/-} and hyt/hyt mice both displayed delayed ossification, reduced cortical bone, a trabecular bone remodeling defect, and reduced bone mineralization, thus indicating that the skeletal abnormalities of congenital hypothyroidism are independent of TSH. Treatment of primary osteoblasts and osteoclasts with TSH or a TSHR-stimulating antibody failed to induce a cAMP response. Furthermore, TSH did not affect the differentiation or function of osteoblasts or osteoclasts in vitro. These data indicate the hypothalamic-pituitary-thyroid axis regulates skeletal development via the actions of T_3 (Bassett et al. 2008).

5.2. Chemokine receptors and chemokine variants in cancer and inflammatory diseases (P4, P1)

5.2.1. CCR6 and metastatic potential in a mouse tumor model (P1)

Chemokines and their receptors play important roles in various aspects of tumoral processes, and evidence was provided for their critical involvement in determining the metastatic destination of tumor cells. We analyzed in vitro and in vivo, how CCR6 expression could alter the behavior of Lewis lung carcinoma (LLC) cells, which were shown to express low levels of the CCR6 ligand, CCL20 (LARC), both in vitro and in

vivo. The expression of CCR6 significantly decreased the number of metastases in immunocompetent C57BL/6 mice, without affecting the tumor-forming ability of LLC cells. This was correlated with a decrease in clonogenicity in soft and hard agar, and with increased adhesion to type-IV collagen. These two observations made in basal conditions were enhanced when CCL20 was added to the assay medium. Thus, expression of CCR6 in tumor cells, associated with the local production of CCL20, decreased the metastatic potential of the LLC line. We propose a model, in which the expression of a chemokine receptor in tumor cells can act as a metastasis-suppressor, or a metastasis-promoting factor, according to the expression, or the absence of expression of the cognate ligand(s) in the tumor (Sutherland et al. 2007).

5.2.2. Activation of G protein signaling by HTLV-1 Tax (P1)

Human T-cell leukemia virus type-1 (HTLV-1) is associated with adult T-cell leukemia (ATL) and neurological syndromes. HTLV-1 encodes the oncoprotein Tax-1, which modulates viral and cellular gene expression leading to T-cell transformation. We reported an interaction between HTLV-1 Tax oncoprotein and the G-protein beta subunit. Interestingly, though the G-protein beta subunit inhibits Tax-mediated viral transcription, Tax-1 perturbs G-protein beta subcellular localization. Functional evidence for these observations was obtained using conditional Tax-1-expressing transformed T-lymphocytes, where Tax expression correlated with activation of the SDF-1/CXCR4 axis. Our data indicated that HTLV-1 developed a strategy based on the activation of the SDF-1/CXCR4 axis in the infected cell; this could have tremendous implications for new therapeutic strategies (Twizere et al. 2007).

5.2.3. Hypoxia enhances CXCR4 expression in human melanoma cells (P4)

Angiogenesis is a crucial step in cancer progression and metastasis. The formation of new blood vessels is essential for the adequate supply of oxygen and serum factors to the growing tumor. Chemokines constitute a family of chemotactic cytokines that can affect many aspects of cancer biology, including the balance between angiogenesis and angiostasis. The influence of environmental factors (cytokines, matrix components, serum factors and O₂ level) on expression of receptors for angiogenic versus angiostatic CXC chemokines in human microvascular endothelial cells has not been extensively investigated. Our semi-quantitative RT-PCR analysis demonstrated that TNF- α and IFN- γ repressed CXCR4 mRNA levels in immortalized human microvascular endothelial HMEC-1 cells after 4h, whereas only TNF- α displayed inhibitory activity in primary human microvascular endothelial cells (HMVEC). CXCR4 mRNA expression was not affected by VEGF, GM-CSF, IL-1 β or various basal membrane matrix components, but was significantly up-regulated after serum starvation and/or hypoxic treatment of the microvascular endothelial cells. The alternative SDF-1/CXCL12 receptor, CXCR7/RDC1, was also up-regulated by hypoxia in HMEC-1 cells, although less consistently than CXCR4. Furthermore, hypoxia and serum starvation were required for cell surface display of CXCR4 and SDF-1/CXCL12 induction of ERK activation in HMEC-1 cells. In contrast, CXCR2 and CXCR3 mRNA levels remained, respectively, low and undetectable under all the conditions tested, and surface expression of CXCR2, CXCR3 and CXCR7 on the HMEC-1 cells could not be demonstrated by FACS. In the human SK-MEL-5 melanoma cell line, CXCR4 mRNA expression was also increased under hypoxic conditions, whereas CXCR2 mRNA levels remained low and levels of CXCR3 and CXCR7 were undetectable. However, immunohistochemical staining of human metastatic melanoma sections demonstrated that CXCR2, CXCR3, CXCR4 and CXCR7 are expressed on tumor cells and, to a lesser extent, on endothelial cells. These results demonstrate that the tumor microenvironment regulates chemokine receptor expression through both cytokine and oxygen levels (Schutyser *et al.*, 2007).

5.2.4. MCP-3/CCL7 delivered by parvovirus reduces tumorigenicity (P4)

Monocyte chemotactic protein-3 (MCP-3/CCL7), a CC chemokine able to attract and activate a large panel of leukocytes including natural killer cells and T lymphocytes could be beneficial in anti-tumor therapy. Vectors were constructed based on the autonomous parvovirus minute virus of mice (MVMp), carrying the human (MCP-3) cDNA. These vectors were subsequently evaluated in the poorly immunogenic mouse melanoma model B78/H1. The infection of the tumor cells with MCP-3-transducing vector at low virus input

multiplicities, but not with wild-type virus, strongly inhibited tumor growth after implantation in euthymic mice. In a therapeutic B78/H1 model, repeated intratumoral injections of MCP-3-transducing virus prevented further tumor expansion as long as the treatment was pursued. The anti-tumor effects of the MCP-3-transducing vector were not restricted to this tumor model since they could also be observed in K1735 melanoma. The depletion of CD4, CD8, NK cells and of interferon- γ in mice implanted with MVMp/MCP-3-infected B78/H1 cells abolished the anti-tumor activity of the vector. The latter data, together with tumor growth in nude mice and reverse-transcriptase (RT)-PCR analyses of MVMp/MCP-3-treated tumors, clearly showed that activated CD4, CD8 and NK cells were indispensable for the anti-neoplastic effect in the B78/H1 tumor. Altogether, our results show that MCP-3-transducing parvovirus vectors may be quite potent against poorly or nonimmunogenic tumors, even in conditions where only a fraction of the tumor cell population is efficiently infected with recombinant parvoviruses (Wetzel *et al.*, 2007).

5.2.5. Platelet factor-4 variant/CXCL4L1 inhibits tumor growth (P4)

Chemokines affect inflammation and cancer through leukocyte attraction and angiogenesis. The platelet factor-4 variant, designated PF-4var/CXCL4L1, is a recently described natural non-allelic gene variant of the CXC chemokine platelet factor-4/CXCL4. Here, we demonstrate that PF-4var/CXCL4L1, a highly angiostatic chemokine, is poorly chemotactic for phagocytes and is inducible in monocytes by inflammatory mediators but remained undetectable in macrophages and neutrophils. In addition, PF-4var/CXCL4L1 production by mesenchymal tumor cells was evidenced *in vitro* and *in vivo* by specific ELISA and immunohistochemistry. PF-4var/CXCL4L1, but not PF-4/CXCL4, was co-induced with the angiogenic chemokine CXCL6/granulocyte chemotactic protein-2 (GCP-2) by cytokines, e.g., IL-1 β and IL-17, in sarcoma cells, but not in diploid fibroblasts. Furthermore, the induction of GCP-2/CXCL6 in endothelial cells by IL-1 β was enhanced synergistically by TNF- α but inhibited by IFN- γ , which synergized with IL-1 β to produce the angiostatic CXCL10/IFN- γ -induced protein-10. These findings indicate that the equilibrium between angiostatic and angiogenic factors during inflammation and tumor progression is rather complex and differs depending on the chemokine, cell type, and stimulus. Selective intervention in the chemokine network may drastically disturb this delicate balance of angiogenesis and tissue repair. Application of angiostatic PF-4var/CXCL4L1 without attraction of protumoral phagocytes may be beneficial in cancer therapy (Vandercappellen *et al.*, 2007).

PF-4var/CXCL4L1 was cloned, and the purified recombinant protein strongly inhibited angiogenesis. Recombinant PF-4var/CXCL4L1 was angiostatically more active (at nanomolar concentration) than PF-4/CXCL4 in various test systems, including wound-healing and migration assays for microvascular endothelial cells and the rat cornea micropocket assay for angiogenesis. Furthermore, PF-4var/CXCL4L1 more efficiently inhibited tumor growth in animal models of melanoma and lung carcinoma than PF-4/CXCL4 at an equimolar concentration. For B16 melanoma in nude mice, a significant reduction in tumor size and the number of small intratumoral blood vessels was obtained with intratumorally applied PF-4var/CXCL4L1. For A549 adenocarcinoma in severe combined immunodeficient mice, intratumoral PF-4var/CXCL4L1 reduced tumor growth and microvasculature more efficiently than PF-4/CXCL4 and prevented metastasis to various organs better than the angiostatic IP-10/CXCL10. Finally, in the syngeneic model of Lewis lung carcinoma, PF-4var/CXCL4L1 inhibited tumor growth equally well as monokine induced by IFN- γ (Mig)/CXCL9, also known to attract effector T lymphocytes. Taken together, PF-4var/CXCL4L1 is a highly potent antitumoral chemokine preventing development and metastasis of various tumors by inhibition of angiogenesis. These data confirm the clinical potential of locally released chemokines in cancer therapy (Struyf *et al.*, 2007).

