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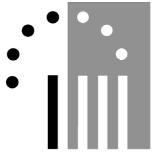

Interuniversity Attraction Poles (IAP)
Phase VI

2007 – 2011

IAP 6-14

G PROTEIN-COUPLED RECEPTORS, FROM STRUCTURE TO DISEASES

Acronym : GPCRs

Coordinator: Marc Parmentier (ULB)

Report 2007-2009

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1. INTRODUCTION

1.1. General information about the network

Title: **G protein-coupled receptors, from structure to diseases**

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

Budget 2007-2011:

	Personnel	**operating costs**	**Equipment**	**Overheads**	**Subcontracting**	**Total**
P1. M. PARMENTIER	840,000	493,334	0	66,666	0	1,400,000
P2. J. VANDEN BROECK	323,571	105,000	0	21,429	0	450,000
P3. J. THEVELEIN	280,000	90,000	61,500	18,500	0	450,000
P4. J. VAN DAMME	270,000	158,571	0	21,429	0	450,000
P5. D. DESMECHT	291,000	89,952	0	19,048	0	400,000
EU1. L. PARDO (European partner)		97,250	**-**	**-**	**-**	97,250
TOTAL						**3,247,250**

1.2. History of the IAP network

The coordinating group and several of the partners have been involved in previous phases of the IAP programs, as follows:

Phase II, IAP 2/15, 1990-1996. Molecular genetics of regulation networks. Involved partner **P1** (Gilbert Vassart).

Phase IV, IAP 4/30, 1997-2001. Molecular genetics and pathology of signal transduction. Involved partners **P1** (Gilbert Vassart, as coordinator), **P2** (Arnold De Loof), **P3** (Johan Thevelein) and **P4** (Jozef Van Damme).

Phase V, IAP 5/30, 2002-2006. G protein-coupled receptors: functional genomics, molecular pharmacology, structure-function studies. Involved partners **P1** (Gilbert Vassart, as coordinator), **P2** (Arnold De Loof), **P3** (Johan Thevelein), **P4** (Jozef Van Damme) and **EU1** (Leonardo Pardo).

The first networks (Phases II and IV) were dedicated to signal transduction in general. The Phase V program was focused on one of the aspects of the previous phases, namely G protein-coupled receptors. The present Phase VI network is the continuation of the Phase V program. The groups most closely involved in GPCR research have been kept, including the European partner L. Pardo, while a new group, D. Desmecht, has been added to bring new expertise in animal models of human diseases.

1.3. Summary of the objectives of the research project

G protein-coupled receptors (GPCR) represent the largest family among membrane receptors. They play a major role in a variety of physiological and pathophysiological processes, and constitute the targets for about 40% of the active compounds presently used as therapeutic agents. Working together in the frame of a previous IAP network, the partners of the present program have played a major role in the characterization of many G protein-coupled receptors in yeast, insects and mammalian species. Based on this previous experience, they have set up a partnership that is studying further both general and specific aspects of this important gene family, with the ultimate goal of improving human health. The partnership is using specific receptors and receptor subfamilies as models, in order to approach the field of GPCRs as a whole, as many of the studied aspects can apply to the entire family. The key models are being studied, on which the partners have built their present expertise, include the glycoprotein hormone and chemoattractant receptors in human and mouse, insect neuropeptide receptors and yeast sugar-sensing receptors.

The first objective of the program is to improve the understanding of structure-function relationships of G protein-coupled receptors. We aim at understanding what are the structural determinants and changes associated with ligand binding, receptor activation and G protein coupling, and how the knowledge of these structural elements can be translated into predictive tools for the selection and design of molecules acting on receptors with a therapeutic potential. Structural models will be built on the crystal structure of inactive bovine rhodopsin (and other rhodopsin structures that will soon become available) for the class A GPCRs of interest. Hypotheses will be raised regarding i) structural elements involved in the binding of natural ligands, antagonists or allosteric modulators, ii) the structural rearrangements associated with receptor activation, and iii) the coupling with G proteins, β-arrestins or other interacting proteins. These hypotheses will be tested by mutagenesis and a set of functional assays. Experimental results derived from different receptor classes will progressively improve the robustness of the structural models, and their predictive character. A topic of special interest will be GPCR dimerization, and how (hetero)dimerization influences the physiological role of receptors, and their use as therapeutic targets.

The second aim of the program will be to characterize the biological role of specific receptors in the physiology of human, mouse, insects and yeasts, and the involvement of human receptors in disease states. The partnership will focus on a set of receptors, many of which have been discovered in the network over the previous IAP phases. They include glycoprotein hormone receptors, chemokine receptors, receptors for neuromodulatory molecules in human and mouse, insect neuropeptidergic receptors and sugar sensing-receptors. They will be studied in terms of signaling pathways, involvement in immune, neural and endocrine functions, and contribution to the development of human diseases. In vitro and in vivo models, and genetically modified organisms will be used for this purpose. Orphan receptors will be studied as a source of future programs in the same area, starting by the identification of their natural ligands, one of the main expertises of the partnership. Through this work, we expect to designate receptors as targets for therapeutic agents fulfilling presently unmet medical needs.

1.4. Summary of the objectives of the partnership

The objective of the partnership is to bring together the complementary expertise of the various groups in order to gather a critical mass able to contribute significantly to the development of the GPCR field at the international level. The combined expertise of the network spans the whole spectrum of GPCR research, from bioinformatic genomic data mining, to in vivo phenotyping of transgenic animals with invalidated or added GPCR genes; from state of the art molecular modeling and molecular dynamics to experimental molecular pharmacology; from analytical and preparative biochemistry to functional identification of novel GPCR agonists; from cell and molecular biology of GPCRs to identification of their roles in a series of diseases (hereditary diseases, aging, cancer, inflammatory and infectious diseases, drug addiction, hypertension, diabetes...). A highly valuable characteristic of the network is also the diversity of the model systems under investigation (from yeast to man, via invertebrates).

2. RESEARCH RESULTS (01/01/2007 - 30/04/2010)

2.1. Workpackage 1. Structural organization of GPCRs

Contributing partners: P1, P2, EU1

2.1.1. Summary description of the objectives

We will construct tridimensional models of our receptors of interest, on the basis of the single crystal structure presently available (bovine rhodopsin) in order to raise hypotheses regarding ligand-receptor interactions, activation mechanisms and oligomeric organization. We will systematically test these models by mutagenesis studies, and the results of these experiments will be used to improve the models. This approach will be applied to most receptor classes studied, and the modeling aspects will be supported by our foreign partner EU1 (L. Pardo, Barcelona). For selected receptors, the models will also be used as basis for the virtual screening of chemical libraries, in order to develop small molecules with agonist or antagonist properties.

2.1.2. Summary description of scientific activities and results

2.1.2.1. Structural models of GPCRs (EU1, P1)

2.1.2.1.1. Glycoprotein-hormone receptors.

The crystal structures of the FSHR in complex with FSH and the TSHR in complex with a thyroid-stimulating autoantibody have 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 (Caltabiano et al. 2008).

Mining of the allowed the identification, by the group of A.J. Hsueh in 2002, Two novel human glycoprotein hormones, which form heterodimers (named thyrostimulin), have been identified in 2002, by mining the human genome. Thyrostimulin is able to activate the human TSH receptor, but not LH and FSH receptors. We have extended the proposed determinants for hormone binding and specificity to thyrostimulin.

2.1.2.1.2. Rhodopsin-like GPCRs.

Within this family, the crystal structures of bovine (Protein Data Bank accession numbers 1F88, 1HZX, 1GZM, 1L9H, and 1U19) and squid (2Z73) rhodopsin bound to the full inverse agonist *cis*-retinal, the ligand-free opsin (3CAP), the ligand-free opsin in its G-protein-interacting conformation (3DQB), the β_1-adrenergic receptor bound to the antagonist cyanopindolol (2VT4), the β_2-adrenergic receptor bound to the partial inverse agonist carazolol (2RH1 and 2R4R), and the A_{2A} adenosine receptor in complex with the antagonist ZM241385 (3EML) have been elucidated. Of special importance is the comparison of the structure of inactive rhodopsin with the recent crystal structure of the ligand-free opsin, which contains several distinctive features of the presumed active state. During the process of GPCR activation the intracellular part of TM6 tilts outwards by 6-7 Å, TM5 nears TM6, and R3.50 adopts an extended conformation pointing towards the protein core, to interact with the highly conserved Y5.58 and Y7.53 in TMs 5 and 7. In addition, the recent structure of opsin bound the carboxyl terminus of the Gα-subunit of transducin shows that the G protein interacts with residues in the inner side of the cytoplasmic TM5 and TM6. These structures have been used to raise hypotheses regarding i) structural elements involved in the binding of natural ligands, agonists, antagonists, inverse agonist, or allosteric modulators; ii) the structural rearrangements associated with receptor activation; iii) the coupling with G proteins; and iv) how (hetero)dimerization influences the physiological role of receptors, and their use as therapeutic targets.

2.1.2.2. Activation mechanisms

2.1.2.2.1. Glycoprotein-hormone receptors (P1, EU1)

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 (Akcurin et al. 2008). 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 (De Leener et al. 2008).

Moreover, a novel mutation in hTSHr, R1.36K, causing hyperthyroidism, has been observed. This finding together with engineered hTSHr mutants allowed us to propose that this residue in TM1 is involved in the transmission of the signal from the ECD-Hinge domain to the heptahelical region of the receptor.

2.1.2.2.2. 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 $D/E^{6.30}$ (Springael et al. 2007).

2.1.2.2.3. Small molecule (ant)agonists to get insight into mechanisms of receptor activation (P1, EU1)

Receptor mapping of nonpeptidic small molecule agonists and antagonists is of crucial importance for understanding the activation mechanisms of GPCRs. We studied functional properties of UCB35625 and J113863, two trans-isomer molecules originally identified as CCR1 and CCR3 bi-specific antagonists. We showed that these molecules bind to additional chemokine receptors and act either as agonists (CCR2, CCR5, CCR8), antagonists (CCR1, CCR3, CCR4) or allosteric modulators (CXCR6) depending on the receptors with which they interact. Interestingly, receptor activation or blockade is highly dependent of molecule symmetry, the borderline case being CX3CR1 receptor for which the isomers act in opposite way (UCB35625 acts as antagonist and J113863 as an agonist). Chemokine receptor 3D-models and mapping of these molecules are currently performed by EU1 to identify the molecular basis of molecules action.

2.1.2.3. Ligand-receptor interactions

2.1.2.3.1. 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 was adapted on the basis of the adrenergic and adenosine receptor structures that became available in the meantime. It was used to determine how chemerin or its C-terminal peptides bind and activate the receptor. Partner P1 has identified 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. Other experiments, using truncated chemerin ligands and blocking antibodies, have demonstrated the existence of a second high affinity binding site for the cystatin domain of chemerin. Only this latter interaction is however able to promote the activation of the receptor.

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. Three mutations affected 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. From the model, $Arg^{5.42}$ 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. Using additional mutants of this residue and peptide variants (i.e. C-terminally amidated peptide), $Arg^{5.42}$ was indeed demonstrated as a partner for the carboxyl group of the chemerin-9 nonapeptide. Mutants of the receptor for investigating the binding site of the cystatin domain have been constructed as well.

Chemerin has been produced at mg amounts in yeast, and crystals have been obtained in collaboration with Raphaël Dutoit (Ceria, Brussels). We will try to resolve the structure of chemerin, and will extend this to prochemerin and truncated forms (cystatin domain) in order to invetigate potentilal structural changes associated with proteolytic processing. The chemerin-9 peptide was 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.

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

Tachykinins are multifunctional neuropeptides that have been identified in vertebrates as well as invertebrates. Neuropeptides related to vertebrate tachykinins have been identified in *Drosophila melanogaster* and are referred to as drosotachykinins, or DTKs. Their C-terminal FXGXRa-motif constitutes a consensus active core region which is well conserved in tachykinin-related peptides from insects and other protostomian invertebrates. Two *Drosophila* G protein-coupled receptors, designated NKD (neurokinin receptor from *Drosophila*; CG6515) and DTKR (*Drosophila* tachykinin receptor; CG7887), display sequence similarities to mammalian tachykinin receptors. We first 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 (FX<u>A</u>XRa) display partial agonism on STKR-mediated Ca^{2+}-responses, but not on cyclic AMP-responses (Poels *et al.*, 2004). STKR therefore seems to differentiate between different types of peptide agonists. Gly- and Ala-containing tachykinins are both encoded in the *Drosophila* tachykinin precursor, thus raising the question whether DTKR can also distinguish between these two tachykinin types. When functionally expressed in insect or mammalian cell lines, DTKR was activated by all DTKs and their effect was dose-dependently inhibited by known tachykinin receptor antagonists, such as spantides (Poels *et al.*, 2007). Ala-containing analogs did not produce the remarkable activation behaviour previously observed with STKR, suggesting different mechanisms of discerning ligands and/or activating effector pathways for STKR and DTKR. Whereas DTKR was hereby shown to be activated by DTKs and was localized by immunocytochemistry in *Drosophila* central nervous system (CNS), agonist-dependent activation and distribution of NKD had not yet been investigated in depth. We have therefore challenged NKD-expressing mammalian and insect cells with a library of *Drosophila* neuropeptides and revealed DTK-6 as a specific receptor agonist that can induce a calcium response in these cells. In addition, we have produced antisera to sequences from NKD protein to analyze receptor distribution. We found that NKD is less abundantly distributed in the central nervous system than DTKR, while NKD was also found in the intestine. In fact, the two receptors are distributed in mutually exclusive patterns in the CNS. The combined distribution of the receptors in brain neuropils corresponds well with the distribution of DTKs. Most interestingly, NKD appears to be activated only by DTK-6, known to possess an Ala-substitution in an otherwise conserved C-terminal core motif (FX<u>A</u>XRa instead of the consensus sequence FXGXRa). Our findings suggest that NKD and DTKR provide substrates for two functionally and spatially separated peptide signalling systems (Poels *et al*., 2009; Van Loy *et al*., 2010).

2.1.2.4. Design of agonists and antagonists (EU1, P1, P2)

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).

Selective opioid receptor ligands can be used as pharmacological tools to distinguish the activity of different types of opioid receptors and may constitute potentially useful therapeutics. In a close collaboration of P2 with Prof. Janecka (Department of Biomolecular Chemistry, University of Lodz, Poland), amino acid substituted opioid receptor ligands have been developed and their corresponding functional properties (agonism, antagonism) tested. We therefore use a screening system that is based on the co-expression of the Ca^{2+}-sensitive bioluminescent protein aequorin and human δ- or μ-opioid receptors. Results indicate that this fast (medium-throughput) screening system can partially replace classical binding studies and the employment of animal tissues in the search for new opioid receptor (ant)agonists. Application of this assay has already led to the identification of several novel, potent and selective μ-opioid receptor agonists (Staniszewska *et al.*, 2008; Perlikowska *et al.*, 2010) and antagonists (Fichna *et al.*, 2006, 2007, 2008). The effectiveness and degradation rates of selected agonists and antagonists are further tested *in vivo* in cooperation with Prof. Do-Rego (University of Rouen, France).

The HIV coreceptor CCR5 is a validated target for both the prevention and therapy of HIV infection. PSC-RANTES, an N-terminally modified analogue of one of the natural chemokine ligands of CCR5 (RANTES/CCL5), is a potent inhibitor of HIV entry into target cells. In collaboration with Oliver Hartley, we set out to engineer the anti-HIV activity of PSC-RANTES into another natural CCR5 ligand (MIP-1β/CCL4), by grafting into it the key N-terminal pharmacophore region from PSC-RANTES. We were able to identify MIP-1β/CCL4 analogues that retain the receptor binding profile of MIP-1β/CCL4, but acquire the very high anti-HIV potency and characteristic inhibitory mechanism of PSC-RANTES. Unexpectedly, we discovered that in addition to N-terminal structures from PSC-RANTES, the side chain of Lys33 is also necessary for full anti-HIV potency (Gaertner et al. 2008).

In another collaborative work involving several groups, we identified by screening inhibitors of the interaction between CXCL12 and the receptor CXCR4. These synthetic compounds from the family of chalcones reduce binding of CXCL12 to CXCR4, inhibit calcium responses mediated by the receptor, and prevent CXCR4 internalization in response to CXCL12. We found that the chemical compounds display an original mechanism of action as they bind to the chemokine but not to CXCR4. The highest affinity molecule blocked chemotaxis of human peripheral blood lymphocytes ex vivo. It was also active in vivo in a mouse model of allergic eosinophilic airway inflammation in which we detected inhibition of the inflammatory infiltrate (Hachet-Haas et al. 2008).

2.1.3. Main achievements in relation to the initial objectives

Construction of models for different GPCRs, including TSHR, FSHR, CCR5, ChemR23 and glucose-sensing receptors.

Structure-function analysis of glycoprotein hormone receptors, CCR5, ChemR23 and insect tachykinin receptors.

Design of small molecule agonists and antagonists for CCR5, CXCR4 and opiate receptors.

2.1.4. Comments (in case of deviations from the initial project workprogramme)

None

2.2. Workpackage 2. Dimerization of GPCRs

Contributing partners: P1, P3

2.2.1. Summary description of the objectives

GPCRs were initially considered to act as monomers. More recent data have shown that most receptors form dimers if not higher order oligomers. The ability of GPCRs to homo- and heterodimerize will likely change many aspects of pharmacology in general, and the partnership has recently demonstrated allosteric interactions between receptor protomers. We will investigate further, for different classes of receptors, the functional consequences of dimerization, in terms of pharmacology, receptor activation, signaling properties and regulatory pathways, and will explore whether these observations apply to receptors expressed at physiological levels in native cell populations. Chemokine, glycoprotein hormone and yeast sugar-sensing receptors will be the first families studied in this frame.

2.2.2. Summary description of scientific activities and results

2.2.2.1. Allosteric interactions within glycoprotein hormone receptor heteromers (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 (unpublished).

2.2.2.2. Allosteric interactions within chemokine receptor heteromers (P1)

Chemokine receptors are presently used as targets for candidate drugs in the frame of inflammatory diseases and human immunodeficiency virus infection. We have shown so far the existence of about 20 different chemokine heteromers by using a variety of techniques (co-immunoprecipitation, complementation assays, BRET and Homogenous time-resolved FRET). We have also identified negative binding cooperativity between the subunits of some heteromers (CCR2/CCR5, CCR2/CXCR4, CCR5/CXCR4, CCR2,/CCR7, ChemR23/CXCR4, ChemR23/CCR7 and OPR3/CCR5). Importantly, we showed that specific antagonists of one receptor can inhibit the binding of chemokines to other receptors as a consequence of heteromerization, 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, Sohy et al, 2009).

CCR2/CCR5, CCR2/CXCR4 and CCR5/CXCR4 heterodimers. We identified negative binding cooperativity between the subunits of CCR2/CCR5, CCR2/CXCR4 and CCR5/CXCR4 heteromers (El-Asmar et al., 2004, Springael et al., 2006, Sohy et al., 2007, Sohy et al., 2009). CCR2, CCR5 and CXCR4 form thus homomers as well as heteromers with one another, raising the question of their natural organization at the surface of immune cells expressing these three receptors endogenously. Using Bi-LC BRET assays, we demonstrated that hetero-oligomeric complexes containing simultaneously the three receptors are formed (Sohy et al., 2009). Importantly, we showed that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heteromerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. Using the air pouch model in mice, we established that this trans-inhibition by antagonists has major consequences on the migration of cells in vivo. We showed that the CCR2 and CCR5 antagonist TAK-779 inhibits both lymphocytes and dendritic cells recruitment into the pouch in response to CXCL12/SDF-1α. This data support the new concept following which small-molecule antagonists can trans-modulate the function of receptors on which they do not bind directly, as the result of their heteromerization, with important implications on the activities of chemokine receptor antagonists in vivo. From a general point of view, allosteric regulation across GPCR oligomeric interfaces is expected to greatly influence the practice of modern 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.

Heterodimerization of CCR7 with CXCR4, CCR5 and CCR2. We also investigated the dimerization status of the chemokine receptor CCR7 and showed using BRET that CCR7 forms heterodimers with CCR2, CCR5 and CXCR4 constitutively. Similarly to what we reported for other chemokine heterodimers, we showed that the heterodimerization of CCR7 with CCR2 is associated with a “symmetrical” negative binding cooperativity, the ligand of each receptor being able to compete for the binding of radiolabelled tracer to the other. In contrast, CCR7 heterodimerization with CCR5 is linked to an “asymmetrical” negative binding cooperativity i.e. specific ligands of CCR7 being able to compete for radiolabelled tracer to CCR5 while CCR5-specific ligands being unable to do so for tracer binding to CCR7. Finally, we showed that CCR7 heterodimerization with CXCR4 does not involve binding cooperativity, the ligands of each receptor composing the dimer being unable to compete for binding of radiolabelled tracer to the other. Among all the chemokine heterodimers we tested, this is the first case of receptors for which heterodimerization is not associated with negative binding cooperativity. In contrast, we showed that the functional response of CXCR4 and CCR5 is strongly reduced upon co-expression of CCR7. This decrease of response is not linked to a reduced expression level of CCR5 or CXCR4 receptor as measured by FACS or saturation binding assay. The molecular mechanism underlying this phenomenon is not known for sure but might involve conformational change of CCR5 and CXCR4 receptors as the result of their interaction with CCR7. Interestingly, this negative effect of CCR7 was not detected in cells coexpressing CCR7 with CCR2 or ChemR23, suggesting that properties of receptors might vary greatly according to the partner with which CCR7 interacts.

We showed, on purified $CD4^+$ T cells and monocytes that negative binding cooperativity takes place between the binding pockets of CCR2, CCR5 and CXCR4, demonstrating the functional interaction of these three receptors in primary leukocytes. We showed that the cross-competition of CCL2/MCP-1 and CXCL12/SDF-1α binding by CCL4/MIP-1β is specifically abrogated in leukocytes derived from a CCR5Δ32/Δ32 donor, demonstrating that the expression of functional CCR5 at the cell surface is required for the negative cooperative effects of CCR5 ligands to occur. In contrast, the negative binding cooperativity between CCR2 and CXCR4 was still detected in these cells, demonstrating that the lack of CCR5 did not disrupt the oligomerization status of the two other receptors. These results support thus the view of a complex organization of chemokine receptor oligomers at the surface of primary leukocytes, depending essentially on their relative expression levels. Importantly, we showed also in primary leukocytes that specific antagonists of one receptor inhibit the binding of chemokines to the others as a consequence of their heterodimerization. This heterologous binding inhibition resulted in a significant functional trans-inhibition of cell chemotaxis ex vivo. Using the air pouch model in mice, we established that the trans-inhibition by antagonists has also major consequences on the migration of cells in vivo. We showed that the CCR2 and CCR5 antagonist TAK-779 inhibits both lymphocytes and dendritic cells recruitment into the pouch in response to CXCL12. These data demonstrate that antagonists of the therapeutically important

receptors like CCR5 and CXCR4 can regulate allosterically the functional properties of receptors on which they do not bind directly, with important implications on the effects of these agents in vivo. We demonstrated more recently the relevance of ChemR23/CXCR4 heterodimerization in primary leukocytes by showing that mouse chemerin competed for mCXCL12 binding on BMDC and that this cross-inhibitory effect is specifically lost in cells generated from ChemR23 KO mices. We are currently testing the relevance of other heterodimers on primary leukocytes that coexpressed endogenously receptors of interest.

2.2.2.3. Oligomerization of chemokine receptors with ChemR23 and opiate receptors (P1)

With the aim of further characterizing functional consequences of chemokine receptors dimerization, we also investigated the dimerization status of ChemR23. Like chemokine receptors, ChemR23 is expressed on leukocytes such as macrophages, dendritic cells as well as on a subset of NK cells. Using BRET, we showed that ChemR23 is able to form heterodimers with chemokine receptors CXCR4 and CCR7 constitutively and that this interaction results in a strong negative binding cooperativity. We also showed on mouse BMDC expressing endogenously ChemR23 and CXCR4, that chemerin competed for CXCL12/SDF-1α binding and that this cross-inhibitory effect is specifically lost in cells generated from mice invalidated for ChemR23, thus demonstrating the functional relevance of ChemR23/CXCR4 dimerization in primary leukocytes. Finally, we studied the binding properties of the CCR5/OPR3 heterodimer and showed that this interaction results also in a negative binding cooperativity. Altogether, these results support the concept that negative binding cooperativity can take place across receptors that bind chemoattractant structurally unrelated and even receptor of distinct physiological function.

2.2.2.4. 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 to similar levels as earlier reported for the Ste2 pheromone receptor in yeast, suggesting receptor oligomerization. The role of dimerization in sugar sensing was however not yet investigated.

2.2.3. Main achievements in relation to the initial objectives

We have demonstrated that heterodimerization of chemokine receptors can lead to a modification of their apparent pharmacology and signaling properties in primary leukocyte populations. Altogether, there are only few reports describing data consistent with heteromultimeric GPCR complexes 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).

2.2.4. Comments (in case of deviations from the initial project workprogramme)

None

2.3. Workpackage 3. signaling cascades activated by GPCRs

Contributing partners: P1, P2, P3, P4

2.3.1. Summary description of the objectives

Besides the classical pathways activated by GPCRs through heterotrimeric G proteins, a number of additional pathways, some of which are G protein-independent have been described. In addition, the range of signaling cascades activated by a given receptor can vary according to the agonist. Signaling cascades will be studied for yeast sugar-sensing, insect neuropeptide and mammalian chemoattractant receptors, focusing on new pathways and the protein complexes involved in signal transduction.

2.3.2. Summary description of scientific activities and results

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

Leukocyte migraton 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 NH_2-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).

2.3.2.2. Spectrum of GPCRs recognized by post-translationally-modified chemokines (P4)

Chemokines regulate leukocyte migration during physiological and pathological conditions. They exert their biological activity through interaction with 7-transmembrane spanning G protein-coupled receptors (GPCR) and are presented on glycosaminoglycans (GAG) linked to endothelial cell layers. Specific chemokines and chemokine receptor affect angiogenesis or are targets for viral mimicry, e.g. by human immunodeficiency virus (HIV). Several enzymes, in particular proteases, have been described to process chemokines at specific sites generating chemokine isoforms that were also identified from natural sources. For some chemokines, e.g. CXCL8 and CCL3L1, posttranslational modification results in enhanced biological activity. For CXCL7 and CCL14 truncation is even mandatory for receptor signaling and chemotactic properties. The activity of many other chemokines is down-regulated by processing and receptor antagonists are generated, e.g. for truncated CCL8 and CCL11. Moreover, some processed chemokines, such as CCL5(3-68) show enhanced affinity for one receptor (CCR5) and reduced interaction with other receptors (CCR1 and CCR3) resulting in differential changes in leukocyte response. These posttranslational mechanisms, in addition to gene duplication, transcriptional and translational regulation of chemokine ligand and receptor expression, GAG binding properties, expression of “silent” receptors and synergistic interaction between chemokines, modulate chemokine activity in a complex manner. The current understanding on the regulation of the chemokine network through posttranslational modification and its consequences for leukocyte migration, angiogenesis and protection against viral infection requires further attention (Mortier et al., 2008).