5.2.6. Role of GCP-2/CXCL6 in collagen-induced arthritis (P4)

Many experimental autoimmune diseases rely on immunization with organ-specific autoantigens in complete Freund's adjuvant (CFA), which contains heat-killed mycobacteria. In most of these models, endogenous interferon- γ (IFN- γ) acts as a disease-protective factor. Mice with a disrupted IFN- γ system are remarkably susceptible to experimental autoimmune diseases, such as collagen-induced arthritis (CIA),

which rely on the use of CFA. The inflammatory lesions of these IFN- γ knockout (KO) mice are characterized by an excessive proportion of neutrophils. Here, we show that the increased severity of CIA in IFN- γ R KO as compared with wild-type mice is accompanied by increased levels of the CXC chemokine granulocyte chemotactic protein-2 (GCP-2), a major neutrophil-attracting chemokine in mice. We demonstrated that the heat-killed mycobacteria present in CFA elicited production of GCP-2/CXCL6 in mouse embryo fibroblast cultures and that this production was inhibited by IFN- γ . Inhibition of GCP-2/CXCL6 production by IFN- γ was STAT-1-dependent. IFN- γ receptor KO mice treated with neutralizing anti-GCP-2/CXCL6 antibodies were protected from CIA, indicating the *in vivo* importance of GCP-2/CXCL6 in the pathogenesis of CIA. Our data support the notion that one of the mechanisms whereby endogenous IFN- γ mitigates the manifestations of CIA consists of inhibiting production of GCP-2/CXCL6, thereby limiting mobilization and infiltration of neutrophils, which are important actors in joint inflammation. These results may also be applicable to other experimental models of autoimmunity that rely on the use of CFA (Kelchtermans *et al.*, 2007).

5.3. Chemerin in inflammation and cancer (P1, P5)

5.3.1. Chemerin and ChemR23 in a psoriasis model in mice (P1)

In order to evaluate the pathophysiological role of ChemR23 and chemerin, we designed a mouse model of skin inflammation mimicking some aspects of psoriasis pathology in human. Chemerin expression was indeed reported in non affected skin of patients with psoriasis and induced in skin by retinoids. In addition, previous reports have shown that psoriasis skins were infiltrated with plasmacytoid dendritic cells. The mouse psoriasis-like model was induced by repetitive painting of the skin by the chemical agent 12-O-tetradecanoyl-phorbol-13-acetate (TPA). This promoted an acute inflammatory response with massive recruitment of neutrophils and lymphocytes, hyperplasia of the epidermis and abnormal proliferation of keratinocytes. Preliminary studies demonstrated differences in the skin inflammatory response in ChemR23 KO mice versus WT mice. Indeed, compared to WT mice, ChemR23 KO mice displayed stronger and more sustained inflammation in the upper dermis as well as an increased proliferation of undifferentiated keratinocytes (measured with keratin K14 and PCNA staining) from day 2 to day 6 after the last TPA application. Chemerin expression increased in the upper layers of the epidermis at day 2 after TPA application in both WT and KO skin. Whereas chemerin expression rapidly decreased in WT skin, sustained chemerin staining remained between day 2 and day 6 in ChemR23 KO mice. Inflammatory cytokines (TNF- α , IL-1- β and IL-6) involved in psoriasis pathology were significantly up-regulated in ChemR23 KO skins, in particular TNF- α . CD11c and CCR5 were also measured as DC markers. CCR5 and CD11c expression was lower in the skin of KO mice, suggesting that the increased skin inflammation can be explained by an impairment of the recruitment of DCs, leading to a delay in the resolution of the inflammatory reaction.

5.3.2. A viral pneumonia model due to respiratory syncytial virus applied to ChemR23 knockout mice (P5, P1)

Respiratory syncytial virus (RSV) is known to infect ~85% of children by age 18 mo, and virtually all children by age 3 y. About 3% of each year's birth cohort is admitted for severe RSV respiratory tract infection every winter in Europe, Australasia and North America. Until recently, the human RSV virus itself was used to infect inbred mice to decipher the pathophysiology of RSV-associated disease. However, RSV infection of mice does not result in any measurable degree of morbidity, evokes a mild mononuclear cell infiltration instead of a profuse granulocytic bronchiolitis, does not result in eosinophil recruitment, never progresses to ARDS and generates viral titers systematically lower than that inoculated. Taken together, these characteristics suggest that using the RSV murine model to mimick the human disease is rather counterintuitive. This is the reason why we developed a model using the natural rodent pneumovirus pathogen, which is also the closest phylogenetic relative of human RSV virus, pneumonia virus of mice (PVM). The crucial advantages of our PVM-associated lung infection include the following: (i) clinical picture – morbidity – consistently mimicking that observed in infants with RSV-associated bronchiolitis ; (ii) dramatic granulocytic and eosinophilic infiltrations that parallel the pathological changes observed in humans

; (iii) clear evidence of widespread viral replication in lung tissue, with incremental recoveries that, at peak, are in excess of 10^8 pfu/g in response to as few as 30 pfu in the inoculum ; and (iv) clear progression to ARDS as reported for ~3% infants with RSV bronchiolitis.

The model consists in the intranasal inoculation of the virus under light anaesthesia, the daily follow-up of body weight, double chamber plethysmographic lung function values and carbon monoxide uptake values, lung histopathology and lung viral titrations on days 5, 6 and 7 pi.

Starting from the J3666 PVM strain, we have established a working viral stock by serial passages in highly susceptible mice. The inoculum inoculated to each mouse consists in 10^3 PFUs diluted in 50 microliter of MEM. This standardized infection results in cumulated mortality rates on days 5, 6 and 7 pi of 0%, 10% and 10% in DBA/2, 0%, 10% and 15% in 129/Sv and 5%, 10% and 20% in C3H/HEN inbred strains respectively. As far as the BW is concerned, two trends are distinguishable. The first is a gradual decrease in BW from day 2 (BALB/c) or day 4 (129/Sv, C3H/HeN, C57BL/6, DBA/2) which peaks on day 7 with a weight loss of ~15%. The second is stable BW (SJL).

Regarding the respiratory pattern, two specific types of reaction are typically observed, depending on the mouse strain inoculated. The first, typical of DBA/2, C3H/HeN, 129/Sv and BALB/c strains consists of a stable minute ventilation up to day 4, followed by a progressive decrease of which the nadir is reached at day 7 and amounts to 50, 50, 65 and 75% of the preinoculation levels in DBA/2, C3H/HeN, 129/Sv and BALB/c respectively. The stable minute ventilation is achieved via a stable respiratory rate/tidal volume combination, whereas the hypoventilation period is always characterized by an increased respiratory rate (120, 130, 115 and 115% respectively) on the one hand, and a dramatically collapsed tidal volume on the other (50, 50, 65 and 70 % respectively). The second type of reaction is shared by C57BL/6 and SJL and consists of a constant minute ventilation throughout the observation period, achieved through a constant respiratory rate/tidal volume combination up to day 4, but via a faster and shallower breathing after. Among the two strains, the C57BL/6 response is obviously more intense (~150% of preinoculation respiratory rate combined with ~60% of preinoculation tidal volume on day 7) than that of SJL (127 and 83% respectively).

Nasal turbinates and tracheae of infected mice display very subtle changes, consisting in degeneration of individual epithelial cells and infiltration of some mononucleated inflammatory cells in the lamina propria. At first sight, the morphologic aspect of cartilaginous as well as membranous intrapulmonary airways is similar among mouse strains, with very modest and rare regressive epithelial lesions (degeneration, necrosis, exfoliation) and luminal exudates, and total absence of epithelial hyperplasia or syncytia formation. Two clearly different histological profiles were identified on the basis of qualitative and quantitative criteria applied to the peribronchial/peribronchiolar cuffs, SJL on the one hand and BALB/c, DBA/2, C57BL/6, 129/Sv and C3H/HeN on the other. In SJL, there is little difference between the lamina propria of infected animals and the normal morphology of murine airways. Specifically, only a few animals exhibited a moderate infiltration with mononuclear inflammatory cells and there was no eosinophilic infiltration. Conversely, in the 5 other strains, most of the animals displayed significant to severe infiltration of the lamina propria with mononucleated inflammatory cells but also with eosinophils. Among the five strains, eosinophils are readily identified in 30% of BALB/c, 70-80% of C57BL/6, C3H/HeN and DBA/2 and virtually in all 129/Sv. Considering the lungs, SJL also differs from the other 5 strains in that the alveolar spaces remain empty, with the exception of a few macrophages, the density of which is comparable to that observable in a healthy lung. In the interstitium, cell density appears slightly elevated, multifocally, in roughly half of the animals. The histopathological diagnosis most compatible with SJL-specific observations is slight mononuclear multifocal interstitial pneumonitis. The lung histological profile of the 5 other strains is qualitatively similar, consisting of the presence of alveolar exudates and diffuse interstitial infiltration. The alveolar exudates is a more or less concentrated mixture of cell debris, pleiomorphic lymphoid cells, neutrophils and morphologically altered macrophages (cytoplasmic vacuolation, pycnosis, caryorhexis). Quantitatively speaking, most of the alveolar spaces are optically empty, but often (~50% of individuals in BALB/c) or rather systematically (C57BL/6, C3H/HeN, DBA/2 and 129/Sv), groups of alveoli are observed

which contain the exudates described above. In general, these alveolar clusters are distributed in a random fashion and in C3H/HeN and 129/Sv, tend to merge, giving the impression of conquering the more peripheral spaces gradually. Overall, the number of alveoli concerned varies between strains, from a few dozen in BALB/c to 15% (C57BL/6, DBA/2 and 129/Sv) or 20% (C3H/HeN) of the lung section area. The lung interstitium, when examined away from the areas of alveolitis, is multifocally (C57BL/6, BALB/c) or diffusely (C3H/HeN, DBA/2, 129/Sv) infiltrated by cells with round nuclei of the monocytoïd or lymphoid type. The histopathological diagnoses most compatible with these observations are multifocal mononuclear alveolitis and moderate (C57BL/6, BALB/c) to severe (C3H/HeN, DBA/2, 129/Sv) diffuse mononuclear interstitial pneumonia.

The time required to reach the peak viral titer differs according to the strain used : 5 days in C3H/HeN, DBA/2 and SJL, 6 days in BALB/c and DBA/2 and 7 days or more in C57BL/6. The 129/Sv strain displays the highest peak viral load (on day 6), and SJL the lowest (on day 5). Among strains of which the peak viral titer is reached on day 5, C3H/HeN and SJL display comparable day 5 to day 7 clearances (minus 1.14 vs 1.09 log. unit), whereas that of DBA/2 is not significant. Thus, SJL combines the shorter time-to-peak viral titer, the lowest peak viral titer and, with C3H/HeN, the best lung clearance. Topological distribution of viral antigens within the lungs by immunofluorescence also generates very different results according to the strain. The 129/Sv strain is distinguished by the presence of virus-positive bronchial/olar epithelial cells, type-1 and -2 pneumocytes and macrophages homogeneously distributed throughout the lungs. The types of virus-positive cells is similar in DBA/2, C3H/HeN, C57BL/6 and BALB/c, but the virus-positive cells occupy an accumulated area of ~60 to ~80% of the samples section area. In SJL, only few and far between virus-positive bronchial cells and subpleural pneumocytes are identified.

Altogether, we established a mouse model of pneumovirus-associated disease that recapitulates the clinical, functional, histological and virological characteristics of the human disease. Significant differences appeared between strains, to the extent that a wide phenotypic spectrum was established for survival rate, clinical and tissue responses (bronchial cuffing, alveolar exudates, interstitial infiltration), eosinophil infiltration (present or not), lung viral peak titer (high, medium, low) and clearance (effective or not). The detection of these subtle between-strain differences shows the ability of the model to catch a wide array of physiopathological aspects. Thanks to the IAP project, this model developed by P5 is currently implemented in the C57BL/6 mouse line knockout for ChemR23 in order to examine the possibility that this receptor participates to the pathophysiology of human RSV disease. A first set of experiments has been obtained with this model, but other series need to be performed before being able to derive conclusions.

Afterwards, this model will be applied to other GPCR knockout models (GPR10 and GIR), while other viral infection models (paramyxovirus and influenzaviruses) will be used on the same mouse lines.

5.4. Mutations of the arginine-vasopressin receptor type 2 (P1)

Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is a recently described genetic cause of hyponatremia in male infants due to activating mutations of the arginine-vasopressin receptor type 2 (AVPR2). Whether this X-linked condition could be detected in the adult or also could affect women is unknown. A large five-generation family was identified in which the recently described AVPR2 mutation that is responsible for NSIAD was segregated. The proband was a 74-yr-old patient who had a syndrome of inappropriate antidiuresis and whose hyponatremia resisted administration of two AVPR2 antagonists. The phenotype of family members who carry the mutation was investigated. Patients with normal serum sodium were subjected to a water-load test. The previously reported activating missense R137C mutation in the AVPR2 gene in three hemizygous male and four heterozygous female individuals was identified. Except in one woman, spontaneous episodes of hyponatremia or abnormal water-load test were identified in all patients with the mutation, whether male or female. Skewed X inactivation was evidenced in the blood of the asymptomatic woman, which is compatible with preferential inactivation of her mutated allele. NSIAD is therefore not limited to male infants. The diagnosis should also be considered in both male and female adults (Decaux et al. 2007).

5.5. QTL analysis in animal models (P5)

Infection of mice with the mouse pneumovirus (PVM) was used as a model of human infections with respiratory syncytial virus (RSV). The main objective of this study was to map quantitative trait loci (QTLs) influencing resistance/susceptibility to pneumoviruses. A whole genome scan was conducted on 245 F2 mice issued from a cross between two inbred strains (susceptible strain 129sv and resistant strain SJL) showing extreme responses to PVM infection. Evolution during infection of the respiratory functions measured by whole body plethysmography, viral titer in the lungs, and body weight losses were used as traits for this analysis. One hundred ninety-six microsatellite markers covering the 19 autosomes and the X chromosome were genotyped on the F2 population with mean marker spacing (mean \pm SD) of 8.2 ± 6.0 cM (range 0.2-29.4) and an average information extraction of 82%. QTL mapping was performed on single traits by linear regression using the web-based software QTL Express. Significance thresholds were determined by permutation and confidence intervals were obtained by bootstrap analysis. At the genome-wide significance level $p < 0.05$, QTLs for resistance/susceptibility to PVM were located on chromosomes 1, 3, 4 and 16. Suggestive QTLs were also located throughout the genome with an expected proportion of true QTL among suggestive peaks of 41%. Interestingly, an overlap was observed between most of these genome-wide significant QTLs and significant or suggestive QTLs detected for these same traits measured before infection. Thus, part of the differences in resistance or susceptibility to PVM could be due to differences in respiratory physiology between the two parental strains and not to anti-viral or immunological phenomenon. However, phenotypic variance was only partially explained by these effects. One explanation could be that differences in resistance to PVM between the parental strains could also be due to numerous QTL not detected in this experiment because of their small effect or because of the non-additive interactions between these loci. Interactions between loci will thus be investigated to try to explain the part of the phenotypic variance not explained by the major effects related to mice respiratory physiology.

WORKPACKAGE 6. IDENTIFICATION OF NOVEL RECEPTORS AND THEIR LIGANDS

Many orphan receptors for which the ligands and function are still unknown are encoded by the mammalian, insect and yeast genomes. Several partners focus on the characterization of these receptors, through the identification of their ligand, and the subsequent delineation of their function. We aim at the identification of human receptors for leukocyte chemoattractants, neuropeptides, glycoprotein hormone-like proteins and glucose, insect receptors for neuropeptides, and nutrient-sensing receptors in yeast, using evolutionary clues in this approach.

6.1. Chemoattractant and neuropeptide receptors (P1)

6.1.1. Functional screening assays.

With the aim of identifying the natural ligands of orphan receptors, we had established cell lines coexpressing $G_{\alpha 16}$, apoaequorin and genes encoding selected orphan G protein-coupled receptors. This library is regularly updated by incorporation new receptors of interest. We have however identified for some receptors a number of problems in this expression system (low expression levels, sequence rearrangements, instability of the cell lines). In order to improve the probability of finding natural ligands for these orphan receptors, specific strategies were developed to better characterize the cell lines expressing them.

We tested the orphan receptors for constitutive activity in the G_q and G_s -stimulated cascades, in order to identify potentially the natural pathways activated by the receptors, and to manage eventually the screening using other, and best suited, functional assays. To be able to monitor the expression at the cell surface of recombinant cells by FACS analysis, we have also fused some of the receptors at their N-terminus to an epitope tag. We also used parental cell lines expressing aequorin and $G_{\alpha 16}$ and allowing to express GPCRs under the control of doxycycline (Tet-On inducible system) and to prevent counter-selection problems linked to the constitutive activity or toxicity of the receptor in the cell line.

Finally, $G_{\alpha 16}$ is known to couple most receptors to the phospholipase C-calcium release pathway, but is not the optimal coupling protein for all receptors. The use of a chimeric G protein, made of the $G_{\alpha q}$ backbone, with the last 5 aminoacids originating from the $G_{\alpha i}$ sequence, can be a better partner to couple to calcium mobilization some receptors naturally linked to the G_i pathway. New parental cell lines, coexpressing aequorin and the $G_{q/i}$ chimera, were therefore used for expressing some receptors.

6.1.2. Characterization of natural or surrogate ligands.

A set of receptors was selected for their structural similarity with chemoattractant receptors and/or their described expression in leucocyte populations. These include HCR, HB954, GPR1, EST6, H963, HSGPCP, GPR31, ETBRLP2, GIR and GPR81. All these receptors have been expressed in cell lines co-expressing apoaquorin and $G_{\alpha 16}$ and in addition, some of them were expressed as well in cells coexpressing apoaquorin and $G_{\alpha q i 5}$.

A collection of fractions from animal tissues (essentially porcine) has been prepared according to a number of standardized extraction and fractionation procedures. Fractions collected after the first step of the separation by HPLC are tested on recombinant cell lines expressing orphan receptors, using the aequorin-based assay, in order to detect potential biological activities.

The procedures routinely used for preparing peptide extracts include the following:

- **organic extracts** were performed in a methanol/water/acetic acid (90:9:1) medium, following homogenization of tissues with a warring blender and ultraturax. After centrifugation, the lipids were removed by extraction with CH_2Cl_2 . Sample were then loaded on a preparative C18 column for separation with an acetonitrile/TFA gradient.

- **aqueous extracts** were performed according to the same protocol, with the exception that the extraction was made in boiling water in order to inactivate proteases and precipitate most large proteins.

- For **lipid extracts**, the tissue homogenization was performed in methanol. After centrifugation, the supernatant was diluted with 20% water and subjected to a solid phase extraction on a C18 phase using acetonitrile for the elution. The eluate was dried using a rotary evaporator and reconstituted in a mixture of methanol, acetonitrile and water (1:1:1). The extracts were separated by HPLC using a C18 column and an acetonitrile gradient in 0.02% formic acid as eluent.

- **Ammonium acetate extracts** were generated in order to avoid the potential degradation of bioactive compounds in acidic conditions during the original extraction procedures described above. For this purpose, tissues were homogenised in a mixture of 20 mM ammonium acetate and methanol (1:1). After centrifugation, the methanol was removed from the supernatant by evaporation. The extracts were further loaded on a C18 column and an acetonitrile gradient in 20 mM ammonium acetate was applied.

These extracts were tested on the selected receptors. As these receptors are expected to respond to inflammatory mediators, extracts were prepared from human biological inflammatory fluids, including pleural fluid, follicular fluid, ascitis secondary to pancreatic or ovary carcinoma, or ovary hyperstimulation, as well as human plasma. These extracts were fractionated through one or two HPLC steps before testing. A number of putative biological activities have been observed, but so far, either they were not confirmed in subsequent purification steps, or they did not result in the identification of the ligand. A new set of receptors is presently being selected for testing.