Interactions between chemokines and enzymes are vital in immunoregulation. Structural protein citrullination by peptidylarginine deiminase (PAD) has been associated with autoimmunity. We identified a novel naturally occurring posttranslational modification of chemokines, that is, the deimination of arginine at position 5 into citrulline of CXC chemokine ligand 10 (CXCL10) by rabbit PAD and human PAD2. Citrullination reduced (≥ 10-fold) the chemoattracting and signaling capacity of CXCL10 for CXCR3 transfectants; however, it did not affect CXCR3 binding. On T lymphocytes, citrullinated CXCL10 remained active but was again weaker than authentic CXCL10. PAD was also able to convert CXCL11, causing an impairment of CXCR3 signaling and T cell activation, though less pronounced than for CXCL10. Similarly, receptor binding properties of CXCL11 were not altered by citrullination. However, deimination decreased heparin binding properties of both CXCL10 and CXCL11 (Loos et al., 2008).

Natural posttranslational citrullination or deimination alters the biological activities of the neutrophil chemoattractant and angiogenic cytokine CXCL8. Citrullination of arginine in position 5 was discovered on 14% of natural leukocyte-derived CXCL8(1-77), generating CXCL8(1-77)Cit5. PAD is known to citrullinate structural proteins, and it may initiate autoimmune diseases. PAD efficiently and site-specifically citrullinated CXCL5, CXCL8, CCL17, CCL26, but not IL-1β. In comparison with CCL8(1-77), CXCL8(1-77)Cit5 had reduced affinity for glycosaminoglycans and induced less CXCR2-dependent calcium signaling and extracellular signal-regulated kinase ½ phosphorylation. In contrast to CXCL8(1-77), CXCL8(1-77)Cit5 was resistant to thrombin- or plasmin-dependent potentiation into CXCL8(6-77). Upon intraperitoneal injection, CXCL8(6-77) was a more potent inducer of neutrophil extravasation compared with CXCL8(1-77). Despite its retained chemotactic activity in vitro, CXCL8(1-77)Cit5 was unable to attract neutrophils to the peritoneum. Finally, in the rabbit cornea angiogenesis assay, the equally potent CXCL8(1-77) and CXCL8(1-77)Cit5 were less efficient angiogenic molecules than CXCL8(6-77). This study shows that PAD citrullinates the chemokine CXCL8, and thus may dampen neutrophil extravasation during acute or chronic inflammation. (Proost et al;, 2008).

During the first line defense of an infected host, circulating neutrophils invade the inflamed tissue, whereas mature neutrophils from the bone marrow pool migrate into the blood circulation and from there reinforce tissue infiltration. The ability to provoke leukocytosis was assessed by intravenous administration of citrullinated CXCL8 in rabbits. Citrullination of CXCL8 significantly increased this chemokine’s ability to recruit neutrophils into the blood circulation. In addition, the competitive binding properties of CXCL8 for the Duffy antigen/receptor for chemokines were impaired upon citrullination. Since the Duffy antigen/receptor for chemokines is an important scavenging receptor for CXCL8 in the blood stream, citrullination may delay CXCL8 clearance from the circulation. Furthermore, the shedding of CD62L (L-selectin) and the upregulation of CD11b (β_2-integrin) protein expression on CXCL18-induced neutrophils were improved by deimination of CXCL8, possibly contributing to the neutrophil egress from the bone marrow. Conversely, surface expression of CD15, the neutrophilic ligand of endothelial selectins, was equally well upregulated by intact and citrullinated CXCL8. These data show that citrullination of CXCL8 enhances leukocytosis, possibly through impaired chemokine clearance from the blood circulation and prolonged presentation to the bone marrow (Loos et al., 2009).

Biological functions of proteins are influenced by posttranslational modifications such as on/off switching by phosphorylation and modulation by glycosylation. Proteolytic processing regulates cytokine and chemokine activities. Overall, chemokines are the first immune modulators reported of being functionally modified by citrullination. These data provide new structure-function dimensions for chemokines in leukocyte mobilization, disclosing an anti-inflammatory role for PAD. Additionally, because citrullination has severe consequences for chemokine biology, this invites to reassess the involvement and impact of PAD and citrullinated peptides in inflammation, autoimmunity, and haematologic disorders.

2.3.2.3. Synergy between chemokines and enhancement of the inflammatory response (P4)

Tissue infiltration by leukocytes is an important phenomenon for 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 GPCR agonists, including complement factor C5a, bacterial peptides (e.g., fMLP), and 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. Thus, it is likely that more than one chemoattractant is present at the site of inflammation. These co-induced chemokines may cooperate to attract leukocytes to the site of infection, thereby enhancing the outcome of an inflammatory response. CC and CXC chemokines co-induced in fibroblasts and leukocytes by cytokines and microbial agents determine the number of phagocytes infiltrating into inflamed tissues. CXCL8 and CXCL12 significantly and dose-dependently increased the migration of monocytes, expressing the corresponding CXC chemokine receptors CXCR2 and CXCR4, toward suboptimal concentrations of the monocyte chemotactic proteins CCL2 or CCL7. These findings were confirmed using different chemotaxis assays and monocytic THP-1 cells. In contrast, the combination of two CC chemokines (CCL2 plus CCL7) or two CXC chemokines (CXCL8 plus 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 CXCR4/CCR2 co-transfectants. It is noteworthy that CCL2 mediated extracelluar signal-regulated kinase ½ phosporylation and calcium mobilization was significantly enhanced by CXCL8 in moncytes, indicating cooperative downstream signaling pathways during enhanced chemotaxis. Moreover, in contrast to intact CXCL12, truncated CXCL12(3-68), which has impaired receptor signaling capacity but can still desensitize CXCR4, was unable to synergize with CCL2 in monocytic cell migration. Furthermore, AMD3100 and RS102895, specific CXCR4 and CCR2 inhibitors, respectively, reduced the synergistic effect between CCL2 and CXCL12 significantly. These data indicate that for synergistic interaction between chemokines binding and signaling of the two chemokines via their proper receptors is necessary (Gouwy et al., 2008).

During inflammatory reactions, endogenously produced cytokines and chemokines act in a network and interact with hormones and neutrotransmitters to regulate host immune responses. These signalling circuitries are even more interfaced during infections, when microbial agonists activate TLR, RLR, and NLR receptors. On the basis of the discovery of synergy between chemokines for neutrophil attraction, we extend this phenomenon between the chemokine MCP-1/CCL2 and the GPCR ligand fMLP or the TLR4 agonist LPS on monocytes. In fact, the bacterial tripeptide fMLP, but not the cytokines IL-1β or IFN-γ, significantly and dose-dependently synergized with CCL2 in monocyte chemotaxis. Furthermore, LPS rapidly induced the expression of IL-8/CXCL8 but not of the CCL2 receptor CCR2 in monocytic cells. In turn, the induced CXCL8 synergized with CCL2 for mononuclear cell chemotaxis, and the chemotactic effect was mediated by CXCR1/CXCR2, because CXCL8 receptor antagonists or antibodies were capable of blocking the synergy, while keeping the responsiveness to CCL2 intact. These data recapitulate in vitro the complexity of innate immune regulation, provide a novel mechanism of enhancing monocyte chemotaxis during bacterial infections with gram-negative bacteria and demonstrate the importance of local contexts in inflammatory and infectious insults (Gouwy et al., 2009).

2.3.2.4. Downstream signalling effects of insect GPCRs (P2)

Microarray experiments are being performed to identify proteins and pathways that act downstream of GPCRs and thus regulate receptor-mediated physiology, development and behaviour in insects. With this technology, differential gene expression patterns are analyzed on a genome-wide scale resulting from defective GPCR signalling. As a pilot experiment, we have conducted microarray hybridization experiments utilizing mutant *Drosophila* strains, such as *rickets* (*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 *i.e.* interrelated processes that previously have been shown to be initiated by the neurohormone bursicon (the ligand for DLGR2). At present, we have identified several transcripts coding for structural proteins, enzymes *etc.* that likely participate in the process of cuticle maturation. In the future, we will also apply a similar approach to decipher signalling pathways initiated by other insect GPCR/ligand couples. Much effort has been put in experimental design and data analysis. The technology has now been fully introduced in the lab of P2 and has already been utilised in different types of experiments (see also 4.6 and 6.4).

2.3.2.5. 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 activates adenylate cyclase through the Gα protein Gpa2. The latter protein is atypical since it functions without a cognate Gβ protein. Two related kelch-repeat proteins, Krh1 and Krh2, are associated with Gpa2 and were suggested to act as Gβ mimics for Gpa2, based on their predicted 7-bladed β-propeller structure. However, we have found that the Krh proteins function as Gpa2 effectors. They directly link Gpa2 to

PKA, thus forming an adenylyl cyclase bypass pathway that enables inputs other than cellular cAMP concentration to affect PKA activity. Because mammalian PKA expressed in yeast is also subject to control by the same bypass pathway, a functionally similar mechanism might exist in mammalian cells (Peeters et al. 2006, 2007).

In yeast the cAMP-PKA pathway is implicated in the regulation of several important properties (storage of carbohydrates trehalose and glycogen, stress resistance, filamentous differentiation). As such it is indispensible for the proper response of the yeast cellular machinery to the nutrient conditions. Yeast cells contain a glucose/sucrose sensing GPCR Gpr1, that detects its extracellular ligand and activates adenylate cyclase (Cyr1) through the Gα protein Gpa2. The increase in cAMP acts as an intracellular second messenger and causes a rapid increase in PKA activity.

In addition to the presence of the GPCR system at the plasma membrane (non-essential part), the cAMP-PKA pathway requires an intracellular system depending on sugar phosphorylation, which may act through the Ras proteins. In this Ras-PKA part of the pathway (essential part), adenylate cyclase is activated by Ras1 and Ras2. The activity of these GTPases is regulated by Cdc25 (Ras-GEF, activating Ras) and Ira1 and 2 (RAS-GAP, inhibiting Ras). We showed that Ras proteins are able to activate adenylate cyclase even in the absence of Gpa2. In contrast, Gpa2 was unable to activate adenylate cyclase in the absence of Ras. In adition, we have shown that glucose phosphorylation is a potent activator of Ras and we have identified several glycolytic intermediates able to trigger dramatic activation of Ras in permeabilized yeast cells.

The intracellular concentration of cAMP has to be stricly controlled. PKA activates the phosphodiesterases Pde1 and Pde2, which break down cAMP to AMP. On the other hand, PKA negatively regulates cAMP synthesis. Despite elaborate efforts of other groups, the mechanism of this feedback inhibition remains unclear. We have obtained evidence that this feedback inhibition targets the regulatory domain of adenylate cyclase for the downregulation of cAMP synthesis. In a strain with an N-terminally truncated version of adenylate cyclase (Cyr1), both *PDE* genes had to be deleted in order to observe cAMP hyperaccumulation. This suggests that hyperaccumulation in strains with attenuated PKA is due to two mechanism: absence of downregulation of Cyr1 activity (feedback inhibition) and absence of activation of the Pde enzymes. Recently, we have also shown that cAMP is actively exported from the yeast cells apparently as part of the PKA feedback-inhibition mechanism. We have identified the cAMP exporter, which is an ABC cassette transporter.

We have also shown that the protein phosphatases PP2A and PP1 are both under direct control of glucose sensing. We revealed that glucose addition to glucose-deprived yeast cells triggers rapid post-translational activation of both PP2A and PP1. Glucose sensing for activation of PP2A was sustained independently by the two glucose-sensing mechanisms that mediate activation of cAMP synthesis: the glucose-sensing GPCR, Gpr1, and the glucose-phosphorylation dependent mechanism. Of the catalytic subunits of PP2A, only Pph21 and Pph22 were involved. Deletion of *PPH21/22* increased PKA activity, and it reduced glucose-induced cAMP signaling apparently due to enhanced PKA feedback inhibition. For regulatory components of PP2A, only the Rts1 regulatory subunit, the carboxymethyltransferase Ppm1 and a second putative regulatory subunit Pig2, were required. In contrast, none of the established single regulatory subunits of PP1 was required for its activation. Our results reveal that the well-known process of glucose activation of the PKA pathway is closely connected with glucose control of PP2A activity. Glucose activation of PP1 activity may play an important role in the glucose regulated processes known to be controlled by this enzyme (e.g. main glucose repression pathway). (Castermans et al. in preparation).

In general, nutrients are able to trigger similar rapid signaling pathways as growth factors and hormones. Although the transceptors of this 'GPCR-controlled nutrient-sensing network' are characterized (see chapter 4.7), the players and mechanisms involved remain largely unknown. In yeast, rapid nitrogen signaling to specific targets of the PKA pathway requires Sch9, the yeast PKB/S6K orthologue. We showed that Pkh1 binds to Sch9 *in vitro*, and that nitrogen signalling through Sch9 depends on Pkh1-3, the yeast orthologues of mammalian PDK1. Moreover, it appeared that it acts through phosphorylation of a Thr-residue in the conserved sequence motif (PDK1 site) in Sch9, and that nitrogen deprivation abolishes this phosphorylation. Moreover, Pkh inactivation as well as mutagenesis of the PDK1 site in Sch9 affect nitrogen signaling and results in a markedly smaller cell size, phenotypes characteristic of cells lacking Sch9 activity (Voordeckers et al. submitted). In mammalian cells, the protein kinase PDK1 controls a myriad of processes through substrate phosphorylation.

2.3.3. Main achievements in relation to the initial objectives

We have identified post-translational modifications of chemokines that affect deeply their biological activities onto their respective receptors. This involves the proteolytic processing of chemokines by aminopeptidase N, and citrillunation of chemokines by enzymatic modification of arginines.

Synergistic effects between chemokines acting on different receptors were demonstrated.

Setting of an experimental design allowing to determine the signalling events downstream of insect receptors and first application to cuticle maturation as a result of bursicon activity.

We have identified in yeast an adenylate cyclase bypass pathway, directly connecting the Gα protein Gpa2 with PKA. We have now obtained convincing evidence that intermediates of glycolysis are potent activators of the Ras proteins which act in concert with the Gpr1-Gpa2 GPCR system to activate adenylate cyclase. We are close to the first complete elucidation of the complex feedback-inhibition mechanism of PKA on the cAMP level in the cell. We have discovered that the glucose-sensing GPCR Gpr1 plays a role in glucose activation of the major cellular protein phosphatases PPA2 and PP1. The yeast PDK1 homologues, Pkh1-3, were shown to play an important role in nitrogen signaling.

2.3.4. Comments (in case of deviations from the initial project workprogramme)

None

2.4. Workpackage 4. Functional characterization of receptors in physiological processes

Contributing partners: P1, P2, P3, P4, P5

2.4.1. Summary description of the objectives

A number of specific receptors, among which several receptors identified by the partners over the previous IAP programs, will be studied in details in order to determine their role in physiological processes. This will involve in vitro analysis of receptor pharmacology and ligand processing, distribution studies, as well as in vivo studies and the design of knock-out and transgenic models. We will study, among others, chemoattractant receptors (ChemR23, FPRL2) and a set of mammalian neuromodulatory receptors, neuropeptide receptors in insects, and the glucose/sucrose sensing GPCR system in yeast and Candida albicans.

2.4.2. Summary description of scientific activities and results

2.4.2.1. 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.

2.4.2.1.1. Distribution studies.

In collaboration with the group of Silvano Sozzani (Brescia), we have shown that blood $CD56^{low}CD16^{+}$ 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, $DC\text{-}SIGN^{+}$ 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 (Luangsay et al. 2009).

2.4.2.1.2. (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. 2008).

2.4.2.1.3. 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 EC_{50} of about 5 nM (Luangsay et al. 2009), 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.

2.4.2.1.4. Knock out model.

Expression of ChemR23 is restricted to macrophages and dendritics 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 animal 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 Ca^{2+} 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 Ca^{2+} 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 (Luangsay et al. 2009). To determine in vivo the physiological role of chemerin in the innate and acquired immune response, several inflammatory models were used as described in WP5.

In vitro studies have demonstrated that chemerin is an adipokine regulating adipocyte differentiation and stimulating insulin-dependent glucose uptake in white adipose tissue (WAT). ChemR23 KO mice display an increased body weight (31.2 ±1.3 g vs 28.2±1.45 g for WT at 7 months) without significant difference in food intake. This is associated with accumulation of inguinal WAT (10.54 ± 0.94 mg/g vs 5.7 ±1.6 mg/g for WT at 7 months). No difference in levels of glucose, cholesterol and triglycerides were shown in basal fasted state. KO mice displayed a larger size of adipocytes. Expression of the lipogenesis marker (GPDH) was increased in KO adipose tissue, suggesting a role of the chemerin/ChemR23 system in lipogenesis. The function in glucose metabolism and insulin signaling in vivo is currently investigated by insulin and glucose tolerance tests and we have initiated a high fat diet-induced obesity model and diet-induced atherosclerosis model.

2.4.2.2 Human and mouse FPRL2 (P1)

2.4.2.2.1. 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 (EC_{50} 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 EC_{50} 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).

With the aim of comparing mouse Fpr2 expression with human FPRL2 we have further tested the presence of Fpr2 transcripts in various mouse leukocyte populations demonstrating Fpr2 expression on mouse bone-marrow-derived DCs and peritoneal macrophages. Activation of DCs with LPS induces up-regulation of Fpr2 transcript levels. We also tested the capacity of mouse F2L to induce recruitment of DCs and macrophages and demonstrated a chemotaxis with a maximum of 10 µM. We have investigated the chemoattractant activity of mouse F2L in vivo in an air pouch assay. A significantly larger number of cells were collected in mouse F2L-injected pouches than in controls. In these cells, neutrophils, DCs and macropahges were detected and over-represented in the mouse F2L-injected pouches. Finally, to test the physiological relevance of F2L activity, we have identified the production of this peptide in mouse spleen by reverse-phase HPLC, and this activity was increased in inflammatory conditions (Devosse et al 2009).

2.4.2.2.2. 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. We investigated the biological activity of F2L on native pDCs. In microchemotaxis Boyden's chambers, F2L promoted chemotaxis of pDCs with a peak at 10 nM. The migration index was comparable with that obtained for SDF-1 (10 nM) used as a positive controls. These data demonstrate the physiological role of F2L on pDC recruitment, through its action on FPRL2. F2L also promoted chemotaxis of monocyte-derived macrophages, peaking for concentrations of 1 to 10 nM (Devosse et al. 2009). Using tissue microarrays (TMA), staining with antibodies against FPRL2 and CD68, we have demonstrated the expression of FPRL2 in 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 Kuppfer cells, or macrophages from lymph nodes or spleen. 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 have therefore investigated the modulation of FPRL2 in various types of cancer. Strong down-regulation of FPRL2 expression was seen in tumor associated macrophages from colon adenocarcinomas. Finally, expression of FPRL2 by eosinophils was demonstrated by RT-PCR and flow cytometry. We showed a chemotactic activity of F2L on eosinophils, comparable to that promoted by eotaxin. However, F2L did not promote degranulation of eosinophils. The F2L-FPRL2 system might therefore be involved in the regulation of lung inflammatory and allergic diseases, such as asthma.

2.4.2.2.3. Characterization of HBP hydrolysis and F2L generation.

In order to study the processing of HBP into the bioactive peptide F2L, we have recently expressed a C-terminally poly(His)-tagged form of human HBP in a yeast system (Sacharomyces cerevisiae). The protein was purified by affinity (nickel column) and size exclusion chromatography. Human full length HBP displays very low agonist activity on FPRL2 and does not antagonize the activity of the F2L peptide. In order to determine which proteases or protease families are able to cleave HBP and release active F2L, human HBP was incubated with a panel of candidate proteases in vitro, and the products were tested on CHO-K1 cells expressing FPRL2. Cathepsin D, an aspartyl endopeptidase active at low pH was able to cleave HBP and generate an efficient agonist of FPRL2. Moreover, incubation of HBP in conditioned medium from activated human macrophages also resulted in the production of an agonist of FPRL2. This process was blocked by pepstatin A, a specific inhibitor of cathepsin D. Cathepsin D is involved in the activation of prohormones and the inactivation of bioactive peptides, and it contributes to inflammatory and degenerative diseases, such as atherosclerosis and Alzheimer's disease. It released in the extracellular space together with other lysosomal hydrolases, by apoptotic and necrotic cells. Such conditions may be favourable to the local production of F2L from HBP released by damaged cells. We will continue to explore the role of cathepsin D in the generation of F2L, by analysing the nature of the bioactive products, by Western blotting and mass spectrometry (Devosse et al. unpublished). Recently, we have identified another protease, neutrophil cathepsin G, as being able to process HBP into peptides active on FPRL2. This will be studied further in the future.

2.4.2.3. Control of CXCL8, CXCL10 and CCL2 expression (P4 and P5)

Chemokine expression is at least in part controlled by nuclear factor kappa B (NF-κB) activation, which in turn depends on the activation of several transduction pathways. Partners P4 and P5 have joined their respective activities in a project dealing with the possible interference of antiviral Mx proteins with the chemo/cytokinic response of virus infected cells/tissues. Specifically, partner P5 has identified a set of mammalian Mx proteins with inserted motifs involved in fine tuning of NF-κB activation (Cornet et al., manuscript submitted for publication). Stable cell lines and transgenic mouse lines expressing control and principal Mx proteins were produced and their chemokine response to TNFα, IL-1β, poly-I/C and influenza viruses is being examined.

2.4.2.4. Purinergic receptors in inflammation (P1)

2.4.2.4.1. Tolerogenic and tumorigenic properties of ATP-treated dendritic cells

ATP has an important pro-inflammatory action as danger signal but can also be associated with immune tolerance. Our lab demonstrated previously that ATP-treated monocyte-derived DCs were expressing proteins with immunosuppressive properties such as indoleamine 2',3'-dioxygenase and thrombospondin-1 (Marteau et al. 2005). Extracellular ATP and prostaglandin E2 (PGE2) are two cAMP-elevating agents inducing the semimaturation of human monocyte-derived dendritic cells (DCs). We have

compared the gene expression profiles induced by ATPγS and PGE2 in human DCs using microarray technology. At 6 h of stimulation, ATPγS initiated an impressive expression profile compared with that of PGE2 (1125 genes compared with 133 genes, respectively). Many target genes involved in inflammation (chemokines, cytokines, receptors,…) have been identified and validated by quantitative RT-PCR experiments. Our data give an extensive overview of the genes regulated by ATPγS and PGE2 in DCs and an important insight into the therapeutic potential of ATP- and PGE2-treated human DCs (Bles et al. 2007).

We also demonstrated that DCs are a source of proangiogenic factors, including VEGF and amphiregulin, an response to ATP stimulation. Human monocyte-derived DCs and mouse bone marrow-derived DCs treated with LPS and ATPγS released high amounts of amphiregulin. Co-injection of DCs stimulated by LPS and ATPγS (or their supernatants) with LLC cells increased the tumor growth in mice as compared to DCs treated with LPS only. Anti-amphiregulin blocking antibodies inhibited theeffect on tumor growth. We demonstrated therefore that ATP can confer tolerogenic and tumorigenic proterties to DCs (Bles et al, Blood, in revision). Antagonists of ATP receptors expressed by DCs may therefore have therapeutic properties.

2.4.2.4.2. Role of extracellular nucleotides in lung inflammation

Extracellular nucleotides regulate ion transport and mucociliary clearance in human airway epithelial cells (HAECs) via the activation of P2 receptors, especially $P2Y_2$. Therefore, $P2Y_2$ 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 $P2Y_2$ (ATPγS, UTP, INS365) and $P2Y_6$ (UDP, INS48823) agonists increased CCL20 release. UTP-induced CCL20 release was insensitive to NF-κB 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, ATPγS, and UDP but not UTP or INS365 stimulated CCL20 release. Likewise, UDP but not ATPγS was also able to increase CCL20 release from monocytes. Pharmacological experiments suggested an involvement of $P2Y_{11}$ or $P2Y_6$ receptors through NF-κB, 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).

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).

Besides the therapeutical role of nucleotides in cystic fibrosis, ATP was also defined as a key mediator of asthma. We evaluated lung inflammation in mice deficient for the $P2Y_2$ purinergic receptor. We observed that eosinophil accumulation, a distinctive feature of lung allergic inflammation, was defective in ovalbumin (OVA)-treated $P2Y_2$-deficient mice compared to OVA-treated wild type animals (Vanderstocken et al, J Immunol, in revision). Interestingly the up-regulation of VCAM-1 adhesion molecule was lower on lung endothelial cells of OVA-treated $P2Y_2^{-/-}$ mice compared to OVA-treated wild type animals. Additionally the level of soluble VCAM-1, reported as an inducer of eosinophil chemotaxis, was strongly reduced in the bronchoalveolar lavage fluid of $P2Y_2$-deficient mice. In contrast we observed comparable infiltration of macrophages and neutrophils in the bronchoalveolar lavage fluid of LPS-aerosolized $P2Y_2^{+/+}$ and $P2Y_2^{-/-}$ mice. Our data defines $P2Y_2$ as a regulator of membrane and soluble forms of VCAM-1 and eosinophil accumulation during lung inflammation.

2.4.2.4.3. Role of $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ in inflammation

Extracellular nucleotides are involved in a wide spectrum of biological responses. In the context of inflammation, they are released after cell lysis or in a pathogen-induced way and are considered as 'host tissue damage or infection" signal. With the aim to evaluate the physiological relevance of these observations, we are currently analysing the consequence of P2Y genes targeting.

The $P2Y_6$ receptor is selectively activated by UDP, and its transcript has been detected in numerous organs, including the spleen, thymus, intestine, blood leukocytes, and aorta. To investigate the biological functions of this receptor, we generated $P2Y_6$-null mice by gene targeting. The $P2Y_6$ knockout (KO) mice are viable and are not distinguishable from the wild-type (WT) mice in terms of growth or fertility. In thioglycollate-elicited macrophages, the production of inositol phosphate in response to UDP stimulation was lost, indicating that $P2Y_6$ is the unique UDP-responsive receptor expressed by mouse macrophages. Furthermore, the amount of interleukin-6 and macrophage-inflammatory protein-2, but not tumor necrosis factor-alpha, released in response to lipopolysaccharide stimulation was significantly enhanced in the presence of UDP, and this effect was lost in the $P2Y_6$ KO macrophages. The endothelium-dependent relaxation of the aorta by UDP was abolished in KO $P2Y_6$ mice. The contractile effect of UDP on the aorta, observed when endothelial nitric-oxide synthase is blocked, was also abolished in $P2Y_6$-null mice. In conclusion, we have shown that $P2Y_6$-deficient mice have a defective response to UDP in macrophages, endothelial cells, and vascular smooth muscle cells (Bar et al. 2008).

ADP and UDP induced transient intracellular Ca^{2+} increase in bone marrow derived and splenic dendritic cells. These effects were abolished in $P2Y_{12}^{-/-}$ DC and $P2Y_6^{-/-}$ DC respectively. Both ADP and UDP, also stimulated FITC-dextran and ovalbumin

endocytosis by DC through macropinocytosis, and these effects were abolished in DC from $P2Y_{12}^{-/-}$ and $P2Y_6^{-/-}$ mice, respectively. Ovalbumin loading in presence of ADP increased the capacity of DC to stimulate T cells from transgenic mice expressing a T cell receptor recognizing ovalbumin peptide, whereas ADP had no effect on the ability of DC to stimulate allogenic T cells. Moreover, following immunisation with ovalbumin, the serum level of anti-ovalbumin IgG1 was lower in $P2Y_{12}^{-/-}$ mice than in wild types. Therefore, these results suggest that the $P2Y_{12}$ and/or the $P2Y_6$ receptors could be target of new types of adjuvant molecules.

Adenosine triphosphate has previously been shown to induce semi-mature human monocyte-derived dendritic cells (DC) through the $P2Y_{11}$ receptor. We showed that in mice, ATP and adenosine inhibited the production of IL-12p70 by bone marrow-derived DC (BMDC). In the absence of $P2Y_{11}$ receptor in mouse, the effects of adenine nucleotides on mouse DCs are mediated by their degradation product, adenosine, acting on the A2B receptor (Ben Addi et al. 2008).

2.4.2.5. VPAC receptors in the control of immune functions (P1)

Vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide with a broad spectrum of biological functions including immunomodulatory properties. VIP effects are mediated through interactions with G protein-coupled receptors (VPAC1 and VPAC2) that are differentially expressed on various cell types involved in innate and acquired immunity. Anti-inflammatory properties of VIP have been described on mouse peritoneal macrophages and bone marrow-dendritic cells (BM-DC) including inhibition of LPS-induced TNF-alpha secretion and an enhanced production of IL-10. VIP was also shown to induce tolerogenic DC when added during their differentiation process and to promote the emergence of regulatory T cells. Human macrophages and dendritic cells were generated after differentiation of monocytes isolated from peripheral blood in presence of recombinant human (rh) M-CSF or rh GM-CSF and rh IL-4, respectively. Cells were then cultured in vitro in the presence of 10^{-8} M VIP and stimulated with 100 ng/ml lipopolysaccharide (LPS). After 24 hours of culture, cells were harvested, and cytokine production (TNF-alpha and IL-10) was evaluated by ELISA. Cell phenotype and VPAC1 receptor expression were determined using flow cytometry after staining with specific antibodies. BM-DC were generated in the presence of recombinant mouse GM-CSF from bone marrow cells. The immunomodulatory effect of VIP was tested on the induction of human and mouse tolerogenic DC. As described for mouse peritoneal macrophages, LPS-induced TNF- alpha production by human macrophages was decreased in the presence of VIP whereas IL-10 production was increased. Those effects were not observed on human monocyte-derived DC (mo-DC), even if mo-DC were differentiated in the presence of VIP. VPAC1 expression was determined on the antigen-presenting cells showing an expression on human monocytes and macrophages but not on mo-DC. Additional experiments were performed on mouse BM-DC generated in the presence or absence of VIP. As for human DC, no effect of VIP was detected on phenotype of cells. In conclusion, immunomodulatory effects of VIP have been confirmed on human macrophages whereas those effects were not observed on either human or murine DCs (unpublished).

2.4.2.6. Glucocorticoid-induced receptor (GIR, GPR83) controls the activity of cholinergic interneurons in the striatum (P1)

Glucocorticoid-induced receptor (GIR or GPR83) 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 D_1-expressing neurons and reduced activity of D2-expressing neurons, while the number of D_1 and D_2 binding sites in the striatum appeared unchanged. The number and distribution of cholinergic neurons in the striatum were not modified in knockout mice. We monitored the acetylcholine secretion in vivo in the dorsal striatum with a microdialysis probe. We observed a decrease in basal acetylcholine secretion in knock-out mice as compared to wild type mice. 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). Induction of early genes such as c-fos or Zif-268 has been proposed to reflect neuronal activity. Zif-268 transcript levels were increased in the caudate putamen of knock-out mice, more particularly in the most superficial parts of the structure, where most GIR-positive cholinergic interneurons are located. Cocaine administration resulted in stimulated locomotion in both genotypes, but more efficiently in KO animals. The treatment with D_1 and D_2 agonists or antagonists also affected the KO and wild-type mice differently. The 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). Plasma corticosteroid concentrations were significantly lower in knock-out mice in basal conditions, and the difference between genotypes became larger in stressful situations (LPS administration, restraint, open field test). The activity of NPY peptides on locomotion were lost in GIR-KO mice. Our results indicate therefore several physiological functions for GIR. The receptor appears to be activated by NPY peptides and to mediate the inhibitory effects of NPY on locomotion. Beside motor effects, GIR is also involved in stress axis regulation, energy homeostasis and depressive-like behaviours (Laurent et al. unpublished).

2.4.2.7. In vivo function of the adenosine 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.

The role of A_{2A} receptors in aortic vascular tone was investigated using isolated aortic rings from A_{2A}WT and KO mice precontracted with phenylephrine and stimulated with adenosine analogs. A_{2A}R KO mice displayed significantly lower aortic relaxation and endothelial function in response to adenosine analogs, suggesting that the A_{2A}R plays an important role in vasorelaxation, probably through an endothelium-dependent mechanism (Ponnoth et al. 2009). It was also observed that coronary A_{2B} adenosine receptors are up-regulated in A_{2A} knockout mice, and that the A_{2A} receptor is involved in the regulation of basal coronary tone through the release of nitric oxide (Teng et al. 2008).

Endogenous adenosine is an important ligand trigger for the cardioprotective effects of postconditioning. The role of the A_{2A} receptor in this process was evaluated. The 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, 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).

The A_{2A} adenosine receptor is involved in the regulation of addiction induced by different drugs of abuse. The role of A_{2A} receptors in the behavioural and neurochemical responses to morphine was tested. Morphine induced a similar enhancement of locomotor activity and antinociceptive responses in A_{2A} KO and WT mice. However, the rewarding effects induced by morphine were blocked and naloxone did not induce place aversion in A_{2A} KO mice. The results support a role of the A_{2A} receptor in the motivational effects of morphine addiction (Castañé et al. 2008).

We showed, by performing perforated-patch-clamp recordings on brain slices, that D_2 and A_{2A} receptors regulate the induction by NMDA of a depolarized membrane potential plateau in through mechanisms relying upon specific A_{2A}-D_2 receptors heteromerization, demonstrating the physiological relevance of these heteromers (Azdad et al. 2009).

Adenosine has been proposed as an endogenous anticonvulsant. A_{2a}R KO mice were not protected against seizures originating from brainstem structures (electroshock-induced seizures), but seizures induced by pentylenetetrazol or pilocarpine were significantly reduced, suggesting that adenosine exacerbates limbic seizures through the A_{2a} receptors (El Yacoubi et al. 2009). Long-term caffeine intake has been reported to decrease the susceptibility to convulsants in mice. Clonic seizures induced by pentylenetetrazol were similarly attenuated in KO mice and following chronic caffeine treatment in WT mice, demonstrating the role of the A_{2A} receptor in the effects of caffeine (El Yacoubi et al. 2008).

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). Peripheral nerve injury produces a persistent neuropathic pain state characterized by spontaneous pain, allodynia and hyperalgesia. The possible involvement of adenosine receptors in the development of neuropathic pain after sciatic nerve injury was evaluated. Partial ligation of the sciatic nerve was performed in A_{2A} KO and WT littermates. Mechanical and thermal allodynia, and thermal hyperalgesia was evaluated by respectively the von Frey filament model, the cold-plate test and the plantar test. A significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia were observed in A_{2A}R deficient mice, demonstrating the involvement of A_{2A}Rs in the control of neuropathic pain (Bura et al. 2008).

The A_{2A} receptor is known to suppress superoxide generation in leukocytes through protein kinase A. We explored the role of A_{2A}R in murine tracheas in the context of a model of asthma (ovalbumin). A_{2A}R was decreased in sensitized mice. Sensitized mice and A_{2A}KO mice displayed increased expression of NADPH oxidase subunits (p47phox and gp91phox), increased tracheal reactive oxygen species (ROS) generation, and reduced tracheal relaxation to adenosine analogs. This study showed that A_{2A}R is involved in tracheal relaxation, and that decrease in A_{2A}R in asthma results in increased ROS generation and decreased tracheal relaxation (Nadeem et al. 2009).

2.4.2.8. In vivo function of the CB1 cannabinoid 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.

The endocannabinoid system is involved in the addictive processes induced by different drugs of abuse. We have tested the role of the CB1 receptor in the pharmacological effects of different classes of drugs. 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 GABAA 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). MDMA (3,4-methylenedioxymethamphetamine) was also tested. Acute MDMA administration increased locomotor activity, body temperature, and anxiogenic-like responses in wild-type mice, but these responses were lower or abolished in CB1 knockout animals. MDMA produced similar conditioned place preference in both genotypes but CB1 knockout mice failed to self-administer MDMA. These results indicate that CB1 receptors play an important role in the acute

prototypical effects of MDMA and are essential in the acquisition of an operant behavior to self-administer this drug (Touriño et al. 2008).

We investigated the effects of genetic and pharmacological blockade of the CB_1 cannabinoid receptor on the behaviour of CD1 mice using different tests of fear and anxiety. The CB_1 receptor antagonist SR141617A (rimonabant) increased anxiety parameters in all settings, while CB_1 receptor knockout mice and mice treated with the selective CB_1 receptor antagonist AM251 did not display significant alterations in anxiety-related behaviour. These data support the existence of a SR141617A-sensitive site that is different from the 'classical' CB1 receptor and that has a pivotal role in the regulation of anxiety (Thiemann et al. 2009). Other data also suggested that, in the hippocampus, WIN 55,212-2 reduces glutamate release from Schaffer collaterals solely via CB1 receptors in the nM concentration range, whereas in μM concentrations, ir suppresses excitatory transmission by an additional mechanism independent of CB1, the blockade of N-type voltage-gated Ca^{2+} channels (Németh et al. 2008). 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).

Serotonergic and endocannabinoid systems are both involved in the pathophysiology of mood disorders and we studied the interactions between these systems. We showed that the CB_1 receptor is necessary for the serotonergic negative feedback in mice. Indeed, in vivo microdialysis experiments revealed increased basal extracellular levels of 5-HT but attenuated fluoxetine-induced increase of 5-HT levels in the prefrontal cortex of CB_1 knockout mice. There was also a reduction in 5-HT transporter in frontal cortex and a reduction in the expression of the $5\text{-}HT_{2C}$ receptor within several brain areas related to the control of the emotional responses, such as the dorsal raphe nucleus (Aso et al. 2009).

We have investigated further the involvement of the CB1 receptor in the responses to stress. Stress is known to cause damage and atrophy of neurons in the hippocampus by deregulating the expression of neurotrophic factors that promote neuronal plasticity. We showed that CB1 knockout mice exhibit an increased response to stress, including increased despair behavior and corticosterone levels in the tail suspension test, and decreased brain derived neurotrophic factor (BDNF) levels in the hippocampus. Local administration of BDNF in the hippocampus reversed the increased despair behavior of CB1 knockout mice, confirming the role played by BDNF in the emotional impairment of these mice (Aso et al. 2008).

Regulation of Ca^{2+} homeostasis plays a critical role in oligodendrocyte function and survival and CB_1 and CB_2 receptors have been shown to regulate Ca^{2+} levels and/or K^+ currents in a variety of cell types. We investigated the effect of cannabinoid compounds on the Ca^{2+} influx elicited in cultured oligodendrocytes by transient membrane depolarization. We showed that cannabinoids inhibit depolarization-evoked Ca^{2+} transients in oligodendrocytes via CB_1 receptor-independent and -dependent mechanisms that involve the activation of PTX-sensitive $G_{i/o}$ proteins and the blockade of Kir channels (Mato et al. 2009). Other data have shown that activation of CB_1 receptors offers neuroprotection against dopaminergic lesion and the development of l-DOPA-induced dyskinesias. Indeed, lesions induced by 6-OHDA and motor deterioration were more severe in CB_1 KO mice, with increased oxidative/nitrosative and neuroinflammatory parameters in the caudate-putamen and cingulate cortex of these mice (Pérez-Rial et al. 2009).

The endogenous cannabinoid system plays important roles in nociceptive information processing in various areas of the nervous system including the spinal cord, but cellular distribution of CB1 in the superficial spinal dorsal horn is poorly defined. We investigated the cellular distribution of CB1 in the rodent spinal dorsal horn by immunocytochemistry. Axonal varicosities revealed a strong immunoreactivity for CB1, but no CB1 expression was observed on dendrites and perikarya of neurons. By colabelling studies, CB1 was found in part of peptidergic (CGRP) and non-peptidergic (isolectin B4) nociceptive primary afferents, glutamatergic and GABAergic spinal interneurons, astrocytic (GFAP) and microglial (CD11b) cells. These findings suggest that the activity-dependent release of endogenous cannabinoids activates a complex signaling mechanism in pain processing spinal neural circuits into which both neurons and glial cells may contribute (Hegyi et al. 2009).

Acetaminophen is the most used analgesic/antipyretic drug. In thermal, mechanical and chemical pain tests, AM-251, a specific CB1 receptor antagonist, abolished the analgesic action of acetaminophen, which was also lost in CB1 receptor knockout mice. Inhibition of FAAH, an enzyme metabolizing acetaminophen into AM-404 suppressed the antinociceptive effect of acetaminophen. In addition, the antinociceptive activity of ACEA, a CB1 receptor agonist, was inhibited by lesion of bulbospinal serotonergic pathways and antagonists of spinal 5-HT receptors. Therefore, acetaminophen-induced analgesia likely involves the FAAH-dependent metabolism of acetaminophen into AM-404, the CB1 receptor, and an endocannabinoid-dependent reinforcement of the serotonergic bulbospinal pathways (Mallet et al. 2008).

During transit through the epididymis, spermatozoa are normally kept immotile and do not attain the ability to become motile until they reach the caudal epididymis. We tested whether endocannabinoids play a role in the epididymis and in suppressing the ability of spermatozoa to become motile. We showed that the levels of the endocannabinoid, 2-arachidonoylglycerol, are high in mouse spermatozoa isolated from the head of the epididymis, where these cells do not move, and decrease dramatically in spermatozoa isolated from the tail. This gradient regulates the activity of the CB1 receptor present on the sperm cells and induces caudal spermatozoa to acquire the potential to become motile ("start up"). Accordingly, the genetic or pharmacological inactivation of CB1 increased the number of motile spermatozoa in the epididymal head (Cobellis et al. 2009).

2.4.2.9. Role of LGR4 and LGR5 in the developing small intestine.

The Leucine-rich repeat G protein-coupled receptors 4 and 5 (LGR4/GPR48; LGR5/GPR49) are orphan members of the family of glycoprotein hormone receptors. We have described the phenotype of mice invalidated for the LGR4 gene, displaying defects in development of the male genitourinary tract (Mendive et al 2006, Lambot et al 2009). LGR5 is a target of the Wnt signaling

pathway. LGR5 KO mice die soon after birth secondary to malformation of the tongue (ankyloglossia), preventing efficient suckling (Morita et al 2004). It has recently been identified as a reliable marker of adult stem cells (SC) in the intestine, skin and other epithelia (Barker et al 2010). However, the precise functions of the two receptors have not been addressed yet. We have investigated the role of LGR4 and LGR5 during ileal development by using LGR4 and LGR5 null/LacZ-NeoR mice.

For LGR5, X-gal staining experiments showed that, after villus morphogenesis, Lgr5 expression becomes restricted to dividing cells clustered in the intervillus region and is more pronounced in the distal small intestine. At day E18.5, LGR5 deficiency leads to premature Paneth cell differentiation in the small intestine without detectable effects on differentiation of other cell lineages, nor on epithelial cell proliferation or migration. Quantitative RT-PCR experiments showed that expression from the LGR5 promoter is upregulated in LGR5-null mice, pointing to the existence of an autoregulatory negative feedback loop in intact animals. This deregulation is associated with overexpression of Wnt target genes in the intervillus epithelium. Transcriptional profiling of mutant mice ileums revealed that LGR5 function is associated with expression of SC and SC niche markers. Together, these data identify LGR5 as a negative regulator of the Wnt pathway in the developing intestine. These results are detailed in Garcia et al 2009.

For LGR4, we are exploring the intestinal phenotype of LGR4 KO mice. In addition to classical immunohistological characterization of intect tissue, we explore the survival and development ex vivo of intestinal crypts from LGR4 KO mice, cultured according to a recently described protocol (Sato et al 2009). Preliminary results show that contrary to LGR5 KO, crypts from LGR4 KO mice do not survive ex vivo. We are currently attempting "rescue" experiments in this system to identify the regulatory cascade(s) controlled by LGR4.

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

Our aim is to unravel the *in vivo* function of particular ligand-receptor couples in the fruit fly. To reach our goals, we utilize a diverse range of genetic tools that are available to study gene function in these insects. We have studied 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. 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. QRT-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. 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.

For the study of *Drosophila* LGRs (leucine-rich repeats containing GPCRs), we are also applying quantitative real-time RT-PCR and *in situ* hybridization analyses to identify the tissues in which these receptors are expressed. Furthermore, we are investigating the possible *in vivo* role(s) of the fly FSH-TSH-like receptor (DLGR1) more in detail. To gain further insight in the precise gene-expression pattern of this LGR, we have also created a reporter plasmid in which the putative promoter of DLGR1 drives Gal4 (a yeast transcriptional factor) expression. Fruit flies in which this construct is stably integrated into the genome (by injection of fly embryos) in the germ cell line allowed us to investigate the *in vivo* expression pattern of DLGR1. Indeed, when these flies are crossed with flies that carry a reporter gene (*e.g*. green fluorescent protein or β-galactosidase) under control of UAS (upstream activating sequence, the DNA-target site for Gal4), gene-expression can be studied in detail. In addition, the function of DLGR1 has been studied by means of RNA interference (RNAi). This is achieved by driving the expression of a specific dsRNA fragment (targeting the mRNA for this receptor to degradation) in different *Drosophila* tissues by means of the Gal4-UAS system. Our results indicate that the transgenic flies with severely down-regulated DLGR1 transcript levels do not develop into adults. Their development appears to be arrested in the third instar larval stage. The larvae seem to be unable to form a pupa. Interestingly, the observed defect can be partially rescued by supplying ecdysteroid hormone via the larval food. This effect may be indicative for insufficient levels of the moulting hormone (20-hydroxyecdysone) in the transgenic LGR1-knockdown larvae. Analysis of hormone titres in these animals indeed confirmed this hypothesis. Further studies are now underway to analyze this receptor knockdown phenotype in detail and to study the role of the (potential) physiological agonist for this receptor which may be responsible for the control of ecdysteroid production and/or of pupariation in larval insects. In the future, we also plan to investigate the *in vivo* function of the orphan *Drosophila* LGRs (*i.e.* DLGR3 and 4), as well as other GPCRs and their corresponding ligands, through RNAi (see also 6.5).

2.4.2.11. Glucose-sensing receptor (yeast and Candida albicans) and 'GPCR-controlled nutrient-sensing network' (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 have shown that sucrose is the most potent nutrient inducer of pseudohyphal growth and 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 dispensible 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.

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. CaGpa2, but not CaGPR1, can complement the yeast mutant for glucose-induced cAMP signaling. Interestingly, expression of the CaGPR1 gene in a yeast gpr1 mutant causes a constitutively high PKA phenotype. The mechanisms involved are under investigation.

In order to sense changes in nutrient availability and be able to elicit a fast intracellular signal leading to an adaptive response, the yeast *Saccharomyces cerevisiae* has evolved three different classes of nutrient-sensing proteins acting at the plasma membrane: GPCRs or classical receptor proteins, which simply detect the presence of nutrients; non-transporting transceptors, i.e. nutrient carrier homologues with only a receptor function; and the recently described transporting transceptors, i.e. active nutrient carriers that combine the functions of nutrient transporter and receptor. Our research focusses on proteins that belong to the first and third category.

A well studied **GPCR system** in *S. cerevisiae* consists of the GPCR glucose/sucrose sensitive 7-transmembrane domain receptor Gpr1, the Gα protein Gpa2 and its regulator of heterotrimeric G protein signaling, Rgs2, which is responsible for glucose and sucrose control of the protein kinase A (PKA) pathway. Previously we have shown that the *C. albicans* Gpr1 and Gpa2 homologues function upstream of the PKA-pathway. Although it has been shown that the cAMP signal in *C. glabrata* is also activated by glucose, but not by sucrose, the ligand of CaGpr1 does not seem to be glucose as CaGpr1 is dispensible 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*.

The active nutrient carriers that combine the functions of nutrient transporter and receptor are called **transceptors**. Until now, the precise action mechanism of these transceptors is unknown. Three examples of such proteins have been characterized in our laboratory: the general amino acid permease Gap1 (Donaton et al., 2004), the ammonium permease Mep2 (Van Nuland et al., 2006) and the phosphate carrier Pho84 (Giots et al., 2003). Addition of essential nutrients, like nitrogen or phosphate, to nitrogen- or phosphate-starved cells, also triggers rapid activation of the PKA pathway but in a cAMP independent manner. However, it has to be noted that this rapid activation requires, in addition to nitrogen or phosphate, the presence of a rapidly-fermentable sugar, such as glucose sensed by the GPCR system. Therefore, we refer to this transceptor-mediated sensing system as the **'GPCR-controlled nutrient-sensing network'**.

Mep1 and Mep2 are ammonium permeases involved in the ammonium-induced activation of the PKA pathway. Whether the involvement of these Mep's in signalling is direct or indirect (intracellular sensing of transported ammonium) was unknown. Several mutants in residues of Mep2 in regions exposed to the cytoplasm resulted in a reduction of both trensport and signalling to a similar extent, suggesting that these properties are coupled. However, with some highly specific mutations signalling was affected to a much larger extent than the corresponding uptake and accumulation of the substrates. Taken together, these results argue against an intracellular sensing mechanism for ammonium, and add further evidence to the role of Mep2 as the actual sensor for these substrates.

Gap1 is a general amino acid permease that imports amino acids for their use as nitrogen source. In addition to its transporting properties, Gap1 triggers activation of the PKA pathway. We have screened 319 amino acid analogues to identify compounds that act on this permease. We identified competitive and non-competitive inhibitors of transport, either with or without agonist action for signaling, and including non-transported agonists. This clearly indicates that transport and signaling properties of transceptors can be uncoupled. Signaling apparently requires a ligand-induced specific conformational change, which may be part of, but does not require the complete transport cycle. We also identified amino acid residues of which the side chain is exposed into the substrate binding site of Gap1 and used this to demonstrate that Gap1 uses the same amino acid binding site for transport and signaling (Van Zeebroeck et al. 2009).

In the absence of amino acids, Gap1 is expressed at the plasma membrane, while upon addition of amino acids the permease is internalized (induced by ubiquitination) and routed through the endosomal pathway to the vacuole/lysosome, where it is broken down. However, three specific dipeptides caused Gap1-dependent persistent activation of the PKA target trehalase. We have found that vacuolar sorting of Gap1 is impaired in a similar way as in the absence of certain functions controlling endosomal trafficking. Ubiquitination is not impaired. Gap1 is accumulated in endosomes apparently as a result of malfunctioning of Vps27-mediated recognition of ubiquitinated Gap1. These and other results indicate that binding of the dipeptides to Gap1 induces a conformational change that interferes with the vacuolar delivery of the permease, resulting in persistent activation of PKA targets. (Rubio-Texeira et al. in preparation).

Intriguingly, a similar phenotype of constitutive activation of the PKA pathway is observed with two short C-terminal truncations of Gap1 (14 and 26 amino acids, resulting in Gap1ΔC6 and Gap1ΔC9 alleles respectively). However, transport activity of Gap1 was only slightly increased in these mutant strains (150% and 130% respectively compared to wild-type). It was demonstrated that PKA is involved in mediating the effects caused by these alleles, since deletion of merely one of the catalytic

subunits of PKA resulted in a wild-type phenotype. In contrast, we showed that the phenotype is not caused by overactivation of Tor or Stt4 or by the inhibition or overexpression of Sch9. Interestingly, the truncated overactive Gap1 alleles can suppress the lethality caused by cAMP deficiency but not PKA deficiency, indicating that they apparently act upstream or at the level of PKA.

2.4.2.12. 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 (AlHV-1) is carried asymptomatically by wildebeest, but causes malignant catarrhal fever (MCF) following cross-species transmission to a variety of susceptible species. The A5 ORF of the AlHV-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 [^{35}S]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 AlHV-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 AlHV-1 in MCF pathogenesis (Boudry et al. 2007).

2.4.3. Main achievements in relation to the initial objectives

The mouse ChemR23 receptor was characterized and a knock out model was obtained. Proteinase 3 and chymase were shown to inactivate chemerin.

A mouse receptor (Fpr2) responding to the F2L peptide was characterized as a functional homolog of human FPRL2. FPRL2 was described on human plasmacytoid dendritic cells, macrophages and eosinophils. Processing of heme-binding protein into F2L by cathepsin D was shown.

Extracellular nucleotides, acting though P2Y receptors, were shown to render dendritic cells tolerogenic, and to behave as proinflammatory mediators in a number of other settings.

The role of the adenosine A2a and cannabinoid CB1 receptors was evaluated in collaboration with various groups.

LGR5 has been identified as a negative regulator of the Wnt pathway in the mouse intestine

LGR4 invalidation affects development of testis efferent ducts and epididymis

We have identified new roles for the glucose/sucrose sensing GPCR, Gpr1, in yeast. We have discovered Gap1 and Pho84 as transceptors for amino acid and phosphate activation of the PKA pathway in yeast and have made them into the best characterized nutrient transceptors in cell biology.

2.4.4. Comments (in case of deviations from the initial project workprogramme)

None

2.5. Workpackage 5. Role of GPCRs in human diseases and animal models

Contributing partners: P1, P4, P5, EU1

2.5.1. Summary description of the objectives

For mammalian receptors, we will further determine their potential involvement in human diseases, with a special focus onto inflammation, cancer, and the neuroendocrine axis. These studies will be conducted both by studying human pathological samples, and by submitting the genetically modified mice to a number of in vivo disease models. These models will be run with the help of a group of pathologists (partner P5). The receptors studied in this frame include glycoprotein hormone, chemoattractant and neuromodulatory receptors. Finally, the influence of allelic variation in GPCR genes or gene clusters in mouse lung disease models will be studied by genetic linkage analysis. If candidate GPCR genes result from this approach, they will be studied more specifically both in human and in mouse models.

2.5.2. Summary description of scientific activities and results

2.5.2.1. Glycoprotein hormone receptors (P1, EU1)

2.5.2.1.1. 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 section 2.1.2.1) and we propose a molecular explanation to the binding characteristics of the p.Ser128Tyr mutant (De Leener et al. 2008).

2.5.2.1.2. 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 (High free T3 and free T4, very low TSH and no TSH receptor antibody). 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 adenyl 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).

2.5.2.1.3. 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 (T3) 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 T3 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 T3 (Bassett et al. 2008).

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

Chemokines, or chemotactic cytokines, and their receptors have been discovered as essential and selective mediators in leukocyte migration to inflammatory sites and to secondary lymphoid organs. Besides their functions in the immune system, they also play a critical role in tumor initiation, promotion and progression. There are four subgroups of chemokines: CXC, CC, CX3C, and C chemokine ligands. The CXC or α subgroup is further subdivided in the ELR+ and ELR- chemokines. Members that contain the ELR motif bind to CXCR2 and are angiogenic. In contrast, most of the CXC chemokines without ELR motif bind to CXCR3 and are angiostatic. An exception is the angiogenic ELR- CXC chemokine CXCL12/SDF-1, which binds to CXCR4 and CXCR7 and is implicated in tumor metastasis. CXC chemokines and their receptors play a role in tumorigenesis, including angiogenesis, attraction of leukocytes to tumor sites and induction of tumor cell migration and homing in metastatic sites (Vandercappellen et al., 2008).

2.5.2.2.1. 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).

2.5.2.2.2. 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).