6.2. Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and IL-8/CXCL8 (P4)

Chemokines and chemokine receptors are well preserved through various mammalian species. However, a few animal chemokines (e.g. regakine-1 and lungkine) exist for which a homologue has not yet been identified. Functional interleukin-8 (IL-8) receptors (IL-8RA and IL-8RB: CXCR1 and CXCR2, respectively) have been described in human, monkey, dog, rabbit and guinea pig. Although three IL-8R homologues have been found in rat, only one of these, rat CXCR2, appears to be functional based on

responsiveness to ligands. Similarly, CXC chemokines induce biological responses through the murine homologue of CXCR2, but the identification of functional rodent CXCR1 homologues has remained elusive. We have identified and characterized the mouse CXCR1 homologue (mCXCR1). Murine CXCR1 shares 68 and 88% amino acid identity with its human and rat counterparts, respectively. Similar to the tissue distribution pattern of rat CXCR1, we found murine CXCR1 mRNA expression predominantly in lung, stomach, bone marrow, and leukocyte-rich tissues. In contrast to previous reports, we determined that mCXCR1 is a functional receptor. We show predominant engagement of this receptor by mouse GCP-2/CXCL6, human GCP-2/CXCL6, and IL-8/CXCL8 by binding, stimulation of GTP γ S exchange, and chemotaxis of mCXCR1-transfected cells. Furthermore, murine CXCR1 is not responsive to the human CXCR2 ligands ENA-78/CXCL5, NAP-2/CXCL7, GRO- α , - β , - γ /CXCL1-3, or rat CINC-1-3. In addition, we show that concomitant elevation of mCXCR1 and its proposed major ligand, GCP-2/CXCL6, is positively correlated with paw swelling in murine collagen-induced arthritis. This report represents the first description of a functional CXCR1-like receptor in rodents (Fan *et al.*, 2007).

6.3. Glucose-sensing receptors (P3)

We have identified a sugar-sensing receptor in intestinal epithelial cells. The expression level of the main glucose transporter in the small intestinal brush border membrane (BBM), SGLT1, is controlled by sugar-levels present in the gut lumen. After a sugar-rich meal, the number of SGLT1 proteins in the BBM increases up to twofold, increasing transport capacity dramatically. In diabetic patients, the SGLT1 levels are 4-fold upregulated in a constitutive manner. Previous research has demonstrated that the sugar levels in the gut lumen, exert their effect on SGLT1 levels through a true sensing mechanism, since both non-metabolisable and non-transportable sugar analogues can mimic the effect of naturally occurring sugars. A GPCR has been proposed to be involved, since G proteins can be detected in the BBM and the cAMP-PKA pathway, a classical target for G proteins was shown to be critically involved.

We have identified a brush border GPCR, that couples to Gs proteins and the cAMP-PKA pathway. Upon inhibition of this receptor, inhibition of the SGLT1 upregulation is achieved in an *ex-vivo* setting (intestinal loop experiments), moreover, immunohistochemically the receptor colocalises with SGLT1 proteins in the BBM. When expressed in a mammalian cell system, the cells display GPCR mediated sugar signalling, while control transfected cells do not.

To characterise the response generated by various sugars, we collaborate with Walter Luyten, (KUL, connected with partner 2). To determine ligand binding, we will make use of radioligand binding techniques, and to determine the exact ligand-binding site, we use both SCAM analysis (*see below*) and computer based modelling in collaboration with Leonardo Pardo (EU1). In addition we will determine the effect of *in vivo* blocking the receptor (feeding experiments), using a compound obtained from Johnson & Johnson with low-bioavailability, that theoretically should only act in the small intestine. In parallel, we will try to establish the same using nanobodies (VHH fragments of camelid antibodies) generated against our GPCR target. An expression system to generate large amounts of the nanobodies is currently under development.

In yeast, glucose and sucrose activation of the PKA pathway through the GPCR Gpr1 is closely connected with the sensing of other essential nutrients, which together with the sugar are required to maintain high PKA activity. The other nutrients are sensed by nutrient transporter-receptors or 'transceptors' that activate PKA without using cAMP as second messenger. In this project we investigate in detail how these transceptors function, what signaling pathway they use and how this pathway integrates with the GPCR-induced cAMP-mediated pathway for activation of PKA.

Transporter-related nutrient sensors, called transceptors, have been discovered in the plasma membrane of eukaryotic cells. The action mechanism of transporting and non-transporting transceptors is unknown. We have screened 319 amino acid analogues for inhibition of amino acid transport by Gap1, a yeast transceptor that triggers activation of the PKA pathway. Among the competitive inhibitors, only a minority acts as agonists of the signaling function of Gap1. This indicates that mere binding of a compound to the amino acid

binding site is not enough to trigger signaling. Several non-transported agonists for Gap1 were identified, mimicking the functioning of non-transporting transceptors. Using SCAM analysis (Substituted Cysteine Accessibility Method) we identified the Ser³⁸⁸ and Val³⁸⁹ residues as being exposed in the amino acid binding site and we show that transported and non-transported agonists use the same binding site as used in the transport process for activation of transceptor signaling. Our results provide the first insight into the action mechanism of transceptors. They have identified the ligand-binding site of Gap1 and indicate that signaling requires a ligand-induced specific conformational change, which may be part of, but does not require the complete transport cycle. (Van Zeebroeck et al. 2008)

6.4. Studies on insect orphan GPCRs (P2)

6.4.1. Reverse pharmacology of orphan GPCRs derived from insect genome data

The main goal of this research section is the molecular and functional characterization of orphan GPCRs derived from insect genome and EST databases. We hypothesize that a number of *Drosophila melanogaster* GPCRs with an as yet unknown function or ligand do perform a (not yet identified) developmental and/or physiological role in the fruit fly. In addition, these GPCRs might yield information concerning the action and evolution of the corresponding orthologs in other metazoans (including vertebrates). We have constructed expression vectors for several uncharacterized fruit fly GPCRs. These receptors show similarity to known peptide, biogenic amine and prostaglandin receptors from mammals. The initial goal will be to search for the natural ligands of these orphan GPCRs by using a suitable cellular expression and detection system. Candidate GPCRs will be co-expressed with apo-aequorin (a Ca²⁺-sensitive photoprotein) and possibly G_α16 (a G-protein that couples most GPCRs to the Ca²⁺ signaling pathway). This allows a relatively easy and fast detection of receptor mediated Ca²⁺-pulses and the approach has already proven to be successful for the deorphanization of many metazoan GPCRs. We own an extensive amine and *Drosophila* peptide library that is used for an initial screening assay. Since our ligand library contains *ca.* 80 % of all known *Drosophila* neuropeptides and amines, and because this reverse pharmacological screening strategy has already proven its use several times, we anticipate to functionally characterize a number of the current, orphan GPCRs. In addition, the analysis of *Drosophila* GPCR mutants (knock-down, knock-out, over- and miss-expression mutants) will yield valuable information concerning their *in vivo* role(s).

6.4.2. Exploring a novel EST database from desert locust CNS

The Desert Locust (*Schistocerca gregaria*) is a notorious agricultural pest that threatens the livelihood of millions of people and therefore is constantly monitored by the FAO (<http://www.fao.org/ag/locusts/>). At the root of this threat is an extreme form of environmentally driven phenotypic plasticity by which one genome can manifest itself in two very different kinds (phases) of animal. At low population densities, locusts usually occur in the *solitarious phase*. Occasionally, however, patchy food resources and increasing population densities can force many solitarious locusts into close contact. This forced crowding triggers a striking phenotypic switch that results in the *gregarious phase*, an anatomically, physiologically and behaviourally distinct insect that actively aggregates into huge destructive swarms, which can migrate over thousands of kilometres. For many decades, the desert locust has been an experimental model for physiological and neurobiological research. In collaboration with the 'W.M. Keck Center for Comparative and Functional Genomics', we recently generated an *Expressed Sequence Tags* (EST) database representing a large number of transcripts expressed in desert locust CNS (<http://titan.biotech.uiuc.edu/locust/>). These sequences are currently being annotated by bioinformatic analyses and will be further employed to generate microarrays for transcriptome-wide experimental studies on this heterometabolous species. *Contig* analysis (of *ca.* 35000 sequences) revealed that the EST database currently represents *ca.* 12700 unique transcripts and constitutes a rich source of novel, high quality macromolecular sequence data, including a variety of neuropeptide precursors, receptors and other components of signal transduction cascades.

In addition, for an initial set of 6 locust GPCR transcripts, we have studied in more detail the tissue distribution and possible gender- and phase-dependent expression by means of Q-RT-PCR. This already led

to the discovery of a putative octopamine receptor that is induced in skeletal (flight) muscles of gregarious phase locusts.

6.5. Orphan LGRs (P1 and P2)

Leucine-rich repeats containing G protein-coupled receptors (LGRs) constitute a unique cluster of transmembrane proteins sharing a large leucine-rich extracellular domain for hormone binding. In mammals, LGRs steer important developmental, metabolic and reproductive processes as receptors for glycoprotein hormones and insulin/relaxin-related proteins. In insects, a receptor structurally related to human LGRs mediates the activity of the neurohormone bursicon thereby regulating wing expansion behaviour and remodelling of the newly synthesized exoskeleton. In the past decade, novel insights into the molecular evolution of LGR encoding genes accumulated rapidly due to comparative genome analyses indicating that the endocrine LGR signalling system likely emerged before the radiation of metazoan phyla and expanded throughout evolution. (Van Loy *et al.*, 2008).

Bursicon bioactivity is essential for tanning of the exoskeleton and for wing spreading behavior that occur in newly emerged adult insects. Previously, we demonstrated that in the fruit fly, *Drosophila melanogaster*, bursicon exists as a heterodimeric cystine knot protein that activates the leucine-rich repeats containing G protein-coupled receptor 2 (DLGR2). By performing similarity based *in silico* searches in genomic and complementary DNA databases, we identified bursicon homologous sequences in several protostomian as well as deuterostomian invertebrates. In the genome of the honeybee, *Apis mellifera*, the coding regions for bursicon cystine knot subunits are organized in a genomic locus of approximately 4 kilobase pairs. Reverse transcription PCR analysis indicates that this region likely codes for two distinct bursicon cystine knot subunits. Our results illustrate the remarkable conservation of bursicon in invertebrate species and provide an avenue for functional analyses of this hormone in a wide range of animal species. (Van Loy *et al.*, 2007).