2.5.2.2.3. 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-1β 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).

2.5.2.2.4. Chemokines delivered by parvovirus reduce 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).

Gliomas are highly malignant brain tumors. The median survival time of patients diagnosed with glioblastoma multiforme is less than 1 year. These tumors are particularly refractory to most conventional anticancer therapies, including surgical resection, radiation therapy and chemotherapy. The limited efficacy of current standard treatments makes it necessary to investigate alternative therapeutic approaches, among which those based on immunostimulation seem promising. One approach relies on cytokines inducing an increased immune response within the tumor. Another involves ex vivo manipulation of effector cells (dendritic cells); for example, stimulating them with specific antigens or promoting their maturation. A hallmark of glioblastoma is the high degree of neovascularization observed within the tumor. Inhibition of angiogenesis might be a powerful strategy for impeding the growth of vascularized tumors. The therapeutic effectiveness of targeting glioma-induced angiogenesis has indeed been demonstrated in various experimental systems. CXCL10 is a potent chemoattractant for natural killer cells and activated T lymphocytes. It also displays angiostatic properties and some antitumor activity. Tumor necrosis factor-α (TNF-α) is a powerful immunomodulating cytokine with tumoricidal activity in various tumor models and the ability to induce strong immune responses. This prompted us to evaluate the antitumor effects of recombinant parvoviruses designed to deliver CXCL10 or TNF-α into a gliobastoma. When Gl261 murine glioma cells were infected in vitro with a CXCL10- or TNF-α-transducing parvoviral vector and were subcutaneously implanted in mice, tumor growth was significantly delayed. Complete tumor regression was observed when the glioma cells were co-infected with both the vectors, demonstrating synergistic antitumor activity. In an established in vivo glioma model, however, repeated simultaneous peritumoral injection of the CXCL10- and TNF-α-delivering parvoviruses failed to improve the therapeutic effect as compared with the use of a single cytokine-delivering vector. In this tumor model,

cytokine-mediated immunostimulation, rather than inhibition of vascularization, is likely responsible for the therapeutic efficacy (Enderlin et al., 2009).

MCP-2/CCL8 was induced at only suboptimal levels in fibroblasts and endothelial cells by IL-1β or IFN-γ, unless these cytokines were combined. IFN-γ also synergized with the TLR ligands peptidoglycan (TLR2), dsRNA (TLR3) or LPS (TLR4). Under these conditions, intact MCP-2/CCL8 (1-76) produced by fibroblasts was found to be processed into MCP-2/CCL8(6-75), which lacked chemotactic activity for monocytic cells. Furthermore, the capacity of MCP-2/CCL8(6-75) to increase intracellular calcium levels through CCR1, CCR2, CCR3 and CCR5 was severely reduced. However, the truncated isoform still blocked these receptors for other ligands. MCP-2/CCL8(6-75) induced internalization of CCR2, inhibited MCP-1/CCL2 and MCP-2/CCL8 ERK signalling and antagonized the chemotactic activity of several CCR2 ligands (MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7). In contrast to MCP-3/CCL7, parvoviral delivery of MCP-2/CCL8 into B78/H1 melanoma failed to inhibit tumor growth, partially due to proteolytic cleavage into inactive MCP-2/CCL8 missing five NH_2-terminal residues. However, in an alternative tumor model, using HeLa cells, MCP-2/CCL8 retarded tumor development. These data indicate that optimal induction and delivery of MCP-2/CCL8 is counteracted by converting this chemokine into a receptor antagonist, thereby losing its anti-tumoral potential (Struyf et al., 2009).

2.5.2.2.5. 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-1β and IL-17, in sarcoma cells, but not in diploid fibroblasts. Furthermore, the induction of GCP-2/CXCL6 in endothelial cells by IL-1β was enhanced synergistically by TNF-α but inhibited by IFN-γ, which synergized with IL-1β 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).

The regulated expression of angiogenic SDF-1/CXCL12 and IL-8/CXCL8 and angiostatic PF-4var/CXCL4L1 and IP-10/CXCL10 chemokines was examined in human colorectal and esophageal cancer. In HCT 116 and HCT-8 colorectal adenocarcinoma cells, the production of IL-8/CXCL8 immunoreactivity was up-regulated by IL-1β, TNF-α, the toll-like receptor (TLR) ligands double-stranded RNA and peptidoglycan and phorbol ester. Increased PF-4/CXCL4 and synergistic IL-8/CXCL8 and IP-10/CXCL10 induction in carcinoma cells after stimulation with IL-1β plus TNF-α or IFN-γ was demonstrated by enzyme-linked immunosorbent assay, quantitative reverse transcriptase polymerase chain reaction or immunocytochemistry. In addition, IL-8/CXCL8 from HT-29 colorectal adenocarcinoma cells was molecularly identified as intact chemokine, as well as NH_2-terminally truncated, more active IL-8/CXCL8(6-77). The presence of PF-4var/CXCL4L1, SDF-1/CXCL12 and vascular endothelial growth factor (VEGF) was evidenced by immunohistochemistry in surgical samples from 51 patients operated on for colon adenocarcinoma (AC), esophageal AC, or esophageal squamous cell carcinoma (SCC). PF-4var/CXCL4L1 was strongly detected in colorectal cancer, whereas its expression in esophageal cancer was rather weak. Staining for SDF-1/CXCL12 was almost negative in esophageal SCC, whereas a more intense and frequent staining was observed in AC of the esophagus and colon. Staining for VEGF was moderately to strongly positive in all 3 types of cancer, although less prominent in esophageal AC. The heterogenous expression of angiogenic (IL-8/CXCL8, SDF-1/CXCL12) as well as angiostatic (IP-10/CXCL10, PF-4var/CXCL4L1) chemokines not only within the tumor and between the different cases, but also between the different tumor cell types may indicate a distinct role of the various chemokines in the complex process of tumor development (Verbeke et al., 2010).

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 servere 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).

Recently, it was found that the variant of PF-4/CXCL4 (PF-4var/CXCL4L1) could exert a more pronounced angiostatic and antitumoral effect than PF-4/CXCL4. However, the molecular mechanisms of the angiostatic activities of the PF-4 forms remain partially elusive. Therefore, we studied the biological properties of the chemically synthesized COOH-terminal peptides of PF-4/CXCL4 (PF-4/CXCL4$^{47-70}$) and PF-4var/CXCL4L1 (PF-4var/CXCL4L1$^{47-70}$). Both PF-4 peptides lacked monocyte and

lymphocyte chemotactic activity but equally well inhibited (25 nmol/L) endothelial cell motility and proliferation in the presence of a single stimulus (i.e., exogenous recombinant fibroblast growth factor-2). In contrast, when assayed in more complex angiogenesis test systems characterized by the presence of multiple mediators, including in vitro wound-healing (2.5 nmol/L versus 12.5 nmol/L), Matrigel (60 nmol/L versus 300 nmol/L), and chorioallantoic membrane assays, PF-4var/CXCL4L1$^{47-70}$ was found to be significantly (5-fold) more angiostatic than PF-4/CXCL4$^{47-70}$. In addition, low (7 μg total) doses of intratumoral PF-4var/CXCL4L1$^{47-70}$ inhibited B16 melanoma growth in mice more extensively than PF-4/CXCL4$^{47-70}$. This anti-tumoral activity was predominantly mediated through inhibition of angiogenesis (without affecting blood vessel stability) and induction of apoptosis, as evidenced by immunohistochemical and fluorescent staining of B16 tumor tissue. In conclusion, PF-4var/CXCL4L1$^{47-70}$ is a potent antitumoral and anti-angiogenic peptide. These results may represent the basis for the design of PF-4var/CXCL4L1 COOH-terminal-derived peptidomimetic anticancer drugs (Vandercappellen et al., 2010)

2.5.2.2.6. 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-γR 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).

Interleukin 17 (IL-17) is a proinflammatory cytokine, produced only by activated lymphocytes, but with a broad cellular host range. The effects of IL-17 on fibroblasts were investigated by analysis of the induction of chemokine and matrix metalloprotease (MMP) mRNA levels by RT-PCR. IL-17 stimulated CC chemokine (MCP-1/CCL2) and CXC (KC, MIP-2) chemokine and TIMP-1 mRNA expression in fibroblastoid L929 cells. In normal mouse embryonic fibroblasts (MEF) this induction profile by IL-17 was extended with the mRNAs encoding the chemokine (GCP-2/CXCL6) and the proteases MMP-3, MMP-9 and MMP-13. The MMP-9 and GCP-2/CXCL6 induction by IL-17 in MEF, and the absence of induction in L929 cells, were corroborated by gelatine zymography and ELISA, respectively. The induction of MCP-1/CCL2 by IL-17 was confirmed in human diploid fibroblasts. We conclude that IL-17 regulates differently chemokine and MMP expression by normal and transformed fibroblasts and is indirectly capable of attracking both monocytes and neutrophils to the inflammatory focus (Qiu et al., 2009).

Since interferon (IFN)-γ inhibits Th17 cell development, IFN-γ receptor knockout (IFN-γR KO) mice develop CIA more readily. A preventive anti-IL-17 antibody treatment inhibited CIA in IFN-γR KO mice. In the joints of anti-IL-17-treated mice, neutrophil influx and bone destruction were absent. Treatment reduced the cellular autoimmune response as well as the splenic expansion of CD11b$^+$ cells, and production of myelopoietic cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-6. IL-17 and TNF-α synergistically induced granulocyte chemotactic protein-2 (GCP-2/CXCL6), IL-6 and receptor activator of NFκB ligand (RANKL) in MEF. This induction was profoundly inhibited by IFN-γ in a STAT-1 (signal transducer and activator of transcription-1)-dependent way. In the abscence of IFN-γ, IL-17 mediates its pro-inflammatory effects mainly through stimulatory effects on granulopoiesis, neutrophil infiltration (via GCP-2/CXCL6) and bone destruction (Kelchtermans et al., 2009).

2.5.2.2.7. Role of chemokines in other diseases

Cerebral malaria (CM) results from the binding of infected erythrocytes and leukocytes to brain endothelia. The precise mechanisms underlying lymphocyte recruitment and activation in CM remain unclear. Therefore, the expression of various chemokines was quantified in brains of mice infected with Plasmodium berghei ANKA (PbA). Several chemokines attracting monocytes and activated T lymphocytes were expressed at high livels. Their expression was almost completely abrogated in IFN-γ ligand and receptor KO mice, indicating that IFN-γ is an essential chemokine inducer in vivo. Surprisingly, the expression levels of chemokines, IFN-γ and also adhesion molecules in the brain were not lower in CM-resistant Balb/c and DBA/2 mice compared to CM-sensitive C57BL/6 and DBA/1 mice, although T lymphocyte sequestration in the brain was significantly less in CM-resistant than in CM-sensitive mice. This difference correlated with a higher up-regulation of CXCR3 on splenic T cells and a higher chemotactic response to CXCL10 in C57BL/6 compared with Balb/c mice. In conclusion, parasite-induced IFN-γ in the brain results in high local expression levels of specific chemokines for monocytes and lymphocytes. The strain-dependent susceptibility to develop CM is more related to the expression of CXCR3 in circulating leukocytes than to the chemokine expression levels in the brain (Van den Steen et al., 2008).

Proliferative vitreoretinopathy (PVR), the most common cause of failure of retinal re-attachment surgery, is characterized by the development of fibrocellular membranes on either side of the retina. The formation and gradual contraction of these membranes cause a marked distortion of the retina and result in complex retinal detachments that are difficult to repair. The presence of α-smooth-muscle actin (α-SMA) expressing myofibroblasts in PVR epiretinal membranes has been previously

reported. Myofibroblasts are found at sites of wound healing and chronic inflammation, and are believed to play a pivotal role in the healing process and in the pathogenesis of fibrosis. Fibrocytes, circulating cells that co-express markers of haematopoietic stem cells, leukocytes and fibroblast products, traffic to sites of tissue injury, differentiate into myofibroblasts and contribute to wound healing and fibrosis. We investigated the presence of fibrocytes and the expression of their chemotactic pathways CCL21/CCR7 and CXCL12/CXCR4 in PVR epiretinal membranes. Sixteen membranes were studied by immunohistochemical techniques. Cells expressing α-SMA, a marker of differentiation of fibrocytes into myofibroblasts, were present in all membranes. Cells expressing the haematopoietic stem cell antigen CD34, the leukocyte common antigen CD45, CCR7, CXCR4, CCL21 and CXCL12 were noted in 50%, 75%, 68.8%, 100%, 80% and 93.8% of the membranes, respectively. Double immunohistochemistry indicated that all cells expressing CD34, CD45, CCR7, CXCR4, CCL21 and CXCL12 co-expressed α-SMA. The number of cells expressing CD34 correlated significantly with the numbers of cells expressing CXCL12 (r_s=0.567; p=0.022) and CCL21 (r_s=0.534; p=0.04). It can be concluded that circulating fibrocytes may function as precursors of myofibroblasts in PVR membranes (Abu El-Asrar et al., 2008).

We hypothesized that besides oro-laryngeal cancer, oral inflammatory states, such as periodontitis, may also influence the chemokine profile of oral fluid. The aim was to characterize the chemokine isoforms in the oral fluid of patients with periodontitis and in the oral fluid of patients with head and neck cancer. It was found that the concentrations of CXCL8, CXCL10, and CCL14 were significantly elevated in the oral fluids of the cancer patients. However, periodontitis did not significantly alter the chemokine levels in oral fluid. Identification of chemokine isoforms by a proteomic approach using a newly developed three-step purification procedure was applied on the oral fluid of head and neck cancer and periodontitis patients and on the conditioned medium from carcinoma cells. Carcinoma cells produced predominantly intact CXCL1, CXCL2, CXCL8, and CCL2, whereas CXCL8 also appeared in a truncated, more active, form. The chemokine profile of head and neck cancer significantly changed after therapy, indicating that it is a useful parameter in clinical practice (Michiels et al., 2009).

The inflammatory response plays an important role in the tissue destruction associated with periodontitis. Bacterial species can regulate the inflammatory responses of host cells, triggered by pathogens. It was hypothesized that, in the field of oral microbiology/immunology, such effects of bacterial interactions on inflammatory host cell responses might also be present. The effects of beneficial, commensal, and pathogenic species on *Aggregatibacter actinomycetemcomitans*-induced IL-8/CXCL8 production by human cells were investigated. The beneficial species, *Streptococcus mitis, Streptococcus salivarius,* and *Streptococcus sanguinis*, were able to lower the IL-8/CXCL8 production triggered by *A. actinomycetemcomitans*. The inhibitory effect was also achieved by the application of streptococcal supernatants. In contrast, the commensal *Streptococcus gordonii* caused no reduction, and the pathogen *Fusobacterium nucleatum* increased IL-8/CXCL8 production by the host cells. These results show that bacterial species can influence the inflammatory responses of host cells triggered by infection with *A. actinomycetemcomitans* (Sliepen et al., 2009).

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

2.5.2.3.1. 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.

After TPA application, chemerin expression became detectable in granule-cell layer of the skin epidermis in both WT and KO mice. However, chemerin staining decreased rapidly in WT skin, whereas sustained expression and positive staining remained between day 2 and day 6 in ChemR23 KO mice. ChemR23 expression increased progressively after TPA painting in WT mice and reached a peak between day 4 and day 7. The stronger inflammatory reaction observed in ChemR23 KO mice was also confirmed by the higher production in the skin of ChemR23 KO mice, of inflammatory mediators, such as TNF-α, IL-1β, iNOS, S100A9, IL-23 and IL-17, described as hallmarks of psoriatic disease. These observations suggest that increased skin inflammation observed in KO mice could be explained by an impairment of the regulation of the immune response leading to a delay in the resolution of the inflammatory reaction. Analysis of the leukocyte populations in skin dermis and in the skin draining lymph nodes confirmed a stronger inflammation in ChemR23 KO mice with higher and sustained infiltration of immune cells in skin dermis and draining lymph nodes

To better understand the mechanisms that regulate skin inflammation, we tested *ex vivo* the effect of chemerin on the regulation of inflammatory cytokines in LPS-stimulated peritoneal macrophages isolated from WT and ChemR23 KO mice. No significant modifications in the expression of proinflammatory cytokines (IL-12p40, IL-23, IL-6, IL-1β, TNF-α) by macrophages was observed between ChemR23 KO and WT mice in response to LPS stimulation. Chemerin may therefore play a regulatory role in the early steps of inflammation by recruiting leukocyte populations with anti-inflammatory properties. Infiltrating immune cells in the skin were determined, showing recruitment of dendritic cell subsets, macrophages, neutrophils and T cells in the dermis and epidermis. Using flow cytometry on dissociated skin cells, we identified a delayed recruitment of a pDC subset

($CD45^+GR1^{int}B220^+mPDCA^+$) in KO mice as compared to WT mice. Plasmacytoid dendritic cells have been proposed as main players in the initiation of psoriasis, but they were also recently described to have have an immunosuppressive activity on T cell proliferation and to inhibit the production of TH-17 cytokines by T cells. These aspects are being studied further.

2.5.2.3.2 Chemerin and ChemR23 in inflammatory lung diseases (P1, P5)

A number of elements suggest a role of chemerin and ChemR23 in inflammatory diseases of the lung. These include the expression of ChemR23 on macrophages, the bronchiolar expression of chemerin, the known function of plasmacytoid dendritic cells in the physiopathology of asthma and viral pneumonia, and the implication of polynuclear neutrophils in the physiopathology of many lung diseases and the processing of prochemerin into active chemerin. We therefore investigated the potential function of chemerin and ChemR23 in the physiopathology of inflammatory lung diseases. Using wild type (WT) and ChemR23 knock out ($ChemR23^{-/-}$), we studied models of asthma, lung fibrosis, acute lung injury, and viral pneumonia.

The asthma model is based on an intraperitoneal sensitization to ovalbumin followed by repeated airway exposure to ovalbumin delivered by an ultrasonic nebulizer. The evaluation parameters of the Th2 immune response associated with this model were serum IgE, bonchiolar and peribronchiolar eosinophilic infiltration, IL-5 and total eosinophil count in the broncho-alveolar lavage. No significant difference was observed between WT and $ChemR23^{-/-}$ mice with the exception of a higher basal level of IgE in the serum of untreated $ChemR23^{-/-}$ mice as compared with WT mice. The lung fibrosis models involved the trans-oral instillation of bleomycin or silica dust. After respectively 1 and 2 months, mice were sacrificed and lung histological analysis was performed using haematoxylin-eosin and Masson’s trichrome staining. Differential cell count, LDH and protein levels were measured in the broncho-alveolar lavage, and a marker of fibrosis, hydroxyproline, was determined in lung homogenates. No significant difference was observed for all these parameters.

2.5.2.3.3. Anti-inflammatory function of chemerin in a mouse acute lung injury model (P1)

To investigate the role of the chemerin/ChemR23 system in innate immune response, a model of acute lung injury induced by the lipopolysaccharide (LPS) was used. After a trans-oral instillation into the trachea of 1 µg LPS and/or 5 µg recombinant mouse chemerin, the animals were evaluated over a time course of 72 hours. At selected time points, mice were sacrificed and a broncho-alveolar lavage was performed. LPS induced a strong inflammatory reaction in the lung, with an influx of neutrophils peaking between 12h and 24h. Chemerin had an inhibitory effect on the inflammatory response from 12h after LPS administration, and decreased neutrophil recruitment by up to 70%. The anti-inflammatory effect of chemerin persisted at 24h (53% inhibition) and 72h (63% inhibition), during the resolution phase, during which the neutrophil infiltration decreased progressively in all groups. As the neutrophil number in BAL fluids peaked between 12 and 24h, we investigated the role of chemerin in WT and $ChemR23^{-/-}$ mice 18 hours after the LPS challenge, in order to determine whether the anti-inflammatory effect of chemerin is indeed mediated through ChemR23. LPS induced a stronger inflammatory response in $ChemR23^{-/-}$ mice, as compared to WT mice, with an increase of neutrophils (50%) and macrophages (94%) in the BAL fluid. We also measured in BAL fluids a set of inflammatory mediators (KC/CXCL1, IL-6, TNF-α and IL-1β), as hallmarks of the lung inflammatory response, in WT and KO mice. All four cytokines were strongly decreased in the group of WT mice receiving chemerin in addition to LPS (78% for KC/CXCL1, IL-6 and IL-1β, 73% for TNF-α). In $ChemR23^{-/-}$ mice, chemerin treatment had no effect on inflammatory cytokines and chemokines in BAL. Altogether, these data clearly demonstrate that chemerin can exert an anti-inflammatory activity in this model, in a strictly ChemR23-dependent manner (Luangsay *et al*. 2009).

2.5.2.3.4. Role of the chemerin/ChemR23 system in a viral pneumonia model due to respiratory syncitial virus applied to ChemR23 knockout mice (P1, P5)

An anti-inflammatory property of chemerin is also supported by results from the model of acute viral pneumonia. This model is induced by an intra-nasal inoculation of the pneumonia virus of the mouse (PVM), the mouse counterpart of the human respiratory syncytial virus (RSV). RSV is known to infect ~85% of children by age 18 mo, and virtually all children by age 3 y. 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 phylogenic relative of human RSV virus, pneumonia virus of mice (PVM). The model consists in the intranasal inoculation of 1000 PFUs of the J3666 PVM strain under light anesthaesia.

$ChemR23^{-/-}$ mice displayed a far more severe viral disease, with higher mortality/morbidity, exacerbated viral replication, delayed viral clearance, and altered inflammatory response with blunted type I IFNs production and enhanced neutrophilic infiltration. As plasmacytoid dendritic cells (pDCs), a dendritic cell subtype expressing ChemR23, play a central function in anti-viral immunity by secreting high amounts of type I interferon, we demonstrated by flow cytometry analysis of single cell suspension from digested lung and broncho-alveolar lavage fluids a lower pDC recruitment in $ChemR23^{-/-}$ mice compared to WT infected mice, confirming *in vivo* the chemoattractant function of the chemerin/ChemR23 axis on this dendritic cell subtype. *In vitro*, we showed that IFN-α synthesis of stimulated pDCs from WT and $ChemR23^{-/-}$ mice by CPG, a TLR9 agonist, did not differ. This latter observation suggests that the lower type I IFN levels measured *in vivo* in $ChemR23^{-/-}$ infected mice could be linked to the lower pDC recruitment.

Myeloid dendritic cells (mDCs), macrophages, and neutrophils also increased during the viral challenge but, in contrast to pDCs, higher values were found in $ChemR23^{-/-}$ than in WT mice, both as the percentage of total cell and as absolute numbers. No

differences were observed between ChemR23$^{-/-}$ and WT mice for NK cells (NK1-1^{+} CD3^{-}), T cells (both CD3^{+} CD4^{+} and CD3^{+} CD8^{+}) and B cells (CD19^{+} CD3^{-}). Finally, a delayed seroconversion was observed in ChemR23$^{-/-}$ compared to WT infected mice. In the context of a higher viral load in ChemR23$^{-/-}$ mice, the fact that recruitment of T and B cells is not increased, and the delayed seroconversion suggest a defect in the acquired immune response as a result of the lower synthesis of type I IFN. Therefore, depletion experiments of pDC using specific antibody and adoptive transferts of WT pDC to ChemR23$^{-/-}$ infected mice are actually ongoing to confirm the central role of the lower pDC recruitment in the differences observed in our model (Bondue et al., submitted).

In conclusion, data from two different models strongly suggest an anti-inflammatory role of ChemR23 and its ligand chemerin in inflammatory and infectious lung diseases. The underlying mechanisms involved in both the LPS-induced acute lung inflammation and viral pneumonia models are presently under investigation.

2.5.2.4. A viral pneumonia model due to respiratory syncytial virus applied to $P2Y_2R$ and $P2Y_6R$ knockout mice (P5, P1)

The aforedescribed viral model using pneumonia virus of mice as a surrogate of human respiratory syncytial virus-associated bronchiolitis-pneumonia was implemented in the $P2Y_2R$ GPCR knockout model. As judged from survival rate and body weight loss/gain, interruption of $P2Y_2R$-subordinated processes significantly diminish innate resistance of the host to pneumoviruses, which opens new avenues in respiratory physiopathology and therapeutics. An exhaustive comparative study of lung functional and morphological alterations, lung cell infiltrations, lung chemokine response and lung virus load in wild-type and $P2Y_2R$ knockout mice is currently underway. Afterwards, a step-by-step dissection of the global $P2Y_2R$ knockout-associated susceptibility pattern into elementary phenotypes will be started, as it was done 18 mo ago to identify the reasons underlying the susceptibility pattern obtained by ChemR23 inactivation. A similar study is being implemented to examine the effect of $P2Y_6R$ inactivation.

2.5.2.5. Viral ARDS models due to influenza A H1N1 and H5N1 applied to ChemR23 knockout mice (P5, P1)

According to the World Health Organization, influenza infects annually 5-15% of the population, causing 3-5 million cases of severe illness and up to 500,000 registered deaths. The persistence of H5N1 viruses in poultry populations over the past six years and their ability to cause fatal infections in humans, along with the recent H1N1 pandemic, have raised fears of a renewed catastrophic outbreak comparable to that of 1918, which caused 0.2% to as much as 8% mortality in various countries around the world and an estimated ~50 million deaths worldwide. Symptoms reported for the standard illness include fever, cough, headache, sore throat, and dehydration, with some reports of diarrhea, vomiting, and bleeding from the mouth or throat. Benign cases do not show all these symptoms. Severe cases include additional signs typical of either secondary bacterial pneumonia or acute respiratory distress syndrome (ARDS). Importantly, these two manifestations are the ones that kill in fatal influenza, whether seasonal or pandemic, due to the 1918 H1N1 strain or to recent H5N1 strains. The catastrophic lethality of the 1918 pandemic makes it paramount to understand the disease pathogenesis of both severe forms. As most secondary bacterial pneumonias can be controlled with antibiotics, prevention and treatment of flu-associated ARDS are the major medical challenges to be addressed in order to reduce the death rate. This requires more knowledge about the pathogenesis of ARDS. In this IAP program, we intend to examine the role (if any) of a series of GPCRs (ChemR23, P2Y2R, P2Y6R, GIR, etc.) in the pathogenesis of influenza A virus-associated ARDS.