WORKPACKAGE 7. OLFACTORY RECEPTORS AND EVOLUTION OF GPCR FAMILIES

This workpackage represents new activities in the network that are being implemented within the partners' teams, in collaboration with external groups belonging to parallel IAP network applications.

7.1. Olfactory receptors (P1, P2)

The structure of olfactory receptors has been known for 15 years, but their functional expression is still a major problem, as mammalian ORs are poorly targeted to the cell surface in heterologous systems. Due to these limitations, only a few mammalian ORs have been characterized functionally to date. One of the most significant improvements reported over the last few years is the demonstration that the transmembrane proteins RTP1, RTP2 (both expressed specifically in olfactory neurons) and REEP1 contribute to the translocation of ORs to the plasma membrane, and promote their functional expression in mammalian cell lines (Saito *et al.* Cell 2004), allowing the design of more reliable functional assays. It is however our hypothesis that additional membrane or soluble proteins are required for the reconstitution of an efficient signaling complex in the knobs of olfactory neuron cilia, complexes that may resemble those found at neuronal synapses, in which receptors, transduction proteins and channels are organized by a number of chaperones and scaffolding proteins. We have started to explore further this hypothesis by using a proteomic approach. We have established a procedure for the homogenization of olfactory mucosa from mouse and the preparation of a fraction enriched in ciliary knobs. This enrichment has been demonstrated by the detection of the $G_{\alpha_{olf}}$ protein, which is part of the signaling machinery proximal to the receptor. In order to purify further the protein complexes containing the receptors, it will be necessary to express a specific tagged olfactory receptor in all olfactory neurons. To this end, we are preparing a construct that will place the expression of a receptor under the control of tetracyclin responsive promoter, and this construct will be used to generate transgenic mice. These mice will be bred with a strain expressing two other transgenes (OMP-TTA and $G_{\gamma 8}$ -TTA), allowing the permanent expression of a given receptor in all olfactory neurons. Such expression is not achieved adequately using less sophisticated approaches, as a result of a complex control of receptor

expression in olfactory neurons, involving apparently the coding sequence of the receptors themselves (Nguyen et al. Cell 131:1009-17, 2007). An OR-enriched fraction will be prepared from these mice by immunoprecipitation with an anti-Tag monoclonal. Once purified, we will screen by 2D-DIGE and 2D-LC the proteins over-represented in this fraction, as compared to a total membrane fraction of the same initial homogenate. Proteins identified by mass spectrometry will be considered as part of signaling complexes and will be tested functionally in reconstituted systems in mammalian cell lines co-expressing ORs, G_{olf} , and the cyclic-nucleotide-gated channel.

7.2. Bioinformatics and evolution of the GPCR gene family (P1, EU1)

We have initiated the analysis of chemoattractant receptor and ligand genes in the presently available vertebrate genomes, in terms of evolution of these gene families. This will be pursued during the next period.

II. NETWORK ORGANISATION AND MANAGEMENT

Network's organization

The partnership is composed of five Belgian academic laboratories, belonging to three Universities, ULB (P1), KUL (P2, P3 and P4) and ULG (P5), and a Spanish group from University of Barcelona as foreign partner (EU1). These groups have complementary expertise in various aspects of GPCR research, and most are involved in diverse aspects of several workpackages. The interactions between partners have included exchanges of researchers, material and expertise, meetings between the partners concerned by each task, and regular contacts by electronic mail. The management of the program is performed by a steering committee made of the promoters and principal group leaders of the network partners.

Network kick-off meeting

The kick-off meeting of the network was organized on June 28, 2007 in the presence of Corinne Lejour. During this meeting, we determined options regarding practical aspects. The opportunity of creating a specific Doctoral School was considered, but given the large number of existing doctoral schools, and the uncertainty regarding their precise prerogatives, we decided not to go in this direction at this stage. This might be reconsidered when the roles of doctoral schools will be clarified. The format of the first plenary meeting was determined. It was also decided that the International meeting would be organized in 2009. This meeting could complement the 2009 plenary meeting. We considered a symposium of three days, with an attendance limited to about 150 to 200 participants. This meeting would cover the various interests of the network, with a number of invited European and extra-European speakers, spots allocated to partners of the IAP network, and speakers selected on the basis of abstract submission. This meeting might be combined with the regular meeting of one of the EU networks in which IAP partners are involved, in order to combine resources and attractiveness, and allowing assembling an international program of high interest. This will be discussed with the steering committee of these networks.

Plenary meetings of the network

A first plenary meeting was organized on February 15, 2008 on the Erasme campus of the ULB. Although it was initially planned in November or December 2007, it has been difficult to find a date on which all principal investigators could attend, due to attendance to international conferences and academic duties. This meeting has gathered all groups, including most senior and junior staff involved in the program, in the presence of Corinne Lejour. The program of this meeting is provided as an annex (Annex 1). Morning and afternoon sessions were dedicated to scientific lectures (eleven talks) in which all groups presented a selection of their overall contribution to the program for the first year. This has been the occasion for the younger investigators (PhD students and post-docs), to train in presenting their data to a specialized and critical audience. In addition, a poster session (16 posters) was organized during the lunch break, in order to provide a broader overview of the activities going on within the network. With over 70 participants, this meeting was successful in providing all researchers in each group with a clear picture of the network activities. It has been the occasion to establish or re-establish personal relationships between the PhD students and post-docs of the various groups, leading to exchange of information, ideas and reagents.

Besides this scientific session, a coordination session of the steering committee took place at the end of the day. This meeting has also been the occasion of summarizing the network's achievements in prevision of this annual report. It was considered that the program was going well for most workpackages. Only workpackage 7, which constitutes a new activity for the network has been initiated, but is not yet running at its expected speed. No major changes in the orientation of the work or in the structure of the partnership have been considered at this stage, as most ongoing research activities were planned at the initiation of the program. The next meeting will be organised by KUL in November or December 2008, and will take place in

Leuven. The partners have been asked to gather suggestions for the organisation of the International meeting that will be organized in 2009.

Interactions between partners

Exchange of material and data

P1-P2.

Transfer of cell lines and expression constructs have been made between Partners 1 (ULB) and 2 (KUL-Vanden Broeck).

Common experiments were performed with candidate ligands of LGRs from different animal species, with the aim of characterizing orphan receptors of this family.

Two common publications have resulted from these interactions (Van Loy et al. 2007 and 2008)

P1-P3.

A common research program was initiated regarding the dimerization of the sugar sensing GPCR Gpr1 in yeast. This involved hosting Sam Van de Velde from P3 (KUL-Thevelein) in the P1 laboratory (ULB), where he worked with Jean-Yves Springael.

P1-P4.

P1 (ULB-Parmentier) has provided to P4 (KUL-Van Damme) chemokine receptor-transfected cells for different research programs:

- CHO cells expressing CXCR3 and CXCR7, characterized by P1 were used by P4 for the study of the proteolytic processing of I-TAC/CXCL11 by CD13 aminopeptidase N (resulting in one co-publication, Proost et al., Blood, 2007).

- CHO cells expressing CXCR3A and CXCR3B were used for the study of the interaction of IP-10/CXCL10 and CD26/ dipeptidylpeptidase IV (Proost et al., Arthritis Res. and Therapy, 2006).

- CHO cells co-expressing CCR2 and CXCR4 characterized by P1, were used by P4 to study chemokine synergy (a manuscript, Gouwy et al., has been submitted regarding this study)

Partners 1 (ULB) and 4 (KUL-Van Damme) are both involved in the EU network Innochem (Innovative Chemokine-Based Therapeutic Strategies for Autoimmunity and Chronic Inflammation). Several scientists and student from both lab participated to the annual meeting of this consortium (El Escorial, Spain, September 27-29, 2007), that was co-organized by J. Van Damme as member of the Steering Committee. Sophie Struyf (P4) was a chairperson. Four posters (two from P4, two from P1) were presented in addition. Paul Proost (P4) participated to the associated training course "Methodological approaches to leukocyte migration" by teaching the "Use of proteomics in chemokine research". This was held in Madrid, Spain, on September 24-26, 2007.

P1-P5.

In the frame of co-experiments regarding the role of specific receptors in anti-viral immune defense, partner P5 (ULG) has provided to P1 (ULB) influenza virus A, pneumovirus and paramyxovirus strains, as well as reference values of normal and pneumonic respiratory function for mouse strain C57BL/6, and reference lethal dose-50 values for highly pathogenic influenza virus A and respiratory syncytial virus strains for mouse strain C57BL/6. P1 (ULB) has provided knock out animals for the ChemR23 receptor, as well as experimental data regarding these mice, for testing their susceptibility to infection by the various viruses. Benjamin Bondue (from P1) has worked for several months in the P5 laboratory for performing the infections and learning the techniques. This interaction is being pursued in 2008.

P1-EU1.

Partner EU1 (UAB-Pardo) has provided P1 (ULB) with the modeling data for CCR5 that have been used in the design of the mutagenesis of the receptor (activation mechanisms) and the interpretation of the data. This has led to a co-publication (Springael et al. 2007). This model also constituted the basis for the screening of ligands in virtual libraries.

Partner EU1 has also provided models of the glycoprotein hormone receptors, in the frame of the analysis of natural mutations responsible for human diseases. This has led to two co-publications (De Leener et al. 2008, Caltabiano et al. 2008).

Partner EU1 also contributed the model of ChemR23 that is being used in the structure-function studies. The results of these studies will feed back the model.

P2-P3.

J. Vanden Broeck (P2) was president of the jury at the public PhD-defence of Tom Peeters (promotor: J. Thevelein, P3): 18/05/2007

J. Vanden Broeck (P2) and P. Van Dijck (P3) are both teachers of the course *Topics uit de moleculaire genetica (KULeuven)*

P2-P4.

Peptide synthesis and protein sequencing activities were performed by P4 (KUL-Van Damme) for P2 (KUL-Vanden Broeck)

J. Vanden Broeck (P2) and J. Van Damme (P4) are both involved as supervisors and evaluators of a PhD student (Annelies Bronckaers) at the Rega Institute (KULeuven)

J. Vanden Broeck (P2) and P. Proost (P4) are both teachers of the course *Structure, Synthesis and Cellular Function of Macromolecules (KULeuven)*

Exchange of personnel

P1-P3: Dimerization of the sugar sensing GPCR Gpr1 in yeast. Sam Van de Velde from P3 has worked at the laboratories of P1 on BRET analysis of Gpr1 dimerization.

P1-P5 : P1's graduate student B. Bondue is currently doing all the protocols related to the detection of GPCRs' role in viral pneumonia in P5's facilities.

P2-P3: Frederik Paulussen and Frank Stolz from P3 are collaborating with Walter Luyten (connected to P2) on the characterisation of ligand specificity and sensitivity of the sugar-sensing intestinal epithelial GPCR.

Website

A website was created for the IAP6-14 network (<http://www.ulb.ac.be/medecine/pai-iri/>). This website includes a list of the partners of the program and links to their own websites, the overview of the program, a list of publications resulting from the program, as well as informations regarding seminars related to GPCRs, the participation of the partners to European networks, and open positions in the network laboratories. This website will be expanded by additional sections over the next months.

III. PUBLICATIONS

1. PUBLICATIONS OF EACH TEAM

Partner 1

- Akcurin S, Turkkahraman D, Tysoe C, Ellard S, De Leener A, Vassart G, Costagliola S (2008). A family with a novel TSH receptor activating germline mutation (p.Ala485Val). *Eur J Pediatr*, In press
- Ballesteros-Yáñez I, Valverde O, Ledent C, Maldonado R, DeFelipe J (2007). Chronic cocaine treatment alters dendritic arborization in the adult motor cortex through a CB1 cannabinoid receptor-dependent mechanism. *Neuroscience* 146:1536-1545.
- Bassett JH, Williams AJ, Murphy E, Boyde A, Howell PG, Swinhoe R, Archanco M, Flamant F, Samarut J, Costagliola S, Vassart G, Weiss RE, Refetoff S, Williams GR (2008). A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. *Mol Endocrinol* 22:501-512.
- Bles N, Horckmans M, Lefort A, Libert F, Macours P, El Housni H, Marteau F, Boeynaems JM, Communi D (2007). Gene expression profiling defines ATP as a key regulator of human dendritic cell functions. *J Immuno* 179:3550-3558.
- Boudry C, Markine-Goriaynoff N, Delforge C, Springael JY, de Leval L, Drion P, Russell G, Haig DM, Vanderplassen AF, Dewals B (2007). The A5 gene of alcelaphine herpesvirus 1 encodes a constitutively active G-protein-coupled receptor that is non-essential for the induction of malignant catarrhal fever in rabbits. *J Gen Virol* 88:3224-33.
- Bura SA, Castañé A, Ledent C, Valverde O, Maldonado R (2007). Genetic and pharmacological approaches to evaluate the interaction between the cannabinoid and cholinergic systems in cognitive processes. *Br J Pharmacol* 150:758-765.
- Corbillé AG, Valjent E, Marsicano G, Ledent C, Lutz B, Hervé D, Girault JA (2007). Role of cannabinoid type 1 receptors in locomotor activity and striatal signaling in response to psychostimulants. *J Neurosci* 27:6937-6947.
- De Leener A, Caltabiano G, Erkan S, Idil M, Vassart G, Pardo L, Costagliola S (2008). Identification of the first germline mutation in the extracellular domain of the follitropin receptor responsible for spontaneous ovarian hyperstimulation syndrome. *Hum Mutat* 29:91-98.
- Decaux G, Vanderghenst F, Bouko Y, Parma J, Vassart G, Vilain C (2007). Nephrogenic syndrome of inappropriate antidiuresis in adults: high phenotypic variability in men and women from a large pedigree. *J Am Soc Nephrol* 18:606-612.
- Eckle T, Krahn T, Grenz A, Köhler D, Mittelbronn M, Ledent C, Jacobson MA, Osswald H, Thompson LF, Unertl K, Eltzschig HK (2007). Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation* 115:1581-1590.
- Gao JL, Guillabert A, Hu J, Le Y, Urizar E, Seligman E, Fang KJ, Yuan X, Imbault V, Communi D, Wang JM, Parmentier M, Murphy PM, Migeotte I (2007). F2L, a peptide derived from heme-binding protein, chemoattracts mouse neutrophils by specifically activating Fpr2, the low-affinity N-formylpeptide receptor. *J Immunol* 178:1450-6.
- Hussey MJ, Clarke GD, Ledent C, Hourani SM, Kitchen I (2007). Reduced response to the formalin test and lowered spinal NMDA glutamate receptor binding in adenosine A_{2A} receptor knockout mice. *Pain* 129:287-294.
- Kellenberger E, Springael JY, Parmentier M, Hachet-Haas M, Galzi JL, Rognan D (2007). Identification of nonpeptide CCR5 receptor agonists by structure-based virtual screening. *J Med Chem* 50:1294-1303.
- Makara JK, Katona I, Nyíri G, Németh B, Ledent C, Watanabe M, de Vente J, Freund TF, Hájos N (2007). Involvement of nitric oxide in depolarization-induced suppression of inhibition in hippocampal pyramidal cells during activation of cholinergic receptors. *J Neurosci* 27:10211-10222.

- Marcet B, Horckmans M, Libert F, Hassid S, Boeynaems JM, Communi D (2007a). Extracellular nucleotides regulate CCL20 release from human primary airway epithelial cells, monocytes and monocyte-derived dendritic cells. *J Cell Physiol* 211:716-727.
- Marcet B, Libert F, Boeynaems JM, Communi D (2007b). Extracellular nucleotides induce COX-2 up-regulation and prostaglandin E2 production in human A549 alveolar type II epithelial cells. *Eur J Pharmacol* 566:167-171.
- Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, Ledent C, Cheng X, Carrier EJ, Mann MK, Giovannoni G, Pertwee RG, Yamamura T, Buckley NE, Hillard CJ, Lutz B, Baker D, Dittel BN (2007). Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat Med* 13:492-497.
- Matos JE, Sorensen MV, Geyti CS, Robaye B, Boeynaems JM, Leipziger J (2007). Distal colonic Na(+) absorption inhibited by luminal P2Y(2) receptors. *Pflugers Arch* 454:977-987.
- Morrison RR, Tan XL, Ledent C, Mustafa SJ, Hofmann PA (2007). Targeted deletion of A2A adenosine receptors attenuates the protective effects of myocardial postconditioning. *Am J Physiol Heart Circ Physiol* 293:H2523-2529.
- Nadeem A, Fan M, Ansari HR, Ledent C, Jamal Mustafa S (2007). Enhanced airway reactivity and inflammation in A2A adenosine receptor-deficient allergic mice. *Am J Physiol Lung Cell Mol Physiol* 292:L1335-1344.
- Parolini S, Santoro A, Marcenaro E, Luini W, Massardi L, Facchetti F, Communi D, Parmentier M, Majorana A, Sironi M, Tabellini G, Moretta A, Sozzani S (2007). The role of chemerin in the co-localization of NK and dendritic cell subsets into inflamed tissues. *Blood* 109:3625-3632
- Pin JP, Neubig R, Bouvier M, Devi L, Filizola M, Javitch JA, Lohse MJ, Milligan G, Palczewski K, Parmentier M and Spedding M (2007). International Union of basic and clinical pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol Rev* 59:5-13.
- Popoli P, Blum D, Martire A, Ledent C, Ceruti S, Abbracchio MP (2007). Functions, dysfunctions and possible therapeutic relevance of adenosine A2A receptors in Huntington's disease. *Prog Neurobiol* 81:331-348.
- Proost P, Mortier A, Loos T, Vandercappellen J, Gouwy M, Ronsse I, Schutyser E, Put W, Parmentier M, Struyf S, Van Damme J (2007). Proteolytic processing of CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signalling and reduces lymphocyte and endothelial cell migration. *Blood* 110:37-44.
- Roh SG, Song SH, Choi KC, Katoh K, Wittamer V, Parmentier M, Sasaki S (2007). Chemerin-A new adipokine that modulates adipogenesis via its own receptor. *Biochem Biophys Res Commun* 362:1013-1018.
- Skrabanek L, Murcia M, Bouvier M, Devi L, George SR, Lohse MJ, Milligan G, Neubig R, Palczewski K, Parmentier M, Pin JP, Vriend G, Javitch JA, Campagne F, Filizola M (2007). Requirements and ontology for a G protein-coupled receptor oligomerization knowledge base. *BMC Bioinformatics* 8:177.
- Sohy D, Parmentier M, Springael JY (2007). Allosteric trans-inhibition by specific antagonists in CCR2/CXCR4 heterodimers. *J Biol Chem* 282:30062-30069.
- Springael JY, de Poorter C, Deupi X, Van Durme J, Pardo L, Parmentier M (2007). The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cell Signal* 19:1446-1456.
- Springael JY, Urizar E, Costagliola S, Vassart G, Parmentier M (2007). Allosteric properties of G protein-coupled receptor oligomers. *Pharmacol Ther* 115:410-418.
- Sutherland A, Mirjolet JF, Maho A, Parmentier M (2007). Expression of the chemokine receptor CCR6 in the Lewis lung carcinoma (LLC) cell line reduces its metastatic potential in vivo. *Cancer Gene Ther* 14:847-857.
- Thiemann G, Fletcher BC, Ledent C, Molleman A, Hasenöhrl RU (2007). The genetic versus pharmacological invalidation of the cannabinoid CB₁ receptor results in differential effects on 'non-associative' memory and forebrain monoamine concentrations in mice. *Neurobiol Learn Mem* 88:416-423.
- Twizere JC, Springael JY, Boxus M, Burny A, Dequiedt F, Dewulf JF, Duchateau J, Portetelle D, Urbain P, Van Lint C, Green PL, Mahieux R, Parmentier M, Willems L, Kettmann R (2007). Human T-cell leukemia virus type-1 Tax oncoprotein regulates G protein signaling. *Blood* 109:1051-60

- Van Loy T, Van Hiel MB, Vandersmissen HP, Poels J, Mendive F, Vassart G, Vanden Broeck J (2007). Evolutionary conservation of bursicon in the animal kingdom. *Gen Comp Endocrinol* 153:59-63.
- Van Loy T, Vandersmissen HP, Van Hiel MB, Poels J, Verlinden H, Badisco L, Vassart G, Vanden Broeck J (2008). Comparative genomics of leucine-rich repeats containing G protein-coupled receptors and their ligands. *Gen Comp Endocrinol* 155:14-21.
- Warnault V, Houchi H, Barbier E, Pierrefiche O, Vilpoux C, Ledent C, Daoust M, Naassila M (2007). The lack of CB1 receptors prevents neuroadaptations of both NMDA and GABA_A receptors after chronic ethanol exposure. *J Neurochem* 102:741-752.