Models of Flu-induced ARDS

As a first step, two experimental models of ARDS due to influenza A virus were developed. Two influenza A viruses of different subtypes, derived from different species and showing no pathogenicity towards mice, were forced to evolve by serial passaging in mouse lungs. The two adapted viruses obtained show practically identical virulence levels, their MLD50 values being very similar. On the basis of this index, they appear to be more virulent than most other viruses used to date in murine models. Their virulence is of the same order of magnitude as those of the A/Vietnam/1203/2004 [H5N1] and A/Vietnam/1204/2004 [H5N1] viruses, whose respective MLD50s are 0.7 and 2.1 PFUs. In both cases inoculation of 10 MLD50 causes biphasic weight loss, culminating at agony with a loss of ~10% (H5N1) or ~25% (H1N1) body weight. Viral amplification is maximal for both viruses on day 4 pi, roughly corresponding to the typical inoculation-to-peak lag of natural murine respiratory viruses. On the other hand, the two viruses adapted in the lungs show replication kinetics differing significantly from what is observed with natural viruses, with a quasi-plateau from day 2 to day 5/6 pi instead of the classical Gaussian profile. Interestingly, this peculiar amplification kinetics profile has been described previously for mice infected with mouse-adapted forms of the A/Puerto Rico/8/34 [H1N1] virus, the A/South Carolina/1/18 [H1N1] virus, and several human H5N1 strains showing high or low pathogenicity. This suggests that this profile is typical of influenza virus amplification by the murine respiratory system. A final common feature of the two viruses is the diffuse alveolar damage dominating both histopathological profiles, which corroborates the pathological data available in the literature. Seasonal human influenza epidemics typically consist of a transient tracheo-bronchitis due to preferential attachment of the virus to the laryngeal, tracheal, and bronchial epithelia. In contrast, those influenza viruses which are highly pathogenic towards man, from the pandemic viruses of 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) to the H5N1 strains isolated from humans since 2003, additionally colonize the bronchiolar and alveolar epithelia, preferentially or not, causing diffuse alveolar damage as an additional primary lesion. The same lesion has been found in experimental animals injected with a recent H5N1 strain. Although both viruses share the same pathogenicity, replication kinetics, and concentration peak and although they both evoke diffuse alveolar damage by the endpoint day, they differ dramatically as regards the ARDS course and pathological signature. Flagrant differences make it easy to distinguish H5N1 infection from H5N1-associated disease. For the H1N1 virus, the disease becomes fatal at a point where the pulmonary edema is much less intense and leaves a

histopathological picture characterized by much more dense inflammatory cell infiltrates, generating cuffs around the bronchioles and blood vessels. Secondly, the H1N1 virus colonizes the epithelia of both the upper and lower airways, without any obvious preference, whereas H5N1 remains confined essentially to the alveoli and terminal bronchioles. Within the alveoli the H5N1 strain shows, unlike the H1N1 strain, a preferential tropism for type II pneumocytes and alveolar macrophages. Lastly, whereas H1N1 remains strictly confined to the respiratory system, the H5N1 virus spreads to other organs. These differences demonstrate unambiguously that the two highly virulent influenza A viruses studied here cause two different forms of ARDS. This suggests that the physiopathological data obtained when studying one virulent strain should not be extrapolated automatically to other strains. The observed differences also suggest that diverse constellations of critical mutations in the viral genome might lead to the same result in terms of mortality. Although this work is the first, to our knowledge, to address the question of possible differences between two fatal diseases caused by influenza A viruses, some evidence pointing in the same direction as the present results has already been reported. For example, the pandemic human strains of 1918, 1957, and 1968 on the one hand, and the recent H5N1 strains on the other, show different tropisms: panepithelial for the former strains and limited to the bronchiolar and alveolar epithelia for the latter strains, a result compatible with our own observations on mouse-adapted viruses. Likewise, panepithelial tropism has been observed for the A/South Carolina/1/18 [H1N1] virus in mice, whereas a preference for the bronchioles and alveoli has been noted for recent H5N1 strains injected into macaques, mice, ferrets, and cats. On the other hand, the observed strict confinement of our H1N1 strain within the respiratory system confirms previously reported data refuting the existence of polysystemic dissemination of non-H5 viruses that are lethal to humans or laboratory animals. Conversely, our observation that the H5N1 strain spreads beyond the respiratory system confirms similar observations on both humans and laboratory animals.

Although it is not certain that other H5N1 and H1N1 viruses infecting other susceptible hosts would show trends similar to those observed here, the results gathered here, when integrated with the diverse pieces of evidence reported elsewhere, suggest that fatal infections caused by different highly virulent influenza A viruses do not necessarily share the same pathogenesis. To be convinced, one has only to see how easy it is to distinguish, in the absence of any virus labeling, the histopathological sections typical of the two strains used here. These different histopathological signatures and different pathogeneses probably reflect the presence of specific sets of virulence markers that will have to be decrypted in order to anticipate the emergence of a pandemic. In this respect, sequence analysis of both strains will lead to insight on specific residues that are relevant for the adaptation and virulence of an influenza strain in a new host.

Furthermore, the differences between these two strains suggest that there might be more than a single, universal cytokine storm underlying fatal influenzal diseases and that it might be advantageous to tailor the therapeutic approach to the influenza virus pathotype.

Effect of ChemR23 inactivation on Flu-induced ARDS

When the aforedescribed fatal ARDS due to H1N1 influenza A virus was implemented in wild-type and ChemR23 knockout mice, significant differences were detected in survival rate, length of survival and body weight evolution. It is deduced that chemerin/ChemR23-subordinated processes play a significant role in the pathophysiology of ARDS due to primoinfections with hypervirulent influenza A virus strains. An exhaustive comparative study of lung functional and morphological alterations, lung cell infiltrations, lung chemokine response and lung virus load in wild-type and ChemR23 knockout mice is currently underway. Afterwards, a step-by-step dissection of the global ChemR23 knockout-associated susceptibility pattern into elementary phenotypes will be achieved to identify the underlying mechanism/s involved.

2.5.2.6. A viral ARDS model due to influenza A H1N1 applied to $P2Y_6$ and $P2Y_2$ knockout mice (P5, P1)

The aforedescribed viral model using influenza A H1N1 virus of mice as a surrogate of human influenza A virus-associated ARDS was implemented in the $P2Y_6R$ GPCR knockout model. As judged from survival rate and body weight loss/gain, interruption of $P2Y_6R$-subordinated processes do NOT alter innate resistance of the host to influenza A subtype H1N1-induced ARDS. As we have shown that H5N1 and H1N1-associated ARDS do not share similar pathogeneses (Garigliany et al., 2010), a similar study is being implemented with murinized H5N1. Again, a similar study is scheduled to examine the effect of $P2Y_2R$ inactivation on H5N1- and H1N1-induced ARDS.

2.5.2.7. A viral pneumonia model due to gammaherpesvirus applied to ChemR23, $P2Y_2$ and $P2Y_6$ knockout mice (P5, P1)

Human gammaherpesviruses are clinically important lymphotropic pathogens represented by Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (alternatively, human herpesvirus 8 [HHV-8]). Due to their narrow host range, developing animal model systems of infection is problematic, and consequently, it is difficult to accurately investigate pathogeneses associated with these viruses. While attempts have been made to investigate the pathogeneses of EBV and KSHV by infecting nonhuman primates, these hosts do not accurately recapitulate human disease, and in some cases, infection results in a lack of detectable virus-specific antibodies or viral-gene transcription. Alternatively, simian homologues of these human viruses do exist (rhesus lymphocryptovirus [RhLCV], similar to EBV; retroperitoneal fibromatosis herpesvirus [RFHV] and rhesus rhadinovirus [RRV], similar to KSHV), and it has been established that infection of rhesus macaques with these viruses mimics human disease more effectively. However, for numerous reasons, primate studies are not always feasible. Nonetheless, such studies have highlighted the importance of infection in the context of a natural host for accurate modeling of infection and pathogenesis associated with the gammaherpesviruses. Murine gammaherpesvirus isolate 68 - officially murid herpesvirus type 4 (MuHV-4) - naturally infects murid rodents, offering a potential alternative model of human gammaherpesvirus infection through

experimental infection of inbred laboratory strains of mice with MHV-68. In this IAP program, we intend to examine the role (if any) of selected GPCRs (ChemR23, $P2Y_2R$, $P2Y_6R$, GIR, etc.) in the pathogenesis of gammaherpesviruses.

To do this, we first established the MHV-68 mouse model in P5's laboratory. Characteristically, infection of our laboratory mice with MHV-68 via the intranasal route caused intense virus replication in nose and lung, and discrete replication in superficial cervical lymph nodes and spleen (measured by virus titration). Concomitant with this lytic infection, the virus established latent infection within lung, superficial cervical lymph nodes and spleen (from infectious center assays). We are currently developing an ex vivo assay consisting in the infection of isolated primary type 2 pneumocytes and follow-up of viral replication by flow cytometry. The model is ready for starting comparative infections of wild-type and GPCR knockdown mice, with the aim to establish whether selected GPCRs are involved or not in the pathogenesis of gammaherpesviroses.

2.5.2.8. Prolactin-releasing peptide receptor in liver inflammatory models (P1, P5)

Prolactin-releasing peptide (PrRP) is the ligand of the receptor GPR10. As its names indicates, PrRP was described originally as a regulator of prolactin release. However, it has since been involved in a growing number of physiological processes, among which the control of feeding behavior, pain and neuroendocrine function. We have generated a knock-out model for GPR10 and these mice were tested across a wide range of behavioral and physiological assays.

We have demonstrated an important role of the PrRP-GPR10 system in the modulation of the various actions of opiates (Laurent et al. 2005). We have also established a central role of GPR10 in the control of stress reactions, with a lower activity of the hypothalamus-pituitary-adrenal axis in knock-out mice in basal conditions and following a number of stressfull conditions. This low corticostreroid tone was associated with a spontaneous chronic inflammatory disease of the liver, leading to steatosis and fibrosis. This correlated with a progressive increase of TNF-α and plasmatic markers of liver damage, ALT (alanine aminotransaminase) and AST (aspartate aminotransaminase). Moreover, GPR10 KO mice displayed a higher sensitivity to an acute inflammatory challenge (LPS/GalN) of the liver. As our animals were not kept in a SPF environment, we could not determine the exact origin of this chronic disease, and its relation to the central control of the hypothalamus-pituitary-adrenal axis. We have introduced the GPR10 knockout line in a SPF environment, and have bred them for a few generations on CD1 and C57 Black/J backgrounds. We have now started experiments that will allow determining whether the inflammatory syndrome is spontaneous or induced by a specific pathogen. From the first data available, the inflammatory phenotype in an SPF environment is much milder, either spontaneously, or in response to LPS/GalN challenge. We will confirm these data, and will further expose these mice to other models of hepatitis (concanavalin A, CCl4, Murine Hepatitis Virus A59, and other pathogens recorded in our non-SPF facility). We will also test whether the inflammatory phenotype is restricted to the liver or applicable to other organs as well.

2.5.2.9. 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).

2.5.2.10. QTL analysis in animal models (P1, 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 ± 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 were thus

investigated to try to explain the part of the phenotypic variance not explained by the major effects related to mice respiratory physiology. Unfortunately, this kind of analysis did not yield better results. Therefore, it was decided to dissect the global susceptibility/resistance phenotypes of interest into their elementary phenotypes (cell permissivities, cytokine levels, chemokine levels, pathogen phagocytosis, etc.) with the aim to identify the elementary phenotype contributing most importantly to the global resistance/susceptibility phenotype. In a further step, a panel of F2s will be produced again, individual crucial elementary phenotypes will be collected and these latter will be enrolled in the QTL analysis.

Dissection of global resistance/susceptibility phenotypes were started with the pair of inbred mice 129/Sv (susceptible) and SJL/J (resistant) for respiratory syncytial virus pneumonia (Anh et al., 2006) and with the pair DBA/2 (susceptible) and C57BL/6 (resistant) for H1N1 influenza virus A-associated ARDS. For respiratory syncytial virus-induced pneumonia, we first showed that the SJL/J's pattern of innate resistance was radiosusceptible-cells-independent (Glineur et al., submitted for publication). Next, we showed that adoptive transfer of SJL/J's alveolar macrophages (AM) by susceptible mouse strains resulted in dramatically increased resistance to the virus, which suggests that AMs play the key role (Dermine et al., manuscript in preparation). Ex vivo AM infection assays are currently developed to examine the possibility that a simple high-throughput test could be validated to screen large cohorts of F2s. Finally, the chemokine cocktail produced by MAs from susceptible and resistant strains upon contact with the virus are being compared to examine whether corresponding GPCRs could be involved.

Dissection of global resistance/susceptibility phenotypes were continued with the pair of inbred mice DBA/2 (susceptible) and C57BL/6 (resistant) for H1N1 influenza A virus-induced ARDS (Garigliany et al., 2010). We first showed that the C57BL/6's pattern of innate resistance was radiosusceptible-cells-independent. Next, we showed that myéloperoxidase, viral replication rate, IFNγ, IL-6, MIP1α (CCL3), MCP-1 (CCL2), TNFα and IL-1β responses were totally different between strains. Ex vivo infections of primary type-2 pneumocytes are currently in progress to examine the possibility that epithelial and/or AM permissivities to the virus might be different. Adoptive transfer experiments are scheduled after.

2.5.3. Main achievements in relation to the initial objectives

A mutation of the FSH receptor was identified as a couse of spontaneous ovarian hyperstimulation syndrome

A gain of function mutation of the arginine-vasopressin receptor type 2 has been identified in adult patients of a large kindred, demonstrating that this cause of nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is not restricte to infants.

Demonstration that HIV Tax protein interferes with cell signaling at the level of G proteins.

Demonstration that chemokines delivered by parvovirus into tumoral cells may reduce tumorigenicity

Demonstration that a variant of CXCL4 is angiostatic and anti-tumoral

Identification of an anti-inflammatoryu role for chemerin/chemR23 axis models of inflammatory diseases in mice, and as a key player in the pathogenesis of respiratory RSV bronchiolitis/pneumonia and H1N1 influenza A-associated ARDS

Development and validation of H1N1 and H5N1 models of influenza A-associated ARDS, and gammaherpesvirus lytic and latent infection

Identification of a role for P2Y2R in RSV bronchiolitis/pneumonia

Identification of the alveolar macrophage as a key player for innate resistance to RSV

2.5.4. Comments (in case of deviations from the initial project workprogramme)

None

2.6. Workpackage 6. Identification of novel receptors and their ligands

Contributing partners: P1, P2, P3, P4

2.6.1. Summary description of the objectives

Many orphan receptors for which the ligands and function are still unknown are encoded by the mammalian, insect and yeast genomes. Several partners will focus on the characterization of these receptors, through the identification of their ligand, and the subsequent delineation of their function. We will focus on human receptors for new 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.

2.6.2. Summary description of scientific activities and results

2.6.2.1. Functional screening assays for mammalian leukocyte and CNS receptors (P1, P4)

With the aim of identifying the natural ligands of orphan receptors, we have established cell lines coexpressing $G_{\alpha16}$, 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. 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_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. 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.

In order to have screening assays which do not depend on G protein signalling, we have implemented the TANGO (Invitrogen) and PathHunter (DiscoverX) assays. Both systems are based on the recruitment of beta arrestin. In the TANGO assay, the TEV protease hooked to β-arrestin cleaves a target site engineered in the C-terminus of the receptor, releasing a Gal4-VP16 transcription factor, which activates transcription of a beta lactamase reporter gene. In the PathHunter system, β-arrestin recruitment promotes the complementation of the two β-gal fragments, resulting in the formation of a functional enzyme. These systems have the theoretical advantage of being insensitive to the activation of endogenous receptors of the cell lines.

A set of receptors were selected for their structural similarity with chemoattractant receptors and/or their expression in leucocyte populations. Q-PCR microfluidic cards containing all orphan GPCRs were designed in collaboration with the spin-off company Euroscreen, and all major leukocyte populations were tested. Other orphan receptors were selected on the basis of their expression in the central nervous system, or in stem cell populations. All these receptors were expressed in cell lines co-expressing apoaequorin and $G_{\alpha 16}$ or G_{qi5}, and some of them in other expression systems as described above.

A collection of fractions from animal tissues (essentially pig and rat) 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 in order to detect potential biological activities. Various procedures are routinely used for preparing peptidic extracts, lipidic extracts and extracts of bioamines and other small molecules. These extracts were then fractionated on preparative HPLC columns (usually C18). For receptors expected to respond to inflammatory mediators, extracts were also 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.

2.6.2.2. Pharmacological activation of GIR by poly-unsaturated fatty acids and NPY peptides.

Peptides of the NPY family have been described to bind and activate GIR expressed in COS-7 cells (NPY(3-36) > PYY (3-36) > NPY > Leu,Pro-NPY), suggesting that GIR is a novel NPY receptor with a Y2-like pharmacology. Independently, in collaboration with Euroscreen, we have shown that several poly-unsaturated fatty acids (PUFA) act on GIR, following their isolation from pig brain. Both ligand families were tested further, following the expression of human GIR (hGIR) in CHO-K1 cells expressing $G_{\alpha 16}$ and apoaequorin. hGIR-overexpressing cells were specifically activated by docosahexanoic acid (DHA), 9-cis retinoic acid (9-cRA), and arachidonic acid but not NPY or PYY(3-36). A cell line expressing the Y2 receptor was activated by NPY and PYY(3-36) but not by DHA or 9-cRA, while control cell lines expressing other receptor did not respond to these ligands. We next assessed the effect of combinations of PUFAs and Y2 agonists on hGIR-overexpressing cells. DHA in combination with PYY(3-36) or NPY induced a stronger activation. In the presence of DHA, the EC_{50} for PYY(3-36) decreased to the nanomolar range and the E_{max} was increased.

We also monitored the internalisation of GIR following activation in COS-7 cells transiently expressing hGIR or a fusion of hGIR tagged with GFP at its C-terminus. Internalisation was monitored either by fluorescent confocal microscopy or FACS. DHA at 10 μM promoted internalisation of hGIR (62% at 45 min), which clustered in a peri-nuclear endosomal compartment. A control receptor similarly tagged with GFP did not internalise in the same conditions. NPY or PYY(3-36) alone did not promote internalisation of hGIR, but combinations of DHA and PYY(3-36) promoted faster internalisation and at lower concentrations than DHA alone. These results suggest that Y2 agonists can activate hGIR, but only in the presence of DHA.

We tested by in situ binding assay whether GIR could bind NPY peptides in mouse brain. Using ^{125}I-PYY(3-36) as a tracer and Leu,Pro-NPY as a Y1 competitor, we could determine Y2-like binding sites in specific brain regions. We observed a significant reduction of ^{125}I-PYY(3-36) binding sites in the hippocampus and amygdala of GIR knock-out mice as compared to wild type animals. Binding in striatum was very low and we were uable to observe differences in this region. Similarly, no difference was seen in septum, where GIR is not expressed.

We also tested whether part of the physiological effects of PYY could be mediated by GIR. Following i.p. injection, PYY(3-36) decreased locomotory activity of wild type mice, but not of GIR knock-out mice, suggesting that GIR is required for the locomotory effects of PYY(3-36) in vivo. Finally we quantified NPY expression in striatum and hypothalamus of wild type and knock-out mice. We observed a significant increase in NPY expression in the striatum (but not hypothalamus) of knock-out as compared to wild type mice.

Altogether, we have shown that GIR is expressed by cholinergic interneurons of dorsal striatum, and that inactivation of this receptor is associated with cholinergic hypoactivity in the striatum (Laurent et al. In preparation). This leads to an alteration of coordinated movement and increased locomotory activity in stressful conditions. We also showed that GIR is activated in a cooperative manner by NPY peptides and polyunsaturated fatty acids, and that the receptor is involved in some of the activities of NPY peptides in vivo.

2.6.2.3. Identification of the natural ligand for an orphan receptor.

Starting from a rat colon extract, a first step of fractionation on a strong anion exchange column in Tris/HCl buffer, using a NaCl concentration gradient, resulted in a potential activity peak for one of the orphan receptors tested. The positive fractions were pooled and used to perform a second dimension on a polycationic exchange column in ammonium acetate buffer. In this second step, we confirmed the presence of a specific activity for the receptor eluted between 150 and 230 mM NaCl. The bioactivity has been further purified through several HPLC steps, and a potential candidate has been identified by mass spectrometry. Overexpression in CHO or HEK cell lines of the candidate ligand led to the production of an inactive polypeptide towards the orphan receptor. Size-exclusion chromatographic experiments suggested that the ligand might be bioactive as an oligomer. Moreover, immunodetection experiments on Western blots indicated that the active ligand could present a specific pattern of glycosylation and/or proteolytic cleavage. We are now analysing the potential proteolytic cleavage of the ligand in the presence of pure proteases and colon extracts. We are also optimizing a deglycosylation procedure for mass spectrometry analysis and bioactivity measurement without any gel electrophoresis step to investigate the requirement of glycosylation for bioactivity.

2.6.2.4. Chemokine receptors (P4 and P1)

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 GTPγS 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).

CXCL12 has been discovered rather as a cytokine that promotes pre-B cell growth, before its chemotactic effect was elucidated. Importantly, mutant mice with a targeted deletion of the CXCL12 gene die perinatally, because of marked defects in cardiac septal formation and vascularization of the gastrointestinal tract. Furthermore, it was found that T-tropic (X4) HIV infection requires binding to a coreceptor, i.e., CXCR4 which is the major functional receptor for CXCL12. Recently, however, the orphan receptor RDC1, now renamed CXCR7, has been identified as a second receptor for CXCL12, breaking up the monogamous relationship between CXCL12 and CXCR4. Modification of the CXC chemokine CXCL12 by PAD that converts arginine residues into citrulline (Cit), reduces the number of positive charges. The three NH2-terminal arginines of CXCL12, Arg8, Arg12 and Arg20, were citrullinated upon incubation with PAD. The physiologic relevance of citrullination was demonstrated by showing co-expression of CXCL12 and PAD in Crohn's disease. Three CXCL12 isoforms were synthesized for biologic characterization: CXCL12-1Cit, CXCL12-3Cit, and CXCL12-5Cit, in which Arg8, Arg8/Arg12/Arg20, or all five arginines were citrullinated, respectively. Replacement of only Arg8 caused already impaired (30-fold reduction) CXCR4 binding and signaling (calcium mobilization, phopshorylation of ERK and protein kinase B) properties. Interaction with CXCR4 was completely abolished for CXCL12-3Cit and CXCL12-5Cit. However, the CXCR7-binding capacities of CXCL12-1Cit and CXCL12-3Cit were, respectively, intact and reduced, whereas CXCL12-5Cit failed to bind CXCR7. In chemotaxis assays with lymphocytes and monocytes, CXCL12-3Cit and CXCL12-5Cit were completely devoid of activity, whereas CXCL12-1Cit, albeit at higher concentrations than CXCL12-3Cit and CXCL12-5Cit (maximal dose 200 nM) could not inhibit infection of lymphocytic MT-4 cells with the HIV-1 strains NL4.3 and HE. In conclusion, modification of CXCL12 by one Cit severely impaired the CXCR4-mediated, but not CXCR7-mediated biologic effects of this chemokine and maximally citrullinated CXCL12 was inactive (Struyf et al., 2008).

2.6.2.5. Studies on physiopathological deorphanization of regakine-1 (P4-P5)

Regakine-1 is a recently discovered CC chemokine (Partner-4) that is constitutively present at high concentrations in bovine plasma and to which neutrophil chemotactic activity has been ascribed (Struyf et al., 2001). Although the natural 7.5-kDa protein of 70 residues contains the four conserved cysteines, it has less than 50% sequence identity with any known human or animal chemokine. No specific receptors for regakine-1 have been identified and no high affinity for the classical receptors on neutrophils (i.e., CXCR1 and CXCR2) was detected for regakine-1. However, a particular characteristic of the plasma-derived regakine-1 resides in its potential to synergize with IL-8, a ligand for both CXCR1 and CXCR2, to chemoattract neutrophils (Struyf et al., 2001). CXCR2 is also functionally expressed on nonhematopoietic cells and accounts for the angiogenic activity of IL-8 and

related CXCR2 agonists. Regakine-1, like other plasma chemokines is expected to play a unique role in the circulation during normal or pathologic conditions. In this IAP project, serum regakine-1 levels were measured in a cohort of calves with RSV-associated ARDS (acute sera) and in recovered survivors (convalescent sera). Regakine-1 levels were qualtitatively similar in acute and convalescent sera, suggesting that its expression/secretion is not altered by RSV infection. A prospective study is now intended, in which the inflammatory response of calves to standardized agonists will be compared before and after inhibition of regakine-1. This could be achieved either by administering a specific antiserum or via development of a specific autovaccine.

2.6.2.6. Glucose-sensing receptors (P3, EU1) and transceptors (P3)

We have identified a known hormone receptor as 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 mimick 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, strongly expressed on the apical side of intestinal epithelial cells, 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 colocalizes 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.

We have characterized the response generated by various sugars and identified several inhibitors with low-bioavailability that could be used to inhibit the receptor in the gut without interference with the same receptor in other cell types. For this work, we collaborate with Walter Luyten, (KUL, connected with partner 2). Using radioligand binding techniques, we showed that the sugar ligands bind to another site on the receptor than the classical hormone-binding site. Computer based modelling was performed in collaboration with Leonardo Pardo (EU1). In addition we will determine the effect of in vivo blocking the receptor (feeding experiments) on weight gain in rats fed with a sugar-rich diet. For this purpose, we will use the inhibitory compounds with low-bioavailability, that theoretically should only act in the small intestine.

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 prroject 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. 2009)

Earlier it was found that the Pho84 phosphate transporter plays an essential role in stimulation of PKA activity in phosphate-starved cells upon addition of phosphate. Similarly as for Gap1, we screened a library of phosphate-containing compounds for the agonist effect on the PKA-controlled response through Pho84. We identified several nontransported phosphate-containing compounds able to activate the signaling function of the Pho84 transceptor. We identified phosphonoacetic acid (PAA) as a competitive inhibitor of phosphate transport and showed that it is unable to activate the signaling function of Pho84. In contrast, glycerol-3-phosphate (Gly3P) acted as a non-transported agonist of the signaling function of Pho84. This indicates that, as in the case of Gap1, induction of a specific conformational change is required to trigger Gap1 signaling but the complete transport cycle is not required. We also identified amino acid residues exposed with their side chain into the amino acid binding site of Pho84 and made use of this to demonstrate that, like Gap1, the Pho84 transceptor uses the same substrate-binding site for transport and signaling (Popova et al. 2010).

Sulfate starvation of fermenting yeast cells also causes downregulation of PKA activity and re-addition of sulfate triggers rapid activation of PKA targets. Recently, we have obtained the first evidence for involvement of the sulfate transceptors Sul1 and Sul2 in this process using nontransported sulfate-containing compounds. Apparently these transceptors will be the first sulfate sensors discovered in cell biology.

2.6.2.7. Studies on insect orphan GPCRs (P2)

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

The main objective of this research section is the molecular and functional characterization of orphan GPCRs derived from various insect genome and EST databases. We hypothesize that a number of fruit fly GPCRs with an as yet unknown function and/or ligand are essential regulators of (not yet defined) developmental and/or physiological processes. In addition, analysis of these GPCRs might yield information concerning the action and evolution of the corresponding orthologs in other metazoans (including vertebrates). We have at our disposal several expression vectors for orphan fruit fly GPCRs that show sequence similarity to known peptide, amine and prostaglandin receptors from mammals. The initial goal is the search for the natural ligands of these orphan GPCRs by means of cellular expression and reporter systems. For this, the GPCR cDNA is co-expressed with apo-aequorin (a Ca^{2+}-sensitive photoprotein) and possibly $G_{\alpha}16$ (a G-protein that couples most GPCRs to the Ca^{2+} signalling pathway). This allows a relatively easy and fast detection of receptor mediated Ca^{2+}-increases, an approach that has already proven to be successful for the deorphanization of several GPCRs of invertebrate origin (*i.e.* mainly of insects and of nematode worms) in the lab (P2). We own an extensive amine and *Drosophila* peptide library (covering *ca.* 80% of all known *Drosophila* neuropeptides) that we use in initial screening assays. This approach has recently led to the identification in the fruit fly of receptors for DTK-6 (*Drosophila* tachykinin-related peptide 6) and MIPs (myoinhibiting peptides; see also below). 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) (see also 4.6).