Partner 2

- Badisco, L., Claeys, I., Van Hiel, M., Clynen, E., Huybrechts, J., Vandersmissen, T., Van Soest, S., Vanden Bosch, L., Simonet, G., & Vanden Broeck, J. (2008) Purification and characterization of an insulin-related peptide in the desert locust, *Schistocerca gregaria*: immunolocalization, cDNA cloning, transcript profiling and interaction with neuroparsin. *J. Mol. Endocrinol.*, *in press*.
- Badisco, L., Claeys, I., Van Loy, T., Van Hiel, M., Franssens, V., Simonet, G., & Vanden Broeck, J. (2007) Neuroparsins, a family of conserved arthropod neuropeptides. *Gen. Comp. Endocrinol.*, 153, 64-71.
- Breugelmans, B., Simonet, G., van Hoef, V., Claeys, I., Van Soest, S., & Vanden Broeck, J. (2008) Quantitative real-time RT-PCR analysis of pacifastin-related precursor transcripts during the reproductive cycle of solitary and gregarious desert locusts. *Insect Molecular Biology*, *in press*.
- Cnops, L., Hu, T-T., Vanden Broeck, J., Burnat, K., Van den Bergh, G., & Arckens, L. (2007) Age- and experience-dependent expression of Dynamin I and Synaptotagmin I in cat visual system. *J. Comp. Neurol.*, 504, 254-264.
- De Loof, A. (2008) Ecdysteroids, juvenile hormone and insect neuropeptides: Recent successes and remaining major challenges. *Gen. Comp. Endocrinol.*, 155, 3-13.
- Fichna, J., do-Rego, J.C., Chung, N.N., Schiller, P.W., Poels, J., Vanden Broeck, J., Costentin, J., & Janecka, A. (2007) Synthesis and characterization of potent and selective μ -opioid receptor antagonists, antanal-1 and antanal-2. *J. Med. Chem.*, 50, 512-520.
- Fichna, J., do-Rego, J.C., Janecki, T., Staniszewska, R., Poels, J., Vanden Broeck, J., Costentin, J., Schiller, P.W., & Janecka, A. (2008) Novel highly potent μ -opioid receptor antagonist based on endomorphin-2 structure. *Bioorg. Med. Chem. Lett.*, *in press*. [Epub 2008 Jan 8]
- Fichna, J., Staniszewska, R., Poels, J., Vanden Broeck, J., & Janecka, A. (2007) μ -opioid receptor ligands lack receptor subtype selectivity in the aequorin luminescence-based calcium assay. *Chem. Biol. Drug. Des.*, 70, 247-253.
- Franssens, V., Simonet, G., Breugelmans, B., Van Soest, S., Van Hoef, V., & Vanden Broeck, J. (2008) The role of hemocytes, serine protease inhibitors and pathogen-associated patterns in prophenoloxidase activation in the desert locust, *Schistocerca gregaria*. *Peptides*, *in press*. [Epub 2007 Dec 7]
- Husson, S.J., Mertens, I., Janssen, T., Lindemans, M., & Schoofs L. (2007) Neuropeptidergic signaling in the nematode *Caenorhabditis elegans*. *Prog. Neurobiol.*, 82, 33-55. Review.
- Larhammar, D., & Vanden Broeck, J. (2007) Peptide hormone and receptor evolution. *Gen. Comp. Endocrinol.*, 153, 147.
- Poels, J., Verlinden, H., Fichna, J., Van Loy, T., Franssens, V., Studzian, K., Janecka, A., Nachman, R.J., & Vanden Broeck, J. (2007) Functional comparison of two evolutionary conserved insect neurokinin-like receptors. *Peptides*, 28, 103-108.
- Rahman, M.M., Breuer, M., Begum, M., Baggerman, G., Huybrechts, J., & De Loof A. (2008) Localization of the phase-related 6-kDa peptide (PRP) in different tissues of the desert locust *Schistocerca gregaria* - Immunocytochemical and mass spectrometric approach. *J. Insect Physiol.*, *in press*. [Epub 2007 Dec 8]
- Reumer, A., Van Loy, T., Clynen, E., & Schoofs, L. (2008). How functional genomics and genetics complements insect endocrinology. *Gen. Comp. Endocrinol.*, 155, 22-30.

- Sas, F., Begum, M., Vandersmissen, T., Geens, M., Claeys, I., Van Soest, S., Huybrechts, J., Huybrechts, R., & De Loof, A. (2007) Development of a real-time PCR assay for measurement of yellow protein mRNA transcription in the desert locust *Schistocerca gregaria*: a basis for isolation of a peptidergic regulatory factor. *Peptides*, 28, 38-43.
- Van de Velde, S., Badisco, L., Claeys, I., Verleyen, P., Xi Chen, Vanden Bosch, L., Vanden Broeck, J., & Smagghe, G. (2007) Insulin-like peptides in *Spodoptera littoralis* (Lepidoptera): detection, localization and identification. *Gen. Comp. Endocrinol.*, 153, 72-79.
- Van Hiel, M. B., Van Loy, T., Poels, J., Vandersmissen, H.-P., Verlinden, H., Badisco, L., & Vanden Broeck, J. (2008) Neuropeptide receptors as possible targets for development of insect pest control agents. *In*: "Neuropeptide Systems as Targets for Parasite and Pest Control". Edited by: Timothy G. Geary and Aaron G. Maule. Landes Bioscience.
- Vandersmissen, T., De Loof, A., & Gu, S.H. (2007) Both prothoracicotropic hormone and an autocrine factor are involved in control of prothoracic gland ecdysteroidogenesis in *Locusta migratoria* and *Schistocerca gregaria*. *Peptides*, 28, 44-50.

Partner 3

- Maidan, M.M., L. De Rop, M. Relloso, R. Diez-Orejas, J.M. Thevelein and P. Van Dijck (2008). Combined inactivation of the *Candida albicans* *GPR1* and *TPS2* genes results in a-virulence in a mouse model for systemic infection. Submitted for publication.
- Nazarko V.Y., J.M. Thevelein and A.A. Sibirny (2007). G-protein-coupled receptor Gpr1 and G-protein Gpa2 of cAMP-dependent signaling pathway are involved in glucose-induced pexophagy in the yeast *Saccharomyces cerevisiae*. *Cell Biology International*. In press.
- Peeters T., M. Versele and J.M. Thevelein (2007). Directly from G α to Protein Kinase A: the kelch repeat protein bypass of adenylyl cyclase. *Trends in Biochemical Sciences* 32, 547-554
- Peeters T., W. Louwet, R. Geladé, D. Nauwelaers, J.M. Thevelein and M. Versele (2006). Kelch-repeat proteins interacting with the G α protein Gpa2 bypass adenylyl cyclase for direct regulation of protein kinase A in yeast. *Proceedings of the National Academy of Sciences (USA)* 103, 13034-13039
- Stasyk, O.G., M.M. Maidan, O.V. Stayk, P. Van Dijck, J.M. Thevelein and A.A. Sibirny (2008). Identification of hexose transporter-like sensor *HXS1* and functional hexose transporter *HXT1* in the methylotrophic yeast *Hansenula polymorpha*. Submitted for publication.
- Van de Velde S. and J.M. Thevelein (2008). cAMP-PKA and Snf1 signaling mechanisms underlie the superior potency of sucrose for induction of filamentation in yeast. *Eukaryotic Cell*. In Press.
- Van Dijck, P. 2008. Nutrient sensing G protein coupled receptors: interesting targets for antifungals? Submitted for publication.
- Van Zeebroeck G., B. Monge Bonini, M. Versele and J.M. Thevelein (2008). Transport and signaling through the amino acid binding site of the yeast Gap1 amino acid transceptor. Submitted for publication.

Partner 4

- Abu El-Asrar, A., Struyf, S., Opendakker, G., Geboes, K. and Van Damme, J. The role of chemokines and their receptors in uveitis. *Int. Ophthalmol.* 27: 321-327.
- Fan, X., Patera, A.C., Pong-Kennedy, A., Deno, G., Gonsiorek, W., Manfra, D.J., Vassileva, G., Zeng, M., Jackson, C., Sullivan, L., Shariff-Rodriguez, W., Opendakker, G., Van Damme, J., Hedrick, J.A., Lundell, D., Lira, S.A. and Hipkin, R.W. Murine CXCR1 is a functional receptor for GCP-2/CXCL6 and interleukin-8/CXCL8. *J. Biol. Chem.* 282: 11658-11666, 2007.
- Kelchtermans, H., Struyf, S., De Klerck, B., Mitera, T., Alen, M., Geboes, L., Van Balen, M., Dillen, C., Put, W., Gysemans, C., Billiau, A., Van Damme, J. and Matthys, P. Protective role of IFN- γ in collagen-induced arthritis conferred by inhibition of mycobacteria-induced granulocyte chemotactic protein-2 production. *Leukoc. Biol.*, 81: 1044-1053, 2007.