Male fruit flies change the behaviour of their female partner by co-transferring accessory gland proteins (Acps), such as the sex peptide (SP), with their sperm. The *Drosophila* sex peptide receptor (SPR) is a G protein-coupled receptor that is activated by the male-derived SP in a group of sensory neurons in the female's genital tract, which results in the initiation of post-mating effects in the female, including the behavioural rejection of males for mating and an increased egg laying rate. While most Acps show a fast rate of evolution, SPRs are highly conserved in insects. We have discovered that SPRs from different insect species are activated by evolutionary conserved myoinhibiting peptides (MIPs). Structural determinants in SP and MIPs responsible for this dual receptor activation have been characterized by analyzing different synthetic peptide analogs. In addition to adult females, *Drosophila* SPR is also expressed in embryonic and larval stages, as well as in the adult male nervous system, whereas SP expression is restricted to the male reproductive system (accessory glands). MIP transcripts occur in both male and female central nervous systems. Therefore, MIPs are probably acting as endogenous ligands of this promiscuous *Drosophila* SPR. Moreover, since SP results from a rapid evolutionary process and is not well conserved among insect species, MIPs most likely represent the more ancestral neuropeptide ligands of the insect "SPRs". During evolution, this receptor has probably been hijacked by the SP to serve an additional function (Poels/Van Loy *et al*., 2010).

2.6.2.7.2. 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, it is constantly monitored by the FAO (http://www.fao.org/ag/locusts/). At the basis 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 (phase transition) that results in the *gregarious phase*, a behaviourally, physiologically and anatomically 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 have generated an *Expressed Sequence Tags* (EST) database representing a large number of transcripts expressed in the central nervous system of the desert locust (http://titan.biotec.uiuc.edu/locust/). These sequences were annotated by bioinformatic analyses and were employed to generate microarrays for transcriptome-wide experimental studies on this heterometabolous species. *Contig* analysis (of ca 35,000 sequences) revealed that the EST database currently represents *ca.* 12,700 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. Microarray analyses of solitarious and gregarious animals revealed a large set of phase-dependently expressed genes. Additional experiments are performed to verify which transcripts are regulated upon crowding in an initial stage of phase transition.

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 qRT-PCR. This already led to the discovery of a putative octopamine receptor that is strongly upregulated in the central nervous system of desert locusts within a few hours of crowding (Verlinden *et al*., 2010). Following the recent discovery that serotonin plays a very important role in the gregarization process of solitarious locusts (Anstey *et al*., 2009), we are also gathering more information about the different serotonin receptors in the locust and try to unravel the 5HT-induced signal transduction cascade that generates the aggregation behaviour of these animals. Therefore, we are currently analyzing different 5HT receptors as well as some of the putative downstream factors, such as the catalytic and regulatory subunits of protein kinase A (PKA). Interestingly, an RNAi-based knock-down of transcripts coding for a putative 5HT1 receptor, which is believed to couple negatively to adenylate cyclase, and for the regulatory subunit of PKA indeed causes very significant effects on the behavioural gregarization rate of solitarious locusts induced by crowding. In addition to the initial behavioural effects, we are also studying phase-dependent effects (situated more downstream in the phase transition process) on growth and reproduction of these animals. In this context, different neuropeptides, such as the neuroparsins, the insulin-related peptide, neuropeptide F and allatotropin, were shown to be involved in these important aspects of locust physiology, such as the

control of vitellogenesis, reproductive organ growth and lipophillic hormone (ecdysteroids and juvenile hormone) synthesis. The putative receptors for some of these regulatory neuropeptides are currently also under investigation.

2.6.2.8. Orphan LGRs (P1 and P2)

Leucine-rich repeats containing G protein-coupled receptors (LGRs) constitute a unique group of seven-transmembrane receptors sharing a large extracellular domain with multiple leucine-rich motifs for hormone binding. In the recent years, novel insights into the molecular evolution of LGR-genes accumulated rapidly due to comparative genome analyses indicating that the LGR signalling system most probably emerged before the radiation of metazoan phyla and expanded throughout evolution (Van Loy *et al*., 2008). Within the framework of this interuniversity network, we (P1-P2 in collaboration) previously identified the first invertebrate LGR-ligand couple *i.e.* the bursicon receptor DLGR2 (Mendive/Van Loy *et al*., 2005). Bursicon bioactivity is essential for tanning of the exoskeleton and for wing spreading behaviour in freshly eclosed adult insects. Furthermore, we identified bursicon homologous sequences in several protostomian as well as deuterostomian invertebrates illustrating the remarkable conservation of bursicon in invertebrate species (Van Loy *et al*., 2007). Frustratingly, no convincing mammalian orthologs of the bursicon hormone have so far been identified, making the search for natural ligands of the LGRs 4-5-6 (the mammalian orthologous receptors of DLGR2) not a trivial task. These studies are now complemented with functional and molecular genetic analyses of mutant and/or transgenic animal models of mammalian (P1) and/or insect (P2) origin (see also 3.4 and 4.6). We also generated expression plasmids for two other *Drosophila* LGRs (DLGRs 3-4). Although no fully convincing ligands have been characterized so far, we demonstrated that these receptors couple via the G_s-mediated cAMP-pathway, at least when expressed in HEK cells (human embryonic kidney cells). Based on this knowledge, we perform intracellular cAMP-level measurements in an attempt to identify ligands for these remaining orphan insect LGRs. The lab of P4 is currently trying to synthesize one of the most likely candidate ligands for DLGR3.

As described above, LGRs constitute a unique group of seven-transmembrane receptors sharing a large extracellular domain with multiple leucine-rich motifs for hormone binding. In the recent years, novel insights into the molecular evolution of LGR-genes accumulated rapidly due to comparative genome analyses indicating that the LGR signalling system most probably emerged before the radiation of metazoan phyla and expanded throughout evolution (Van Loy et al., 2008). Within the framework of this network we identified the first invertebrate LGR-ligand couple *i.e.* the bursicon receptor DLGR2 (Mendive/Van Loy et al., 2005). Bursicon bioactivity is essential for tanning of the exoskeleton and for wing spreading behavior in freshly eclosed adult insects. Furthermore, we identified bursicon homologous sequences in several protostomian as well as deuterostomian invertebrates illustrating the remarkable conservation of bursicon in invertebrate species (Van Loy et al., 2007). Frustratingly, no convincing mammalian orthologs of the bursicon hormone have so far been identified, making the search for natural ligands of LGRs 4-5-6 (the mammalian orthologous receptors of DLGR2) not a trivial task. We are pursuing the search for LGR4 & 5 natural agonist(s) by functional assays involving receptor-expressing cells with the aequorin reporter system and fractionation of various tissue extracts.

2.6.3. Main achievements in relation to the initial objectives

We have characterized an allosteric interaction between two classes of ligands (NPY peptides and polyunsaturated fatty acids) acting on the orphan receptor GIR (in progress, unpublished).

We have purified the natural ligand of an orphan receptor and identified it by mass spectrometry. Although there is little doubt regarding this characterization, the recombinant ligand is not active, and a post translational modification of the ligand is likely. We are presently characterizing the required modifications.

Description of a CXCR1-like receptor in rodents responding to GCP-2/CXCL6.

Identification of several new peptide receptors in invertebrates

We have obtained strong evidence that a known hormone-sensing GPCR plays a role as sugar-sensing GPCR on the apical side of intestinal epithelial cells in the gut. We have discovered Gap1 and Pho84 as transceptors for amino acid and phosphate activation of the PKA pathway in yeast and have made them into the best characterized nutrient transceptors in cell biology. (P3)

2.6.4. Comments (in case of deviations from the initial project workprogramme)

None

2.7. Workpackage 7. Olfactory receptors and evolution of GPCR families

Contributing partners: P1, P3, EU1

2.7.1. Summary description of the objectives

The partnership involves groups specialized in yeast, insect and mammalian receptors. This will bring an evolutionary dimension to the program, with parallel studies of receptor classes in different systems. We intend to interact with another IAP network dedicated to Bioinformatics in order to allow in depth studies of receptor gene families in the growing number of full genomes available in the databases. Correlation between structural and functional evolution of receptor and ligand gene families will be studied in this context. We will also reinitiate an avenue of research dedicated to olfactory receptors. Following years of

unsuccessful attempts, the reliable functional expression of olfactory receptors became achievable over the recent years. We will build on this recent evolution, and will start a proteomic program in order to identify new proteins involved in the organization of the signaling complex in olfactory neurons, both in mouse and in insects. The evolutionary aspects of olfactory receptors, associated proteins, and ligand specificity will be considered as well.

2.7.2. Summary description of scientific activities and results

2.7.2.1. Olfactory receptors (P1, P2)

The structure of olfactory receptors has been known for 20 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 signalling 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 adenylate cyclase III, $G_{\alpha olf}$ and CNGA2 proteins, which are part of the signalling machinery proximal to the receptor. In order to purify further the protein complexes containing the receptors, it is necessary to express a specific tagged olfactory receptor in all olfactory neurons. To this end, we have developed a transgenic mice that place the expression of a receptor labelled with a F5-tag under the control of tetracyclin responsive promoter, and bred these mice with a strain expressing two other transgenes (OMP-TTA and Gγ8-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). We first verified the expression of the transgenes using immunofluorescence and, as expected, we observed that, in mice expressing the three transgenes, all olfactory neurons are positive for EGFP as well as for the epitope-tagged olfactory receptor. The comparison with the labelling obtained for adenylate cyclase type III (ACIII) also indicated that the receptor is mainly present in the cilia of olfactory neurons. It is well established that each olfactory neuron only expresses a single type of OR and that all olfactory neurons expressing the same OR project their axons to the same glomerulus in the olfactory bulb. We thus wanted to verify if this was also true in our transgenic mice. To tackle this question, we used RT-PCR to evaluate expression of endogenous OR in olfactory neurons and immunofluorescence to visualize the axon projections in the olfactory bulb. We found that in mice expressing the eugenol receptor in all olfactory neurons, the level of transcripts encoding endogenous ORs was dramatically reduced as compared to wild-type mice, and in most cases we were unable to detect any mRNA coding for endogenous receptors. Thanks to the EGFP reporter, we also observed that in transgenic mice, olfactory neurons project their axons to all glomeruli of the olfactory bulb.

In parallel, we performed setup experiments to try to identify by a proteomic approach OR accessory proteins. While preparing OR-enriched fractions from the transgenic mice by immunoprecipitation, we found that immunodetection of the receptor was more efficient with a specific anti-human eugenol receptor polyclonal antibody than with an anti-Tag monoclonal antibody. We are therefore also developping pull-down experiments to study potential interactions between the C-terminus of the eugenol receptor and accessory proteins. Indeed, as the anti-human eugenol receptor polyclonal recognizes an epitope localized in the C-terminus of the receptor, it is possible that the binding of the antibody would prevent the interaction with endogenous proteins potentially belonging to signalling complexes. We now plan to 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 or as compared to samples prepared from wt mice. Proteins identified by mass spectrometry will be considered as part of signalling complexes and will be tested functionally in reconstituted systems in mammalian cell lines co-expressing ORs, G_{olf}, and the cyclic-nucleotide-gated channel. We will also search for accessory proteins involved in cell surface targeting of OR by studying cellular localisation (using microscopy) of OR in cell lines co-expressing the newly identified accessory proteins and various olfactory receptors.

2.7.2.2. Bioinformatics and evolution of the GPCR gene family (P1, P2, P4, EU1)

We have analysed the conservation of GPCRs of interest and their ligands across species, starting with ChemR23 and its protein ligand, chemerin, as a preliminary step to study the biological functions of this new leukocyte chemoattractant system in animal model systems. Prochemerin (Ensembl ID ENSMUSG00000009281) and ChemR23 (Ensembl ID ENSMUSG00000042190) amino-acid sequences from vertebrate species were aligned with ClustalW and a dendrogram was constructed. For chemerin, we had to screen specifically the genome databases in order to identify the orthologs in fish species. Two different genes were recovered in fish species, as often encountered as a result of whole genome duplication that occurred in the ancestry of modern fishes. Orthologs of chemerin and ChemR23 were also unambiguously found in primate, mammalian, bird and amphibian species. The six cysteines presumably involved in disulfide bridging of chemerin were strictly conserved in all species. In addition, the C-terminal nonapeptide of the mature protein, previously shown to be important for the biological activity of human chemerin, was highly conserved (YFPGQFAFS) in all mammalian species. This suggests that the interaction with the cognate receptor and the mechanisms allowing generation of bioactive chemerin are well conserved across species. However, in frog and fish species, the C-terminus was shorter and more divergent, suggesting that the proteolytic processing necessary in human and mouse is not conserved in these species.

2.7.2.3. Nutrient transceptors as possible evolutionary precursors of GPCR's (P3)

With respect to evolution, it seems plausible that nutrients were used by cells before signaling molecules and thus that transporters existed before receptors. In early evolution transporters have evolved the capacity to recognize extracellular molecules and to respond with a conformational change (allowing the passage of the molecule into the cell). Receptors in principle do the same: they detect an extracellular molecule and in response they change their conformation. Hence, it seems plausible that in evolution receptors have arosen from nutrient transporters. This idea has recently received strong support with the discovery first of nontransporting transporter homologs with a receptor function and more recently of active transporters with an additional receptor function. These two categories of transporter-related receptors are now called 'transceptors'. They appear to represent examples of intermediate forms in the evolution from nutrient transporters to receptors. This has allowed us to propose a direct evolutionary line from regular nutrient transporters to regular receptors, such as G-protein coupled receptors (Thevelein and Voordeckers 2009). The general amino acid permease Gap1 and the phosphate transporter Pho84 in yeast have become the best characterised model systems of transporting transceptors (see section 4.7). However, the signaling mechanism is currently unknown. We have obtained several pieces of preliminary evidence that the crucial protein synthesis initiation factor eIF2 and its guanine nucleotide exchange factor eIF2B may function as signal transducers in the activation of the PKA pathway by the nutrient transceptors. This would explain why nutrient activation of the PKA pathway is always closely correlated with stimulation of protein synthesis and cell growth. Since eIF2 is a G-protein, it would also indicate that the nutrient transceptors actually function as G-protein coupled transceptors. This would provide another striking argument for the evolution of receptors from nutrient transporters.

2.7.3. Main achievements in relation to the initial objectives

We have constructed a mouse model in which a tagged receptor is expressed in all olfactory neurons. Two antibodies are available against this receptor, one directed at the N-terminal tag, the other at the natural C-terminus. These antibodies are being used to immunoprecipitate the receptor together with associated proteins, with the aim of identifying proteins belonging to the signaling complexes in olfactory cilia.

Our discovery of nutrient transceptors that activate the PKA pathway, possibly using a G-protein coupled signal transduction mechanism, provides the strongest evidence up to now for nutrient transporters as evolutionary precursors of G-protein coupled receptors. (P3)

2.7.4. Comments (in case of deviations from the initial project workprogramme)

The planned analysis of GPCR evolution though bioinformatics has started slowly.

The search for partners of olfactory receptors has progressed well though the generation of the tools necessary for the sudy (transgenic mouse lines, antibodies). The amount of material represent presently the main limitation for the next steps. We are working on the efficiency of preparation of cilia, and immunoprecipitation procedures.

3. NETWORKING

3.1. Major joint activities

3.1.1. Joint experiments

Many collaborations between the partners of the network have been pursued during the first three years of the program, in terms of receptor modeling and structure-function analysis, determination of receptor oligomerization, determination of the role of the receptors in physiological processes and diseases, and characterization of new receptors. The joint experiments are illustrated by the co-publications of the network for the 2007-2010 period, which are listed in Annex II, and a number of other joined activities are presently being pursued. This includes among others the characterization of the role of various receptors in inflammatory and viral diseases (P1 and P5), the characterization of chemokine variants (P1 and P4), the search for natural ligands for receptor families shared by insects and mammalian species (P1 and P2), the modelling of receptors of interest (EU1, P1, P3).

3.1.2. Added value

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.

A kick-off meeting of the network was organized on June 28, 2007. Thereafter, yearly plenary meetings were organized on February 15, 2008 (Erasme campus, ULB), November 28, 2008 (KULeuven) and February 5, 2010 (Erasme campus, ULB). These meetings have gathered all senior and most junior staff involved in the program from all groups. The program of these meetings is provided as Annex III. Morning and afternoon sessions were dedicated to scientific lectures in which all groups presented a selection of their overall contribution to the program for the 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 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 each time, these meetings were 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.

The flow of information and reagents established through personal contacts have had a positive effect on the scientific output of the individual teams. In particular, the close contacts between the partners have resulted in common publications with significant impact. These would not have been possible without the network. In addition, several publications have greatly benefited from these interactions, even if it did not materialize as co-publication. This is the result of an effective "fertilizing effect", in particular, in relation to molecular modelling, collaboration between the groups working with vertebrate and invertebrate receptors and between those working with chemokine receptors.

The International meeting will be organized in 2010. This meeting will complement the 2010 plenary meeting and will be organized in Barcelona by Leonardo Pardo (EU1). We consider a symposium of three days, with an attendance limited to about 150 to 200 participants. This meeting will 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.

In addition participants of the network met on the occasion of a number of International meetings, including Keystone and Gordon conferences dedicated to GPCRs or chemokine receptors.

3.1.3. Interactions between partners

3.1.3.1. Exchange of material and data

The main exchanges of material and data that have been made between partners are listed here. These exchanges were made in the frame of co-experiments that have led (or will likely lead) to co-publications, but also as the result of network representing a priviledged source of material, information and protocols.

P1-P2.

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

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

Exchange of researchers, information and materials for the study of LGRs

Tom Van Loy, from KUL-Vandenbroeck group, started working for 1,5 years as a postdoctoral fellow at ULB, in the context of the search for the natural agonist of LGR4 and LGR5.

Four common publications have resulted from these interactions (Van Loy et al. 2007, 2008, 2010, Poels et al. 2010)

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 (resulting in one co-publication, Gouwy et al., 2008)

- CHO cells expressing CXCR4 and CXCR7, characterized by P1 were used by P4 for the study of the citrullination of SDF-1/CXCL12 (resulting in one co-publication, Struyf et al., 2009).

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 students from both labs participated to the annual meetings of this consortium. The meeting in El Escorial (Spain, September 27-29, 2007) 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. At the next meeting in Ermenonville (France, April 23-25, 2009) Tamara Loos (P4) and Denis Soho (P1) gave an oral presentation and in addition 4 posters were presented.

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) influenzavirus 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 influenzavirus 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.

Partner P1 (Parmentier – ULB) provided partner P5 (Desmecht – ULg) knock out mice for the ChemR23, P2Y2 and P2Y6 receptors, as well as detailed procedures for the genotyping of these mice. Production of corresponding founder populations at ULg is ongoing for feeding worckpackage 5, thus for performing new infections with H1N1 and H5N1 influenza A, Sendai and murid herpesvirus-4 viruses for each wild-type/knock-down paired lines. Benjamin Bondue (from P1) is working on a regular basis in the P5 laboratory for performing the infections, collecting the plethysmographic respiratory values and for titrating infected lungs. Parner 5 provided virus, and specific antibodies to P1 laboratory for performing adoptive transfer experiments and immunofluorescence/Western blotting studies.

This led to a first paper in collaboration, that was submitted by B. Bondue in 2009. Inversely, Els Van de Paar (from P5) is working on a regular basis in P1 laboratory for performing quantitation of a series of specific cyto- and chemokines (MIP1α, MCP1, IL-6, IFNγ, TNFα and IL-1β) in mouse lungs infected with respiratory syncytial and influenza A viruses. A second paper in collaboration will be submitted shortly by Mrs Van de Paar.

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 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 or member of the jury at the public PhD-defence of Tom Peeters (2007), Patrick Van Dormael (2008) and Griet Van Zeebroeck (2009) (promotor: J. Thevelein, P3).

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

J. Vanden Broeck (P2) is involved as an associated user of the confocal microscope system of P3;

P2-P4.

Peptide synthesis (various insect peptides) and protein sequencing activities were performed by P. Proost (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, PhD defence, 17/09/2009) 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)

P2-EU1.

Structural data on insect receptors and glycoprotein-like hormones have been exchanged between Partner 2 (KUL-Vanden Broeck) and the group of Leonardo Pardo (EU1) in a collaborative work that should result in a common publication.

P3-EU1

EU1 has performed modeling studies on the possible interaction of sugar ligands into the ligand-binding site of the intestinal sugar-sensing GPCR discovered by P3.

P4-P5

A bank of acute and convalescent bovine sera gathered by partner P5 in the context of viral pneumonias in cattle (ULg-Desmecht) has been transferred to the group of Jo Van Damme (partner 4) for starting a large screening of circulating regakine (CC chemokine) levels. The first data analysis of Regakine serum levels in virally infected cattle did not allow to conclude that this chemokine could serve as a parameter for diagnosis of viral disease state. A continuation of this collaboration testing other bovine diseases is programmed.

Partner P4 (Van Damme, KUL) provided partner P5 (Desmecht, ULg) results of regakine-1 values in peripheral blood of cohorts of healthy and pneumonic calves (from the bank of antisera sent by P5 to P4 in 2008). Partner P5 provided P4 laboratory wild-type and stable transgenic Vero cells expressing human MxA, porcine Mx1α, bovine Mx1 and several other mutated bovine Mx1. Partner 4 already provided preliminary results of IL-8, IP-10 and MCP-1 secretion by aforementioned cell lines in response to IL-1β, poly-I/C, LPS, PMA, ConA and TNFα. Anne Cornet (from P5) and Sophie Struyf (P4) are collaborating in this area, and Anne Cornet is working sporadically in P4 laboratory. A paper in collaboration dealing with the discovery of anti-inflammatory effects of selected Mx proteins is intended.

3.1.3.2. Exchange of personnel

P1-P2. In the context of the collaboration between P1 and P2 on Leu-rich repeat containing GPCRs (LGRs), Dr. Tom Van Loy (P2) was a guest researcher in the lab of P1 (G. Vassart) in the period May-September (2009), before obtaining a postdoctoral position in the lab of P1 from the Francqui Foundation (an exchange programme between the Dutch- and French-speaking communities in Belgium) that started on October 1st (2009).

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. Benjamin Bondue (P1's graduate student) is currently doing all the protocols related to the detection of GPCRs' role in viral pneumonia in P5's facilities (ULg).

P5>P1. Els Van de Paar (P5's graduate student) is currently doing all the protocols related to the detection of GPCR's role in H1N1 influenza A-associated ARDS in P1's facilities (ULB).

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 and the development of inhibitors with low bio-availability.

P5>P4. Anne Cornet (P5's post-doc) is currently doing a part of the protocols aimed at examining the effect of Mx proteins on expression of GPCRs' ligands in P4's laboratory (KUL).

4. POSITION OF THE IAP NETWORK

4.1. Cutting-edge research

4.1.1. Scientific highlights of the network

The main achievements are listed below with indication of the partners involved and corresponding publications. These achievements were made in several areas, including structural analyses, characterization of novel receptors, determination of the function of receptors in physiological and pathological processes, and the development of agonists and antagonists.

Demonstration of the conformational coupling between the extracellular domain and the orthosteric binding site of a GPCR, showing that drugs targeting this diverse surface could function as allosteric modulators with high subtype selectivity. **EU1** (UAB). Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi HJ, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L, Kobilka BK (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. **Nature** 463:108-112.

Demonstration of allosteric interactions between different receptors taking part to oligomeric complexes in recombinant systems and in native cells. Contribution with other experts in the field to the determination of criteria required to consider receptor heterodimers as new pharmacological entities. **P1** (ULB). 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, Urizar E, Costagliola S, Vassart G, Parmentier M (2007). Sohy D, Yano H, de Nadai P, Urizar E, Guillabert A, Javitch JA, Parmentier M, Springael JY (2009). Hetero-oligomerization of CCR2, CCR5 and CXCR4 and the protean effects of "selective"-antagonists. **J Biol Chem** 284:31270-31279. Allosteric properties of G protein-coupled receptor oligomers. **Pharmacol Ther** 115:410-418. 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.

Identification of the natural polypeptidic ligand of a human orphan GPCR. Provided the distribution and other available data regarding this receptor, this is expected to constitute a important discovery. It may also lead to the identification of related orphan receptors. The full characterization of this system is however expected to take some more time. Because this report will be posted on the web, we have choosen not to mention the name of the receptor and ligand. **P1** (ULB). Unpublished.

Deorphanization of several GPCRs from insect and other invertebrate species. **P2** (KUL-Vanden Broeck). Janssen T, Husson SJ, Lindemans M, Mertens I, Rademakers S, Ver Donck K, Geysen J, Jansen G, Schoofs L (2008). Functional characterization of three G protein-coupled receptors for pigment dispersing factors in Caenorhabditis elegans. **J Biol Chem** 283,15241-15249. Janssen T, Meelkop E, Lindemans M, Verstraelen K, Husson SJ, Temmerman L, Nachman RJ, Schoofs L (2008). Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. **Endocrinology** 149, 2826-2839. Lindemans M, Liu F, Janssen T, Husson S, Mertens I, Gäde G, Schoofs L (2009). Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in Caenorhabditis elegans. **Proc Natl Acad Sci USA** 106: 1642-1647.

Characterization of the interaction between a peptidic and a lipidic ligand acting on a previously orphan mammalian receptor (GIR/GPR83). **P1** (ULB). Unpublished.

Development of an annotated EST database for desert locust CNS. **P2** (KUL-Vanden Broeck). **http://titan.biotec.uiuc.edu/locust/**.

Trancriptome-wide analysis in function of locust phase polyphenism (which is an important textbook example of an extreme phenotypic plasticity process). **P2** (KUL-Vanden Broeck). **http://titan.biotec.uiuc.edu/locust/** and unpublished.

Use of molecular and reverse genetics tools to unravel receptor/ligand gene expression and functioning in an *in vivo* invertebrate context. **P2** (KUL-Vanden Broeck). Lindemans M, Liu F, Janssen T, Husson S, Mertens I, Gäde G, Schoofs L (2009). Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in Caenorhabditis elegans. **Proc Natl Acad Sci USA** 106: 1642-1647. Poels J, Van Loy T, Vandersmissen HP, Van Hiel MB, Van Soest S, Nachman RJ, Vanden Broeck J (2010). Myoinhibiting peptides are the ancestral ligands of the promiscuous Drosophila sex peptide receptor. **Cell Mol Life Sci** (in press).

Discovery that a regular hormone-sensing GPCR functions as a sugar-sensing GPCR at the apical side of intestinal epithelial cells in the mammalian gut. **P3** (KUL-Thevelein). Not described in the present report and unpublished.

Establishment of yeast Gap1 and Pho84 as the best characterized nutrient transceptors in cell biology. **P3** (KUL-Thevelein). Popova Y, P Thayumanavan, E Lonati, M Agrochão and JM Thevelein (2010). Transport and signaling through the phosphate-binding site of the yeast Pho84 phosphate transceptor. **Proc Natl Acad Sci USA** 107: 2890-2895.

Firm establishment of the new concept that nutrient transporters can have an additional receptor function for activation of regular signal transduction pathways. **P3** (KUL-Thevelein). Van Zeebroeck G, B Monge Bonini, M Versele and JM Thevelein (2009). Transport and signaling via the amino acid binding site of the yeast Gap1 amino acid transceptor. **Nature Chemical Biology** 5: 45-52. Thevelein JM and K Voordeckers (2009). Functioning and evolutionary significance of nutrient transceptors. **Molecular Biology & Evolution** 26: 2407-2414.