- Proost, P., Mortier, A., Loos, T., Vandercappellen, J., Gouwy, M., Ronsse, I., Schutyser, E., Put, W., Parmentier, M., Struyf, S. and Van Damme, J.roteolytic processing of I-TAC/CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. *Blood*, 110: 37-44, 2007.
- Schutyser, E., Su, Y., Yu, Y., Gouwy, M., Zaja-Milatovic, S., Van Damme, J. and Richmond, A.yoxia enhances CXCR4 expression in human microvascular endothelial cells and human melanoma cells.ur. *Cytokine Netw.* 18: 59-70, 2007.
- Struyf, S., Burdick, M.D., Peeters, E., Van den Broeck, K., Dillen, C., Proost, P., Van Damme, J. and Strieter, R.M.latelet factor-4 variant chemokine CXCL4L1 inhibits melanoma and lung carcinoma growth and metastasis by preventing angiogenesis. *Cancer Res.* 67: 5940-5948, 2007.
- Vandercappellen, J., Noppen, S., Verbeke, H., Put, W., Conings, R., Gouwy, M., Schutyser, M., Proost, P., Sciot, R., Geboes, K., Opdenakker, G., Van Damme, J. and Struyf, S. Stimulation of angiostatic platelet factor-4 variant (CXCL4L1/PF-4var) versus inhibition of angiogenic granulocyte chemotactic protein-2 (CXCL6/GCP-2) in normal and tumoral mesenchymal cells. *J. Leukoc. Biol.* 82: 1519-1530, 2007.
- Wetzel, K., Struyf, S., Van Damme, J., Kayser, T., Vecchi, A., Sozzani, S., Rommelaere, J., Cornelis, J.J. and Dinsart, C. MCP-3 (CCL7) delivered by parvovirus MVMP reduces tumorigenicity of mouse melanoma cells through activation of T lymphocytes and NK cells. *Int. J. Cancer*, 120: 1364-1371, 2007.

Partner 5

- Faísca P, Desmecht D (2007). Sendai virus, the mouse parainfluenza type 1: a longstanding pathogen that remains up-to-date. *Res Vet Sci.* 82:115-125.

Partner EU1

- Bakker R.A., A. Jongejan, K. Sansuk, U. Hacksell, H. Timmerman, M.R. Brann, D.M. Weiner, L. Pardo, R. Leurs (2008). Constitutively active mutants of the histamine H₁ receptor suggest a conserved hydrophobic asparagine-cage that constrains the activation of class A GPCRs. *Molecular Pharmacology*, 73, 94-103.
- Caltabiano G., M. Campillo, A. De Leener, G. Smits, G. Vassart, S. Costagliola, L. Pardo (2008). The specificity of binding of glycoprotein hormones to their receptors. *Cellular and Molecular Life Sciences*, in press.
- De Leener A., G. Caltabiano, S. Erkan, M. Idil, G. Vassart, L. Pardo, S. Costagliola (2008). Identification of the first germline mutation in the extracellular domain of the follitropin receptor responsible of spontaneous ovarian hyperstimulation syndrome. *Human Mutation*, 29, 91-98.
- Deupi X., N. Dölker, M.L. López-Rodríguez, M. Campillo, J.A. Ballesteros, L. Pardo (2007). Structural Models of Class A GPCRs as a Tool for Drug Design: Insights on Transmembrane Bundle Plasticity. *Current Topics in Medicinal Chemistry*, 7, 999-1006.
- Dölker N., X. Deupi, L. Pardo, M. Campillo (2007). Charge-charge and cation- π interactions in ligand binding to G protein-coupled receptors. *Theoretical Chemistry Accounts*, 118, 579-588.
- Pardo, X. Deupi, N. Dolker, M.L. López-Rodríguez, M. Campillo (2007). The role of internal water molecules in the structure and function of the rhodopsin family of G protein-coupled receptors. *ChemBioChem*, 8, 19-24.
- Smit M.J. , H.F. Vischer, R.A. Bakker, A. Jongejan, H. Timmerman, L. Pardo, R. Leurs (2007). Pharmacogenomic and structural analysis of constitutive G-protein coupled receptor activity. *Annual Review of Pharmacology and Toxicology*, 47, 53-87.
- Springael J.Y., C. De Poorter, X. Deupi, J. Van Durme, L. Pardo, M. Parmentier (2007). The activation mechanism of the chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cellular Signalling*, 19, 1446-1456.

2. COPUBLICATIONS

P1-P2

Van Loy T, Van Hiel MB, Vandersmissen HP, Poels J, Mendive F, Vassart G, Vanden Broeck J. Evolutionary conservation of bursicon in the animal kingdom *Gen Comp Endocrinol*. 2007 Aug-Sep;153(1-3):59-63. Epub 2006 Dec 27.

Van Loy T, Vandersmissen HP, Van Hiel MB, Poels J, Verlinden H, Badisco L, Vassart G, Vanden Broeck J. Comparative genomics of leucine-rich repeats containing G protein-coupled receptors and their ligands. *Gen Comp Endocrinol*. 2008 Jan 1;155(1):14-21. Epub 2007 Jul 4.

P1-P4

Proost, P., Mortier, A., Loos, T., Vandercappellen, J., Gouwy, M., Ronsse, I., Schutyser, E., Put, W., Parmentier, M., Struyf, S. and Van Damme, J. Proteolytic processing of I-TAC/CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. *Blood*, 110: 37-44, 2007.

P1-EU1

Caltabiano G., M. Campillo, A. De Leener, G. Smits, G. Vassart, S. Costagliola, L. Pardo (2008). The specificity of binding of glycoprotein hormones to their receptors. *Cellular and Molecular Life Sciences*, in press.

De Leener A, Caltabiano G, Erkan S, Idil M, Vassart G, Pardo L, Costagliola S. Identification of the first germline mutation in the extracellular domain of the follitropin receptor responsible for spontaneous ovarian hyperstimulation syndrome. *Hum Mutat*. 2008 Jan;29(1):91-8.

Springael JY, de Poorter C, Deupi X, Van Durme J, Pardo L, Parmentier M (2007). The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cell Signal* 19:1446-1456.

Annex 1: Program of the first annual meeting of the consortium**PAI/IUAP 6/14 : 2007 meeting****February 15, 2008**

Venue: Campus Erasme (building F, room F2.103, see map)

8h45-9h00: Welcome-coffee**9h05: Introduction: *Marc Parmentier*****9h15-10h15: KUL, group “Van Damme”**

- *Anneleen Mortier*
NH2-terminal processing of CXCL11/I-TAC by D13/Aminopeptidase N
- *Jo Vandercappellen*
The role of CXCL4/PF-4 and CXCL4L1/PF-4var in tumor biology

10h15-11h15: KUL, group “Thevelein”

- *Griet Van Zeebroeck*
Functioning of the yeast Gap1 amino acid transceptor (transporter/receptor)
- *Karin Voordeckers*
Novel mechanisms of PKA regulation in yeast

11h15-11h45: Coffee break**11h45-12h45: UA Barcelona, group “Pardo”**

- *Gianluigi Caltabiano*
The specificity of binding of glycoprotein hormones to their receptors
- *Xavier Deupi*
Structures of the β_2 adrenergic receptor vs. rhodopsin: how different is "different"?

12h45-14h15: Lunch and poster session**14h15-15h15: KUL, group “Vanden Broeck”**

- *Jeroen Poels*
Analysis of GPCRs using *Drosophila melanogaster* as a model system
- *Tom Van Loy*
Genomics, evolution and function of LGRs

15h15-15h45: ULG, group “Desmecht”

- *Daniel Desmecht*
Phenotypic characterization of susceptibility/resistance of inbred mice to viral pneumonia

15H45-16h15: Coffee break**16h15-17h15: ULB, IRIBHM**

- *Sabine Costagliola*
Structure and evolution of primate glycoprotein hormone receptors
- *Jean-Yves Springael*
Functional consequences of chemokine receptor heterodimerization

17h15-18h00: Management meeting

PAI/IUAP 6/14 : 2007 meeting
February 15, 2008
Posters

ULB

M Isabelle Garcia. Precocious Paneth cell differentiation in the small intestine of the mice knock-out for the orphan LGR5/GPR49 receptor.

Nathalie Bles. Gene expression profiling defines ATP as a key regulator of human dendritic cells functions.

Denis Sohy. Allosteric trans-inhibition in chemokine receptor oligomers

Tiffany Loy. Identification of residues within the transmembrane domain of glycoprotein hormone receptors implicated in dimerization and/or activation processes.

Anne De Leener. Identification of the first germline mutation in the extracellular domain of the FSHR responsible for spontaneous ovarian hyperstimulation syndrome.

Thalie Devosse. F2L as a new endogenous chemotactic ligand specific for FPRL2

Aude Guillabert. Role of neutrophil proteinase 3 and mast cell chymase in chemerin proteolytic regulation

KUL: Jo Van Damme

Mieke Gouwy. Amplification of the inflammatory response by chemokine synergy

Paul Proost: Post-translational modification of chemokines by proteolytic cleavage

Tamara Loos. TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in autoimmune arthritis

Sofie Struyf. Inhibition of melanoma and lung carcinoma growth and metastasis by the angiostatic chemokine CXCL4L1/platelet factor-4 variant

KUL: Johan Thevelein

Dries Castermans. Interdependency of the G-proteins Ras and Gpa2 for adenylate cyclase regulation in yeast.

Yulia Popova. Functioning of Pho84 as a phosphate transceptor in yeast.

Johan Kriel. Suppression of cAMP but not PKA requirement by an overactive allele of the Gap1 transceptor

ULG: Daniel Desmecht

Stéphanie Glineur. Immunodepression does not affect genetic resistance of SJL mice to respiratory syncytial virus.

Martin Dermine. Respiratory syncytial virus replication in type 2 pneumocytes from genetically susceptible and resistant inbred mice.

Mallory Draye. Mapping QTLs underlying susceptibility of inbred mice to infection with Paramyxoviruses.