A main achievement is the novel finding that minor posttranslational modification, (i.e. citrullination) of chemokines has a significant impact on their biological activity. **P4** (KUL-Van Damme) and **P1** (ULB). Proost P, Loos T, Mortier A, Schutyser E, Gouwy M, Noppen S, Dillen C, Ronsse I, Conings R, Struyf S, Opdenakker G, Maudgal PC, Van Damme J (2008). Citrullination of CXCL8 by peptidylarinine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. **J Exp Med** 205:2085-2097. Loos T, Mortier A, Gouwy M, Ronsse I, Put W, Lenaerts JP, Van Damme J, Proost P (2008). Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. **Blood** 112:2648-2656. Struyf S, Noppen S, Loos T, Mortier A, Gouwy M, Verbeke H, Huskens D, Luangsay S, Parmentier M, Geboes K, Schols D, Van Damme J, Proost P (2009). Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4. **J Immunol** 182:666-674.

The demonstration that genetic chemokine variants (i.e. PF-4var/CXCL4L1) are more potent than the authentic molecules, and that proteolytic processing of chemokines affect greatly their biological activity. **P4** (KUL-Van Damme) and **P1** (ULB). Struyf S, Burdick MD, Peeters E, Van den Broeck K, Dillen C, Proost P, Van Damme J and Strieter RM (2007). Platelet factor-4 variant chemokine CXCL4L1 inhibits melanoma and lung carcinoma growth and metastasis by preventing angiogenesis. **Cancer Res** 67: 5940-5948. Proost P, Mortier A, Loos T, Vandercappellen J, Gouwy M, Ronsse I, Schutyser E, Put W, Parmentier M, Struyf S and Van Damme J (2007). 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.

Delineation of the signaling pathway downstream of yeast GPCRs. **P3** (KUL-Thevelein). Peeters T., W. Louwet, R. Geladé, D. Nauwelaers, J.M. Thevelein and M. Versele (2006). Kelch-repeat proteins interacting with the Gα protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. **Proc Natl Acad Sci USA** 103, 13034-13039.

Identification of LGR5, a receptor expressed in a number of adult stem cell populations, as a negative regulator of the Wnt pathway in mouse intestine. **P1** (ULB). Garcia MI, Ghiani M, Lefort A, Libert F, Strollo S, Vassart G (2009). LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. **Dev Biol** 331:58-67.

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4.1.2. Benchmarking

The **P1-P4** tandem is clearly at the forefront of chemokine receptor research. Marc Parmentier (P1), P. Proost and Jo Van Damme (P4) are repeatedly invited to international scientific meetings dealing with chemokine receptors, including Gordon Conferences and Keystone conferences. **P4**, with the help of **P1**, is holding a leading international position with regard to posttranslational modifications of chemokines (citrullination by peptidyl arginine deiminases, proteolytic processing by CD13, CD26, matrix metalloproteases,...). P4 provided significant contributions to the role of chemokines in angiogenesis (CXCL4, CXCL4L1, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11), parvoviral delivery of chemokines to study their role in tumor biology. P4 discovered that synergistic interactions between chemokine receptor ligands enhance the inflammatory response and has studied the role of chemokines in auto-immune diseases, inflammatory eye diseases, bacterial and viral (HIV) infections.

P1 is also one of the main labs in the world in the field of glycoprotein hormone receptors. Gilbert Vassart is frequently invited to write chapters in textbooks on the molecular biology and genetic variants of TSH and FSH receptors. He is also regularly invited to international meetings dealing with endocrine signaling or GPCRs in general. Another group in **P1** (led by Jean-Marie Boeynaems) is also known worldwide for its studies dealing with the family of P2Y nucleotide receptors. This group has characterized in the past many of the receptors in this subfamily, and has more recently generated the knockout models for these receptors, which are presently being studied.

P1 and **P2** have been leading groups in the deorphanization of receptors for many years, respectively in mammalian species and invertebrate species. The worldwide pace of deorphanization of mammalian GPCRs has slowed down dramatically over the past 5 years, despite the fact that about a hundred receptors do not have known ligands. This may be due to the fact that some of the remaining orphan receptors have no ligands and behave as constitutive regulators of signaling pathways. Others may be exclusively coupled to unconventional signaling cascades, or are activated by unknown biological mediators of very low abundance. Partner **P1** has deorphanized ChemR23 and FPRL2 in 2003 and 2005 respectively, and has characterization further these receptors. The ligand of an orphan mammalian receptor has been identified during the first part of the present program, but

the post-translational modifications required for the activity of the polypeptidic ligand are not yet determined. Provided the distribution of this receptor and the other available data, we expect this characterization will be an important contribution of this network. P1 has also characterized in collaboration with Euroscreen, lipidic ligands of GIR/GPR83. P2 has continued over the last three years to identify novel receptors in invertebrate species, and has kept a leading role in this area.

The lab of **P2** is more generally acting at the international forefront of the invertebrate physiology and molecular neuroendocrinology scene and addresses important and original scientific questions by employing advanced technological procedures. It has been very productive (with a total of >90 articles published in internationally peer reviewed journals, since 2007). Different members of the group have frequently been invited at international conferences and/or have written invited review articles on topics related to the research theme of the IAP-network. J. Vanden Broeck is councillor of the 'European Society for Comparative Endocrinology'.

The Lab of Molecular Cell Biology of Johan Thevelein (**P3**) is among the leading laboratories in the field of cellular nutrient-sensing world-wide. Whereas nearly all other research groups concentrate on sensing of one specific nutrient, this research group has established the best characterized multi-nutrient sensing system in eukaryotic cell biology. This group is publishing research papers in top multi-disciplinary journals, which are highly cited. Johan Thevelein has been invited repeatedly at international conferences to present the progress in the nutrient sensing field, including nutrient-sensing GPCR's and to write reviews on this subject for top international journals. The research group is also partner in several international projects, including two EC projects, and has running research projects with several large companies worldwide.

P5 is one of the few groups mastering models of viral lund diseases. The mouse model of fatal RSV pneumonia available in the network is the only that recapitulates the human fatal disease (Anh et al., 2006, from P5), and is only available in two labs in the world. Mouse models of fatal influenza are not new, but this network is the only research structure in which two models of fatal flu due to different subtypes and exhibiting very different pathogenesis were made available (Garigliany et al., 2010, from P5). This offers a unique context to test the network's set of mouse lines invalidated for GPCRs. The alveolar macrophage population appears to be the key player explaining the innate resistance of the SJL/J mouse line to fatal RSV (unpublished data). Preliminary results gathered in P5's lab show that SJL/J's alveolar macrophages are unique in their control of MIP-1α expression upon viral infection, which suggests that corresponding receptors are involved. This aspect of innate resistance is promising because unprecedented.

The European partner, **EU1**, is one of the leading laboratories in modelization of GPCRs. Leonardo Pardo has also spent a sabattical year in the group of Brian Kobilka, one of the very few leading groups worldwide in the field of GPCR crystallisation. L. Pardo is very frequently invited to international GPCR meetings.

From the analysis of its scientific output, the network is clearly in a very productive phase. The interactions between the members of the network has helped to boost GPCR research in Belgium, to maintain it at a world-class level, and to increase international recognition of the network as a centre of excellence in the field.

4.1.3. Perspectives of the network's research domain for the coming 5 to 10 years

The field of GPCR will certainly remain a hot topic for the coming five to ten years. Globally, GPCRs constitute presently the targets of 30 to 40% of the currently used therapeutic agents. This figure is not expected to shrink in the predictable future, as many GPCRs characterized over the last two decades have triggered drug development programs that often have not led yet to marketed drugs, but will likely do so in the future. This is for example the case for chemokine receptors, which are more and more attractive for the pharmaceutical industry, provided the increasing knowledge of their implication in diverse inflammatory disorders. We can predict that the field will evolve along the following lines:

- Structural information will accumulate at an increasing pace. Rhodopsin in its inactive state has remained for about ten years the only crystal structure available for the whole GPCR family. Over the last few years, a number of additional structures became available, including various states of rhodopsin, as well as a few receptors for bioamines and adenosine. It is likely that the methods used for solving the structure of these few receptors will be applicable to a growing number of others. There will therefore be a very exciting time during which a growing set of structural data will accumulate for various families and subfamilies of GPCRs, paving the way to the understanding of ligand-receptor interactions, receptor activation mechanisms, assembly of complexes with downstream signaling molecules, and rational design of future drugs acting on this receptor family.

- This expected explosion of structural data will feed and support modeling approaches, rendering them more rational and more accurate. Provided the expected growth of the predictive power of this approach, structural modeling will play an increasing role in the understanding of all aspects of GPCR research.

- The understanding of the functional correlates associated with the complexes in which GPCRs take part will also constitute a growing field of interest. This will include the homo- and hetero-oligomerization concept of GPCRs, which has gained considerable ground over the last few years. It will also involve the association of GPCRs with other classes of membrane (other receptor families, adhesion proteins) or soluble proteins (chaperones, scaffolding proteins, trafficking partners, signaling enzymes, cytoskeleton adaptors, ...), forming transient or stable signaling complexes in different cell types (i.e. synapes in neurons, immunological synapses in immune cells, ...). However, the understanding of how these complexes affect the function of each receptor will require the development of specific analytic tools, new biophysical techniques and modeling approaches.

- There are still many orphan receptors in human and mammalian model organisms. The difficulty of assigning them a ligand and a function has however increased considerably, as all "easy" orphan receptors have now been characterized. There will be a

need for new approaches to characterize the remaining orphans. Some of these orphan receptors do most likely have a ligand, but these unknown ligands are either produced at very low levels in specific and rare conditions, or extremely short lived, or membrane-bound proteins, making it difficult to identify them by classical purification procedures. Other receptors might trigger only unconventional signaling cascades, without activating any of the G proteins. Such receptors will require the design of new screening assays adapted to such unconventional signaling. Still other receptors might function only in hetero-oligomeric complexes with other GPCRs or other membrane or soluble proteins, requiring a combinatorial approach to identify them. Finally, orphan GPCRs might affect signaling cascades without being regulated by ligands. GPR3, which is studied by P1, constitutes one of these potentially "ligand-free" receptors, whose regulation might rely on expression, trafficking and degradation. The characterization of these orphan receptors is certainly worth the investment, despite the growing difficulty, as this activity will continue to expand our knowledge of physiology and pathophysiology by bringing to light unsuspected agonists and regulatory mechanisms. However, the range of tools and approaches to characterize them will have to be broadened considerably.

- For invertebrate species, deorphanization will remain a important avenue of research. Provided the rapid pace at which new genomes are being sequenced, there will be an increasing contribution of comparative genomics, and in the frame of GPCRs, comparative pharmacology. This will pave the way for a growing number of biotechnological applications in the field of agriculture and pest control. Comparative genomics is also expected to give more insight into the evolution of GPCR and peptide agonist families. Combining expertise of vertebrate, invertebrate and unicellular specialists should therefore be pursued.

- Sensory receptors (olfactory, gustatory) are attracting growing interest. Functional characterization of these receptors has improved considerably over the last five years, with the identification of several protein families allowing more efficient functional expression of these receptors in recombinant systems. This field is approaching, with a considerable delay, what the pharmacology of other GPCR families has been for about two decades. The possibility of testing sensory receptors in campaigns of high throughput screening is now at hand, with a likely impact on the perfume and food industry. However, the delineation of the combinatorial response of the olfactory receptor repertoire to odorants (about 400 receptors in human and 1000 in other mammalian species), and how this complex information is integrated in the central nervous system is still a major issue.

- The discovery that nontransporting nutrient transporter homologues and especially actively transporting nutrient transporters can function as nutrient sensors has renewed interest in the field of nutrient sensing. The mechanisms of many classical nutrient sensing systems, such as glucose sensing by pancreatic beta cells, are being reevaluated based on these insights. There will be continued strong interest in the mechanisms of nutrient sensing for cellular growth control. However, in our view the current overbias on the TOR pathway is putting the majority of the research field on the wrong track.

- Severaly GPCRs have been shown to be involved in developmental processes. This included GPR3, LGR4 and LGR5, which are studied in the network. With the parallel development of the stem cell field, there will be a growing interest of understanding the role of known and presently orphan receptors in shaping the fate and differentiation of stem cells. It is expected that this field will expand rapidly.

- Besides, there will be a continuous need for a better understanding of the role of the various GPCR classes in physiology and pathogenesis, in order to drive the development of novel drugs acting on this important family of targets. This calls for the continuation of detailed in vivo studies in transgenic animals with gain or loss of function mutations in the agonist-receptor couples, and the search of mutations responsible for human genetic diseases.

4.1.4. Recognition of the network as a critical mass at national and international levels

The combined expertise of the network spans the whole spectrum of GPCR research, from bioinformatic genomic data mining, to in vivo phenotyping of transgenic animals with invalidated or added GPCR genes; from state of the art molecular modeling and molecular dynamics to experimental molecular pharmacology; from analytical and preparative biochemistry to functional identification of novel GPCR agonists; from cell and molecular biology of GPCRs to identification of their roles in a series of diseases (hereditary diseases, aging, cancer, inflammatory and infectious diseases, drug addiction, hypertension, diabetes...). A highly valuable characteristic of the network is also the diversity of the model systems under investigation (from yeast to man, via invertebrates). Altogether, building of this critical mass has boosted several conceptual and technological aspects of research in the participating groups. If one looks around the world in the GPCR field, IAP6/14 is probably unique in putting together such diverse and complementary expertises. The continuous complementary interactions between the various partners have helped to keep the network's research at the frontline.

4.2. International role

4.2.1. Collaborations with European partners within the network

The EU partner has contributed very efficiently to the network activities. His expertise in GPCR and protein modelization was very complementary to those of the partners. The added value of having the European partner in the network is best illustrated by common publications. There are a number of ongoing collaborations that will likely lead to additional co-publications in the future years. This includes the characterization of how various agonists and antagonists of CCR5 bind to the receptor, and how this translates into stabilization of the active or inactive states of CCR5 (P1 and EU1). P3 is also collaborating with EU1 on the modelling of the sugar-sensing GPCR in the mammalian gut.

EU1 will also host in Barcelona the international meeting organized by the network in November 2010, in combination with its annual meeting. The budget of the EU partner was used to support PhD students and post-docs involved in the common research programs.

4.2.2. International activities

Participation in European and international research projetcs

The Belgian partners of the network have entertained collaborations with foreign laboratories, outside the network. This has contributed to promoting the visibility of the network on the international scene. As examples, **P1** (ULB) has co-authored, for the 2007-20010 period, 59 publications with foreign groups from France, Spain, Italy, The Netherlands, Great Britain, Germany, Switzerland, Sweden, Hungary, Japan, USA, Canada. **P2**, (KUL-Vanden Broeck) has a wide network of international collaborations and interactions, and has co-authored many publications with groups from France, The Netherlands, Germany, Sweden, Poland, Greece, USA, Canada, Taiwan, China, South Africa. **P3** collaborates and has co-authored publications with international partners, e.g. in Sweden, Argentina, South Africa, Brazil, USA, Canada, Australia. **P4** has long standing collaborations with groups in Italy (shared with P1), USA and Germany, which has resulted in many co-publications.

This program is also linked through several of its members to FP6 European consortia dedicated to the same or related topics. This includes a (now ended) STREP program "GPCRs" involving partner **P1** (as coordinator) and **EU1**, an integrated project "INNOCHEM", which includes partners **P1** and **P4**, a network of excellence "EADGENE" incorporating partner **P5**, the "NEMO" and recently granted "CORNUCOPIA" projects as well as the Marie-Curie Training Network "CANTRAIN" involving partner **P3**. **P2** is also involved in a Bilateral exchange project Flanders-Poland (BIL05/17), and in Collaborative research contract with two U.K. universities (Cambridge and Leicester). All groups have welcomed numerous international visitors, and host foreign PhD students and post-docs

Organisation of international symposia

P1: Marc Parmentier, Organizer and chairman of a symposium on GPCRs. SBS meeting, Lille, France, April 27-30, 2009.

P2: Jozef Vanden Broeck was symposium organiser or member of the organising committee of:

Symposium organised at the XXIII International Congress of Entomology (ICE2008) in Durban, (South Africa, 6-12/07/2008): "(Neuro)hormonal control of reproduction" (Symposium organisers: Hoffmann K. & Vanden Broeck J.);

"2nd Symposium on receptor pharmacology and evolution" (Leuven, 04/02/2009) (Symposium organisers: Vanden Broeck J. & Janecka A.);

"Symposium on reproductive endocrinology" at the 25th Conference of European Comparative Endocrinologists (CECE2010) (Pécs, 2010) (Symposium organiser: Vanden Broeck J).

International Symposium on Crop Protection in Gent (ISCP, organised each year).

24th Conference of European Comparative Endocrinologists in Genova, Italy (2008).

25th Conference of European Comparative Endocrinologists in Pécs, Hungary (2010).

P3, Johan Thevelein, Organizer of the 27th Small Meeting on Yeast Transport and Energetics, Blankenberge, Belgium. 4-9 Sept 2009.

P4, Jozef Van Damme has organized an international symposium on "Cytokines and Chemokines: post-transcriptional regulation", March 22-24, 2010, St. Sorlin, France.

As stated above, an International meeting on GPCRs will be organized by the network in November 2010. EU1, Leonardo Pardo, will be the host of this meeting in Barcelona. It will be a three day symposium that will cover the interests of the network (GPCR structure, novel receptors in vertebrates, invertebrates and yeast, functional characterization as drug targets).

Invitations to give lectures at international conferences

P1, ULB

Gilbert Vassart was invited at the following meetings:

"TSH receptor function", UK Society For Endocrinology BES, March 5-8, 2007, Birmingham, UK.

"GPCR-complexes and GPCR complexity", 32th Conference Hormones and Cell Regulation, Sept 14-16, 2007, Mont St Odile, France.

"Constitutive Activity of Wild Type GPCRs: Physiological and Evolutionary Significance", Keystone Symposium on G Protein-Coupled Receptors, April 2008, Killarney, Ireland.

«Control of thyroid size in development », Second ESPE Advanced Seminar in Developmental Endocrinology "Thyroid Development and its Disorders", April 22-25, 2008, Paris, France.

"Orphan GPCRs", GPCR meeting, March 5th 2009, Barcelona, Spain.

"Constitutive Activity of wild type GPCRs: role in physiology, evolution and diseases", International Symposium on Signal Transduction and Disease, September 27-30, 2009, Aachen, Germany.

Plenary Lecture, International Congress of Endocrinology. February 2010, Kyoto, Japan.

"GPCRs and genetic disorders", From molecules to systems, February 8-9, 2010, Montpellier, France.

International Thyroid Conference. September 2010, Paris, France.

European Society Pediatric Endocrinology. September 22-25, 2010, Prague, Czech Republic.

Marc Parmentier was invited at the following meetings:

"Leucocyte chemoattractants: new molecules and new concepts" Third ASPET GPCR Colloquium, Washington DC, USA, April 26-28, 2007

"Dimerization of chemokine receptors and functional consequences". Colloque Cytokines, Le Croisic, France, May 14-16, 2007.

"Functional characterization of novel leucocyte chemoattractant receptors as potential drug targets". G protein-coupled receptors in Drug discovery. Lisbon, Portugal, May 21-23, 2007.

"Leucocyte chemoattractants: new receptors and how their work". 32nd Meeting on Hormone Regulation and Cell Regulation. Mont St-Odile, France, September 13-16, 2007.

Innochem, El Escorial, Spain, September 27-30, 2007.

"New data on G protein-coupled receptors". Collège de France, Paris, France, March 27, 2008

« Ligands and functions of FPRL-2 », Workshop "FPRs and their ligands in inflammation". London, June 13, 2008

« Receptor oligomerization: does it matter practically », Gordon Conference on Chemotactic Cytokines, Aussois, France, Sept 21-26, 2008.

« Chemerin and ChemR23: Dendritic cell recruitment and anti-inflammatory actions », SBS meeting. Lille, France 27-30 avril 2009.

« Increasing complexity of GPCR complexes and signaling networks », ESF Exploratory Workshop on "GPCR signalling systems: A new avenue for drug discovery ?", November 24-25 2009, Paris, France

Gordon Research Conference on Chemotactic cytokines, Italy, May 30-June 5, 2010.

Sabine Costagliola was invited at the following meetings:

34rd Meeting of the European Thyroid Association, Sept 2009, Lisbon, Portugal

« Gonadotropin receptors dimerization », "2nd International Conference on Gonadotropins and Receptors", 6-9 juillet 2008, Theobald's Park, Hertfordshire, United Kingdom

« Functional Significance of Oligomerization and Negative Cooperativity in Glycoprotein Hormone Receptors », Keystone Symposium « Structural Biology & Activation Mechanisms of Membrane Receptors", 16-21 septembre 2008, Cambridge, UK

« Constitutively active TSHR mutants display profound alteration of their allosteric behavior for TSH binding », "33rd Meeting of the European Thyroid Association", 20-24 septembre 2008, Chalkidiki, Greece.

Jean-Marie Boeynaems, « New pharmacotherapeutic potentials of P2Y receptors revealed by the study of knockout mice », Purines 2009, July 23-25 2009, Fukuoka, Japon

Didier Communi, « Involvement of P2Y4 receptor in inflammation and angiogenesis », "Purines 2008", June 29-July 2 2008, Copenhagen, Denmark

P2, KUL, Jozef Vanden Broeck.

Several members of the P2 lab group are invited on a regular basis to give plenary or state-of-the-art lectures at international conferences and symposia. **Jozef Vanden Broeck** was invited to give lectures at the:

Invertebrate Neuropeptide Conference (INC2007), Luang Prabang (Laos), 14-19 January 2007.

Invertebrate Neuropeptide Conference (INC2008), Gamboa (Panama), 7-11 February 2008.

Symposium on biotechnology for locust control, Rabat (Morocco), 1-3 July 2008.

International Congress of Entomology (ICE2008), Durban (South Africa), 6-12 July 2008.

Invertebrate Neuropeptide Conference (INC2009), Khajuraho (India), 11-15 January 2009.

International Congress of Orthopterology, Antalya (Turkey), 21-25 June 2009.

International Congress of Comparative Endocrinology (ICCE), Hong Kong (China), 22-26 June 2009.

Invertebrate Neuropeptide Conference (INC2010), Merida (Mexico), 14-18 February 2010.

P3, KUL, Johan Thevelein

Keynote Lecture, 'Microbial stress: from Molecules to Systems', Semmering, Austria, 7 – 10 May

FEMS 2009, 3rd Congress of European Microbiological Societies, Göteborg, Sweden, 28 June – 2 July

8th Meeting of the Slovenian Biochemical Society with International Participation and 5th Congress of the Slovenian Genetic Society with International Participation, Otoček, Slovenia, 20 – 23 Sept.

Symposium: 'Microbial cell factories and biocatalysis', Lund, Sweden, 31 May

28th International Specialised Symposium on Yeast, Bangkok, Thailand, 15 – 18 Sept.

28th SMYTE meeting, New Delhi, India, 23 – 27 Sept.

P4, KUL, Jozef Van Damme

Jo Van Damme was chair of a Chemokine Workshop during the Keystone Symposium on Chemokines and Leukocyte Trafficking", Keystone, USA, January 13-18, 2008.

Jo Van Damme and Sofie Struyf had a Teaching assignment at the University of Brescia in the Academic Year 2007-2008

"Immunopathology of Chemokines: from protein discovery to biological functions" (3 h)

Sofie Struyf was invited to give a lecture at the following meeting:

"Regulation of chemokine activity by proteases" at the Annual meeting of the European Macrophage and Dendritic Cell Society on "The Diversity and Plasticity of the Innate Immune Response", Brescia, Italy, September 18-20, 2008.

Paul Proost was invited to give lectures at the following meetings:

"CD13/aminopeptidase N-processed I-TAC/CXCL11 has reduced CXCR3 and CXCR7 binding and signaling properties that result in impaired lymphocyte chemotaxis and reduced inhibition of endothelial cell migration" at the Keystone Symposium on Chemokines and Leukocyte Trafficking", Keystone, USA, January 13-18, 2008.

"Natural non-proteolytic posttranslational modification of chemokines reduces in vitro and in vivo activity" at the Gordon Conference on Chemotactic Cytokines, Aussois, France, September 21-26, 2008.

"Natural citrullination of the chemokines CXCL8/IL-8 and CXCL10/IP-10 results in reduced inflammatory activity" at the 2nd European Congress of Immunology, Berlin, Germany, September 13-16, 2009.

“Citrullination of chemokines as a regulatory mechanism for biological activity” at the Gordon Conference on Chemotactic Cytokines, Lucca, Italy, May 30 – June 5, 2010.

EU1, UAB, Leonardo Pardo has been invited to the following meetings:

Molecular mechanisms for agonist-induced activation of rhodopsin-like GPCRs. 16th Camerino-Noordwijkerhout symposium. Camerino, Italy, 2007

Deciphering mechanisms of GPCR activation. 13th International Conference on Retinal Proteins. Barcelona, Spain, 2008

Agonists, inverse agonists, and allosteric modulators of G protein-coupled receptors. Frontiers in Medicinal Chemistry. Barcelona, Spain, 2009

Agonists, inverse agonists, and allosteric modulators of G protein-coupled receptors. 8th Annual Congress: G Protein Coupled Receptors in Drug Discovery. Berlin, Germany, 2010

Deciphering mechanisms of GPCR activation. Keystone Symposia on Molecular and Cellular Biology. Breckenridge, Colorado, 2010

4.3. Durability of the IAP

As stated above, GPCR will certainly remain a hot topic for the coming years. As GPCRs are major targets for development of drugs (about 40% of current active drugs are targeting GPCRs), the pharmaceutical industry maintains a strong effort in applied research in this field. It is our opinion that, in order for this field to lead to further important developments with application to human health, but also to food and perfume industry and agriculture, an even stronger effort should be devoted to basic research. The IAP network, with its exceptionally wide spectrum of expertise, is expected to continue to play an important role in GPCR basic research.

The partners, and the network as a whole, will however have to adapt their activities to the evolution of the field (as described under 4.1.3.). This will involve a growing focus on structural data. The partnership has so far not involved much in this direction. This was justified by the well recognized difficulty to solve the structure of membrane proteins in general. These aspects were therefore tackled only by a few highly specialized groups. With the recent evolution of this field however, and its expected explosion in the coming years, it is now tempting to our network to conribute to this future development. Partner P1 has recently entered into a parallel network involving several Belgian labs and a number of foreign collaborators, with the aim of solving the structure of a set of GPCRs. The approach is based on the generation of single chain monoclonal antibodies (nanobodies) from camels, which will be used as tools for purifying and co-crystallize receptors. Despite the recent start of this program, it has led to a first success. Jan Steyaert (VUB) has received from Brian Kobilka purified β2 receptor, has generated nanobodies against the receptor bound to an agonist, and a resulting nanobody was used by Brian Kobilka to determine the crystal structure of the active form of the β2 receptor (data presented at the recent Keystone meeting on GPCRs). We intend to expand this approach to other receptors of interest, and some of the laboratories involved in this program could be included in a future IAP network.

The growing availability of structural data will put modeling approaches as a necessary component of many aspects of GPCR research. The partner EU1, who has the expertise to accomodate in real time all new data in this field, will therefore have a important role to play in the future. Also, with the accumulation of full genome sequences for a growing number of species, and the description of an increasing number of variants in human, bioinformatics and evolutionary aspects will become more and more important, and we will continue to develop the network in this direction.

Characterization of novel receptors will remain a major avenue of research for mammalian species, invertebrate species and yeast. As stated above, the strategies used to characterize new receptors will have to adapt, in order to take into account various hypotheses (receptor heterodimers, membrane-bound ligands, constitutive receptor without ligands, coupling to unconventional ccascades, low abundance or short-lived ligands, ...). Each of these hypotheses will require specific technical approaches, and accumulation of distribution and functional data in order to select these approaches adequately. For vertebrate receptors, we will pursue our focus onto receptors of the immune system, central nervous system, but also on receptors expressed in various stem cell populations.

The network will also pursue its contribution to the characterization of the role of various receptor families s in physiological processes and diseases. This will involve a steady usage of animal models, including knockout and transgenic mice, but also of other models such as zebrafish, which is much faster to handle. This will involve a growing importance of disease models, brought in by partner P5, but will also require the recruitment of other specific models outside the network (as it is done currently). The various partners will pursue and possibly expand their contacts with pharmaceutical and biotechnology companies interested in the applications of their research activities in the frame of human health, agriculture, and the food and perfume industries, according to the receptor families.

5. OUTPUT

5.1. Selection of 10 publications or co-publications

Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, Fung JJ, Choi HJ, Thian FS, Kobilka TS, Puglisi JD, Weis WI, Pardo L, Prosser RS, Mueller L, Kobilka BK (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. Nature 463:108-112. (IF: 31.4)

Bondue B, Glineur S, De Nadai P, De Henau O, Communi D, De Vuyst P, Desmecht D and Parmentier M (2010). Essential role of ChemR23 for plasmacytoid dendritic cell recruitment and anti-viral immunity in a mouse model of viral pneumonia. J Exp Med, under revision.

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. (IF: 6.3)

Lindemans M, Liu F, Janssen T, Husson S, Mertens I, Gäde G, Schoofs L (2009). Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in Caenorhabditis elegans. Proc Natl Acad Sci USA 106: 1642-1647. (IF: 9.4)

Loos T, Mortier A, Gouwy M, Ronsse I, Put W, Lenaerts JP, Van Damme J, Proost P (2008). Citrullination of CXCL10 and CXCL11 by peptidylarginine deiminase: a naturally occurring posttranslational modification of chemokines and new dimension of immunoregulation. Blood 112:2648-2656. (IF: 10.4)

Peeters T., W. Louwet, R. Geladé, D. Nauwelaers, J.M. Thevelein and M. Versele (2006). Kelch-repeat proteins interacting with the Gα protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. Proceedings of the National Academy of Sciences (USA) 103, 13034-13039. (IF: 9.4)

Popova Y, P Thayumanavan, E Lonati, M Agrochão and JM Thevelein (2010). Transport and signaling through the phosphate-binding site of the yeast Pho84 phosphate transceptor. Proc Natl Acad Sci USA 107: 2890-2895. (IF: 9.4)

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. (IF: 10.4)

Proost P, Loos T, Mortier A, Schutyser E, Gouwy M, Noppen S, Dillen C, Ronsse I, Conings R, Struyf S, Opdenakker G, Maudgal PC, Van Damme J (2008). Citrullination of CXCL8 by peptidylarinine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation. J Exp Med 205:2085-2097. (IF: 15.6)

Van Zeebroeck G, B Monge Bonini, M Versele and JM Thevelein (2009). Transport and signaling via the amino acid binding site of the yeast Gap1 amino acid transceptor. Nature Chemical Biology 5: 45-52. (IF: 14.6)

5.2. Appeal of the IAP

We feel that the best way to ensure visibility and appeal of the network is to produce the best possible science, to publish them in the best journals, and to disseminate the results in top international meetings. The groups and the collaborative activities are certainly highly visible in the GPCR field and their subdomains (chemokines for P1 and P4, glycoprotein hormones for P1, modelling and structure for EU1, insect endocrinology for P2, yeast metabolism for P3, mouse disease models for P5).

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 the participation of the partners to European networks, and open positions in the network laboratories.

The network is also also involved in dozens of collaborations with excellent research groups, mainly in Europe, North America and Asia, but also in Africa and South America. This contributes also greatly to the visibility of the groups and the network and the output in terms of publications. Students obtaining their PhD in the network choose very often to spent part of their subsequent career in foreign laboratories, and the excellent reputation of the network partners allows them to obtain post-doc positions in world-class groups in top Universities and Research Centers (Oxford and Cambridge in UK, Harvard, UCSF, UCSD, Rockefeller, Memorial Sloan-Kettering Cancer Center in USA, among others).

Visibility of the network is also achieved through the valorization of previous and present GPCR research by the biotechnology and pharmaceutical industry. Three biotech companies have been created as the result of partner P1 activities in GPCR research. Euroscreen SA (http://www.euroscreen.com/) is a spin-off company created in 1994, which is active in the development of screening tools and services for the pharmaceutical industry, and has developped his own programs of drug development). Chemcom (http://www.chemcom.be/), which is working on applications of olfactory and taste receptors (belonging to the GPCR family) intends to become a major player in the discovery of products and tools relevant to chemosensory technologies: e.g. taste, olfaction and other sensory modalities. TecnoScent (http://www.tecnoscent.com/), is a biotech company dedicated to applications of human olfaction. Collaborations are maintained between these companies and P1, while partnerships have also been established with other companies (among which UCB).

5.3. PHD and postdoc training

Number of PhD students and postdocs financed by IAP and not financed by IAP trained in the last 3 years

5.3.1. Ph D students

5.3.1.1. Financed by IAP

Eight PhD students have been supported by the IAP in the different groups:
P1. Edgard Jacoby, Aude Guillabert
P2. None
P3. Griet Van Zeebroeck
P4. Katrien Deroost, Kathleen Michiels, Hannelien Verbeke, Eva Moelants
P5. None
EU1. Angel Gonzalez

5.3.1.2. Not financed by IAP

Altogether 85 PhD students have contributed to the program since 2007, as follows in the different groups:

P1. Abdehakem Ben Addi, Galadrielle Biver, Nathalie Bles, Benjamin Bondue, Laurence Bosmans, Dorothée Cammarata, Olivier De Henau, Cédric Depoorter, Thalie Devose, Larissa Di Pietrantonio, Michael Horckmans, Khaoula Ichiba, Tiffany Loy, Stéphane Mievis, Davide Pintani, Denis Sohy, Audrey Ssutherland, Maxime Zoenen.

P2. In the lab of **P2**, 17 PhD students are currently being trained under supervision of the different PI's and postdocs, while, since 2007, 14 others have obtained their PhD (Liesbeth Badisco, Kurt Boonen, Bert Breugelmans, Annelies Bronckaers, Carmen Francis, Ana Gutierrez, Kevin Heylen, Steven Husson, Tom Janssen, Marleen Lindemans, Ank Reumer, Tim Vandersmissen, Matthias Van Hiel, Heleen Verlinden). In addition, the lab has performed an extremely important educational role by providing lab training to a large number of bachelor and master students in different educational programmes of the university (mainly biology, biochemistry and biotechnology, biophysics, bioinformatics). As the current director of the educational programme of Biology at K.U. Leuven, J. Vanden Broeck has also established a 'Master of Biology' programme in English for international students.

P3. Joke Serneels, Wendy Louwet, Johan Kriel, Katrien Verschooten, Frederik Paulussen, Wim Schepers, De Graeve Stijn, Vanmiddelem Jan, Swinnen Steve, Bram Stynen, Steven Haesendonckx, Ben Souffriau, Katrijn De Brucker, Sarah Marinelli, Thiago Pais, Mekonnen Demeke, Yudi Yang, Jurgen Vandamme, Tom Den Abt, Marlies Kimpe, Tessa Moses, Lorena Lopez, Ken Peeters, Georg Hubmann, Hilde Van Houtte, Harish Nag, Sona Kucharikova, Frédérique Van Hauwenhuyse, Ines Delorge.

P4. Tamara Loos, Anneleen Mortier, Sam Noppen, Sandra Li, Jo Vandercappellen

P5. Karin Cloquette, Hussein Zezafoun, Nidal Al-Zamel, Mutien-Marie Garigliany, Els Van de Paar, Martin Dermine, Stéphanie Glineur, Nicolas Antoine-Moussiaux, Philippe Vanden Bergh, Thomas Fett

EU1. Jessica Sallander, Ivan Rodriguez

5.3.2. Post-docs

5.3.2.1. Financed by IP

Nine post-doctoral researchers have been supported by the IAP in the different groups:
P1. Isabelle Garcia, Anne Lefort, Roxana Mustata, Tom Van Loy
P2. Tom Van Loy, Heleen Verlinden
P3. Dries Castermans
P4. Nele Berghmans
P5. Anne Cornet
EU1. Gianluigi Caltabiano

5.3.2.2. Not financed by IAP

Altogether 49 post-doctoral researchers and staff scientists have contributed to the program, as follows in the different groups:

P1. David Communi, David Communi, Patricia De Nadai, Jolyn Johnson, Ingrid Langer, Souphalone Luangsay, Mélanie Picard, Bernard Robaye, Laurie Rouger, Jean-Yves Springael, Olivier Vosters

P2. 10 postdoctoral researchers have been associated to the lab and have performed studies that were to some extent related to the theme of the network, although they were not financed by the IAP-programme.

P3. Tine Schaerlaekens, Elke Nevoigt, Françoise Dumortier, Beatriz Bonini, Hélène Tournu, Yulia Popova, Peter Verhaert, Nelson Avonce, Marta Rubio-Texeira, Sofie Saerens, Dries Castermans, Yutaka Haitani, Maria Foulquie, Griet Van Zeebroeck, Alessandro Fiori

P4. Jo Van Damme, Sofie Struyf, Paul Proost, Mieke Gouwy, Philippe Van den Steen

P5. Annabelle Decreux, François Cornet, Soumya Pastoret, Benoit De Muynck, Patrick Stiernet, Laurent Zecchinon

EU1. Xavier Deupi, Arnau Cordomi

5.4. Young emerging research teams

The team of Daniel Desmecht (P5, ULG) is new in the consortium. This strength of this team is the development of animal models of diseases, particularly in the field of viral diseases of the lung. The first collaborations of this team was with partner PI (ULB), in the frame of the characterization of the knockout model of ChemR23, a receptor for the chemoattractant protein chemerin, which is recruiting immature plasmacytoid (and myeloid) dendritic cells, macrophage populations and NK cells. Studies with animal models take time, but the interaction was very fruitful. It allowed to demonstrate an important role of the chemerin/ChemR23 system in the pathogenesis of inflammation and viral lung diseases. A first co-publication has been submitted to J Exp Med and and is presently under revision for meeting the reviewers comments. Besides, a number of other viral models are being pursued on the ChemR23 model, and we have started to aplly the same models to mice invalidated for three other receptors studied by P1 and involved in the regulation of the immune system. In addition, P5 has established collaborations with

other groups of the consortium, particularly P4, and this will certainly lead in the future to co-publications as well. We feel therefore that the integration of this new partner has been very successful and has brought to the network an essential expertise.

ANNEX 1. Publications 2009-2010

1. Publications of each team

1.1. P1, ULB, Marc Parmentier

Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosisio D, Madonna S, Fortugno P, Gonzalvo-Feo S, Franssen JD, Parmentier M, De Pità O, Girolomoni G, Sozzani S (2009). Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. J Exp Med 206:249-258.

Aso E, Renoir T, Mengod G, Ledent C, Hamon M, Maldonado R, Lanfumey L, Valverde O (2009). Lack of CB1 receptor activity impairs serotonergic negative feedback. J Neurochem 109:935-944.

Azdad K, Gall D, Woods AS, Ledent C, Ferré S, Schiffmann SN (2009). Dopamine D2 and adenosine A2A receptors regulate NMDA-mediated excitation in accumbens neurons through A2A-D2 receptor heteromerization. Neuropsychopharmacology 34:972-86.

Bondue B, Glineur S, De Nadai P, De Henau O, Communi D, De Vuyst P, Desmecht D and Parmentier M (2010). Essential role of ChemR23 for plasmacytoid dendritic cell recruitment and anti-viral immunity in a mouse model of viral pneumonia. In revision

Cobellis G, Ricci G, Cacciola G, Orlando P, Petrosino S, Cascio MG, Bisogno T, De Petrocellis L, Chioccarelli T, Altucci L, Fasano S, Meccariello R, Pierantoni R, Ledent C, Di Marzo V (2010). A Gradient of 2-Arachidonoylglycerol Regulates Mouse Epididymal Sperm Cell Start-Up. Biol Reprod 82:451-458.

De Poorter C, Barstsoen K, Parmentier M and Springael JY (2010). Functional consequences of ChemR23 heteromerization with the chemokine receptors CXCR4 and CCR7. Submitted.

Devosse T, Guillabert A, D'Haene N, Berton A, De Nadai P, Noel S, Brait M, Franssen JD, Sozzani S, Salmon I, Parmentier M (2009). Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. J Immunol 182:4974-4984.

Di Virgilio F, Boeynaems JM, Robson SC (2009). Extracellular nucleotides as negative modulators of immunity. Curr Opin Pharmacol 9:507-513.

El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM (2009). Adenosine A(2A) receptor deficient mice are partially resistant to limbic seizures. Naunyn Schmiedebergs Arch Pharmacol 380:223-232.

Garcia MI, Ghiani M, Lefort A, Libert F, Strollo S, Vassart G (2009). LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. Dev Biol 331:58-67.

Hegyi Z, Kis G, Holló K, Ledent C, Antal M (2009). Neuronal and glial localization of the cannabinoid-1 receptor in the superficial spinal dorsal horn of the rodent spinal cord. Eur J Neurosci 30:251-262.

Lambot MA, Mendive F, Laurent P, Van Schoore G, Noël JC, Vanderhaeghen P, Vassart G (2009). Three-dimensional reconstruction of efferent ducts in wild-type and Lgr4 knock-out mice. Anat Rec 292:595-603.

Langer I, Tikhonova IG, Boulègue C, Estève JP, Vatinel S, Ferrand A, Moroder L, Robberecht P, Fourmy D (2009). Evidence for a direct and functional interaction between the regulators of G protein signaling-2 and phosphorylated C terminus of cholecystokinin-2 receptor. Mol Pharmacol 75:502-513.

Luangsay S, Wittamer V, Bondue B, De Henau O, Rouger L, Brait M, Franssen JD, de Nadai P, HuauxF, Parmentier M (2009). Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. J Immunol 183: 6489-6499.

Mato S, Alberdi E, Ledent C, Watanabe M, Matute C (2009). CB1 cannabinoid receptor-dependent and -independent inhibition of depolarization-induced calcium influx in oligodendrocytes. Glia 57:295-306.

Meis S, Hamacher A, Hongwiset D, Marzian C, Wiese M, Eckstein N, Royer HD, Communi D, Boeynaems JM, Hausmann R, Schmalzing G, Kassack MU (2010). NF546 [4,4'-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-alpha,alpha'-diphosphonic acid) tetrasodium salt] is a non-nucleotide P2Y11 agonist and stimulates release of interleukin-8 from human monocyte-derived dendritic cells. J Pharmacol Exp Ther 332:238-247.

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Poels J, Van Loy T, Vandersmissen HP, Van Hiel MB, Van Soest S, Nachman RJ, Vanden Broeck J (2010). Myoinhibiting peptides are the ancestral ligands of the promiscuous Drosophila sex peptide receptor. Cell Mol Life Sci (in press).

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Struyf S, Noppen S, Loos T, Mortier A, Gouwy M, Verbeke H, Huskens D, Luangsay S, Parmentier M, Geboes K, Schols D, Van Damme J, Proost P (2009). Citrullination of CXCL12 Differentially Reduces CXCR4 and CXCR7 Binding with Loss of Inflammatory and Anti-HIV-1 Activity via CXCR4. J Immunol 182:666-674.

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Van Hiel MB, Van Wielendaele P, Temmerman L, Van Soest S, Vuerinckx K, Huybrechts R, Vanden Broeck J, Simonet G (2009). Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions. BMC Mol Biol 10: 56.

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Marchal E, Vandersmissen HP, Badisco L, Van de Velde S, Verlinden H, Iga M, Van Wielendaele P, Huybrechts R, Simonet G, Smagghe G, Vanden Broeck J (2010). Control of ecdysteroidogenesis in prothoracic glands of insects: a review. Peptides 31: 506-519.

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1.3. P3, KUL, Johan Thevelein

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1.4. P4, KUL, Jozef Van Damme

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2. Co-Publications

P1-P4. Struyf S, S Noppen, T Loos, A Mortier, M Gouwy, H Verbeke, D Huskens, S Luangsay, M Parmentier, K Geboes, D Schols, J Van Damme and P Proost (2009). Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4. J Immunol 182: 666-674.

P1-P2. Van Loy T (P1), Vandersmissen HP, Poels J, Van Hiel MB, Verlinden H, Vanden Broeck J (P2) (2010). Tachykinin-related peptides and their receptors in invertebrates: a current view. Peptides, 31: 520-524.

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P1-P5. Bondue B, Glineur S, De Nadai P, De Henau O, Communi D, De Vuyst P, Desmecht D and Parmentier M (2010). Essential role of ChemR23 for plasmacytoid dendritic cell recruitment and anti-viral immunity in a mouse model of viral pneumonia. In revision

ANNEX 2. Co-Publications of the network 2007-2010

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ANNEX 3. Programs of the annual meetings

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 profilling 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.

PAI/IUAP 6/14 : 2008 meeting
Friday November 28, 2008
Venue: "Huis Bethlehem", Schapenstraat 34, B-3000 Leuven (see map)

9h00-9h20 Welcome-coffee

9h20 Introduction: ***M. Parmentier***
9h30-10h00 WP1: Structural organization of GPCRs

- ***Caltabiano Gianluigi (EU1, University of Barcelona)***
  Thyrostimulin, how does it bind to TSHr? Work in Progress

10h00-11h00 WP3: Signaling cascades activated by GPCRs

- ***Tamara Loos (P4, KUL)***
  Gene regulation and posttranslational modification of CXCL10/IP-10 -quantity versus quality
- ***Mieke Gouwy (P4, KUL)***
  Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events

11h00-11h30 Coffee break

11h30-13h00 WP4: Characterization of receptors in physiological processes

- ***Bles Nathalie (P1, ULB)***
  Angiogenic properties of human dendritic cells through P2Y activation
- ***Rubio-Texeira Parta (P3, KUL)***
  Constitutive-like activity induced by specific dipeptides in the yeast Gap1 amino acid transceptor
- ***Kriel Johan (P3, KUL)***
  Constitutively active alleles of the yeast Gap1 amino acid transceptor

13h00-14h00 Lunch at the "STUK kunstencentrum", Naamsestraat 96, B-3000 Leuven

14h00-15h00 Poster session and networking

15h00-16h00 WP5: Role of GPCRs in human diseases and animal models

- ***Garigliany Mutien-Marie (P5, ULg)***
  H5N1 vs. H1N1 lethal flu in mice : similar or distinct diseases?
- ***Luangsay Souphalone (P1, ULB)***
  Role of chemerin and its receptor ChemR23 in inflammatory disease model

16H00-16h30 Coffee break
Meeting of principal investigators with Véronique Feys (BELSPO)

16h30-17h30 WP6: Identification of novel receptors and their ligands

- ***Poels Jeroen (P2, KUL)***
  Analysis of GPCRs using *Drosophila melanogaster* as a model system
- ***Verlinden Heleen (P2, KUL)***
  GPCR transcript profiling during phase transition and RNA interference in the desert locust, *Schistocerca gregaria*

17h30-18h15 Management meeting

PAI/IUAP 6/14 : 2008 meeting
Friday November 28, 2008
Posters

WP1: Structural organization of GPCRs

Cases Montserrat et al. (EU1, UAB). The steep way to the small GPHr ligand's discovery.

WP2: Dimerization of GPCRs

Springael Jean-Yves et al. (P1, ULB). Allosteric interactions regulate the function of chemokine receptor oligomers

Zoenen Maxime et al. (P1, ULB). Inverse relation between constitutive activity and negative cooperativity in mutants of glycoprotein hormone receptors.

WP3: Signaling cascades activated by GPCRs

Kimpe Marlies et al. (P3, KUL). **Mechanism of Gap1 amino acid transceptor signaling in yeast**

Schepers Wim et al. (P3, KUL). **Nutrient-induced phosphorylation of the PKA target trehalase in yeast**

Struyf Sofie et al. (P4, KUL). Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding, with loss of inflammatory and anti-HIV-1 activity via CXCR4.

Proost Paul et al. (P4, KUL). Citrullination of CXCL8 by peptidylarginine deiminase alters receptor usage, prevents proteolysis, and dampens tissue inflammation.

Loos Tamara et al. (P4, KUL). Gene regulation and posttranslational modification of CXCL10/IP-10 -quantity versus quality.

Gouwy Mieke et al. (P4, KUL). Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events.

Cornet Anne et al. (P5, ULg). The antiviral Bos taurus Mx1 GTPase interferes with NF-kappaB-dependent pathways.

WP4: Characterization of receptors in physiological processes

Robaye Bernard et al. (P1, ULB). Knock-out mice reveal a role for P2Y6 receptor in macrophages, endothelial cells and vascular smooth muscle cells

Marchal Elisabeth et al. (P2, KUL). Q-RT-PCR profiling of Cyp genes in the biosynthetic pathways of 20-hydroxyecdysone and juvenile hormone in the desert locust.

WP5: Role of GPCRs in human diseases and animal models

Bondue Benjamin et al. (P1, ULB). Anti-inflammatory function ChemR23 in viral pneumonia and acute lung injury

Dermine Martin et al. (P5, ULg). SJL-specific innate resistance to mouse respiratory syncytial virus correlates with viral proteins accumulation in alveolar macrophages during the early phase of infection.

WP6: Identification of novel receptors and their ligands

Garcia Marie-Isabelle (P1, ULB). Altered cell lineage differentiation in fetal lgr5-null mice

Vandersmissen Hans Peter et al. (P2, KUL). Characterizing *Drosophila* LGR1: expression profiling and initial RNAi study.

Van Loy Tom et al. (P2, KUL). Characterization and distribution of NKD, a receptor for *Drosophila* tachykinin-related peptide 6.

PAI/IUAP 6/14 : 2009 meeting
Friday February 5, 2010
Venue: Campus Erasme, 808 route de Lennik, 1070 Bruxelles
Bat F, Room F2-303 (see map)

9h00-9h10 Welcome

9h10-10h40 WP3: Signaling cascades activated by GPCRs

- ***Jurgen Vandamme (P3, KUL)***

Phosphorylation targets in feedback-inhibition of Gpr1-induced PKA activation on cAMP production

- ***Yulia Popova (P3, KUL)***

Transport and signaling through the phosphate binding site of the Pho84 phosphate transceptor

WP4: Characterization of receptors in physiological processes

- ***Marie Garcia (P1, ULB)***

Role of the orphan Leucine-rich repeat G-coupled receptor Lgr4 in mouse postnatal intestinal development

10h40-11h10 Coffee break

11h10-12h10 WP4: Characterization of receptors in physiological processes

- ***Paul Proost (P4, KUL)***

CXCL8/interleukin-8-induced leukocyte migration is affected by chemokine truncation and citrullination

- ***A. Cornet (P5, ULg)***

Bos taurus anti-influenza Mx1 GTPase controls NF-kB activation by interacting with TRAF6

12h10-14h00 Lunch, Management meeting, Poster session and networking

14h00-16h00 WP1: Structural organization of GPCRs

- ***Leonardo Pardo (EU1, UAB, Barcelona, Spain)***

Molecular mechanisms of ligand-regulated activation and oligomerization of G protein-coupled receptors

WP5: Role of GPCRs in human diseases and animal models

- ***Benjamin Bondue (P1, ULB)***

Role of chemerin and its receptor ChemR23 in the physiopathology of viral pneumonia

- ***Hannelien Verbeke (P4, KUL)***

Expression of angiostatic PF-4var (CXCL4L1) counterbalances angiogenic impulses of IL-8/CXCL8 and SDF-1/CXCL12 in esophageal and colorectal cancer

- ***Martin Dermine (P5, ULg)***

Elucidating the innate resistance opposed by SJL mice to respiratory syncytial virus: first clues pointing at alveolar macrophages

16H00-16h30 Coffee break

16h30-17h30 WP6: Identification of novel receptors and their ligands

- ***Matthias (Boris) Van Hiel (P2, KUL)***

Leu-rich repeats containing G protein-coupled receptors in the fruit fly Drosophila melanogaster

- ***Heleen Verlinden (P2, KUL)***

Biogenic amines, G protein-coupled receptors and their possible role in the behavioural polyphenism of locusts

17h30 Closure

PAI/IUAP 6/14 : 2009 meeting
Friday February 5, 2010
Posters

WP2: Dimerization of GPCRs

Jean-Yves Springael et al. (P1, ULB). Hetero-oligomerization of CCR2, CCR5 and CXCR4 and the protean effects of "specific" antagonists

et al. (P1, ULB). Title.

WP3: Signaling cascades activated by GPCRs

Marlies Kimpe et al. (P3, KUL) Gap1 amino acid signaling and translation initiation in S. cerevisiae.

Griet Van Zeebroeck et al. (P3, KUL) Transport and signaling through the amino acid binding site of the yeast Gap1 amino acid transporter.

Marta Rubio et al. (P3, KUL) Effects of agonists of Gap1-dependent activation of PKA targets on downregulation of amino acid permease activity.

Steven Haesendonckx et al. (P3, KUL) Role of Pkh protein kinases in rapid nutrient induced PKA activation in yeast.

WP4: Characterization of receptors in physiological processes

Olivier Vosters et al. (P1, ULB) Immunomodulatory properties of vasoactive intestinal peptide observed on macrophages but not on dendritic cells.

Mieke Gouwy et al. (P4, KUL) CC chemokine ligand-2 synergizes with the nonchemokine G protein-coupled receptor ligand fMLP in monocyte chemotaxis, and it cooperates with the TLR ligand LPS via induction of CXCL8.

Jo Vandercappellen et al. (P4, KUL) The angiostatic chemokine platelet factor-4 variant (CXCL4L1/PF-4var) and its COOH-terminal derived peptide (CXCL4L1/PF-4var47-70) strongly inhibit angiogenesis and suppress tumor growth in vivo.

Rut Vleugels et al. (P2, KUL) RNAi study in the desert locust, Schistocerca gregaria: Knockdown of PKA and PKG

WP5: Role of GPCRs in human diseases and animal models

Hannelien Verbeke et al. (P4, KUL) Expression of angiostatic PF-4var (CXCL4L1) counterbalances angiogenic impulses of IL-8/CXCL8 and SDF-1/CXCM12 in esophageal and colorectal cancer.

Stéphane Mievis et al. (P1, ULB) Effects of adenosine A2A receptor invalidation in a transgenic mouse model of Huntington's disease.

Benjamin Bondue et al. (P1, ULB) Anti-inflammatory function of chemerin in LPS-induced acute lung injury.

Els Van De Paar et al. (P5, ULg) Profiling of cytokine response in influenza-resistant C57BL/6 and influenza-susceptible DBA/2 inbred mouse lines.

WP6: Identification of novel receptors and their ligands

Pieter Van Wielendaele et al. (P2, KUL) Neuropeptides involved in the control of reproduction and hormone biosynthesis in locusts

Hans Peter Vandersmissen et al. (P2, KUL) Unraveling the expression pattern of Drosophila LGR1 using the GAL4/UAS system