



Interuniversity Attraction Poles (IAP) Phase VI

2007 – 2011

IAP 6-14

G PROTEIN-COUPLED RECEPTORS, FROM STRUCTURE TO DISEASES

Acronym : GPCRs

Report 2008

March 25, 2009

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NETWORK COMPOSITION

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WORKPACKAGE 1. STRUCTURAL ORGANIZATION OF GPCRS

1.1. Structural models of GPCRs (EU1, P1)

1.1.1. Glycoprotein-hormone receptors.

The glycoprotein hormone receptor (GpHR) family is peculiar because, in contrast to other GPCRs, a large N-terminal extracellular ectodomain is responsible for hormone recognition. We have identified certain 'hot spot' positions both at the hormone and the concave inner surface of the receptor that play attractive and repulsive interactions for binding and specificity, respectively (De Leener et al. 2008; Caltabiano et al. 2008)

Mining of the human genome permitted to identify, by the group of A.J. Hsueh in 2002, two novel human glycoprotein hormones, which form heterodimerization (named thyrostimulin), and are able to activate human TSH receptors, but not LH and FSH receptors. We have extended the proposed determinants for hormone binding and specificity to thyrostimulin.

1.1.2. Rhodopsin-like GPCRs.

During 2008, the crystal structures of the β_1 -adrenergic receptor; the adenosine A_{2A} receptor; and opsin, both in its ligand-free and in its G-protein-interacting conformation, have been published. 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. In addition, the recent structure of opsin bound the carboxyl terminus of the $G\alpha$ -subunit of transducin shows that the G protein interacts with residues in the inner side of the cytoplasmic TM5 and TM6. These structures, together with the previously reported crystal structures of rhodopsin and the β_2 -adrenergic receptor, 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.

1.2. Activation mechanisms

Glycoprotein-hormone receptors (P1, EU1)

We have published the report regarding the identification of the first germline mutation in the extracellular domain of the follitropin receptor responsible for spontaneous ovarian hyperstimulation syndrome, which was described in the previous report. This study allowed to study the relation between binding and activation of glycoprotein hormone receptors, on the basis of the 3D models (De Leener et al. 2008).

Besides, we have described a new mutation of the TSH receptor causing 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 reported a Turkish family with a novel TSHr gene mutation with distinct features all consistent with ADNAH. Thyroid function tests of the proband were as follows: free T3: 13.1 pg/ml (N: 1.8-4.6); free T4: 5.1 ng/dl (N: 0.9-1.7); TSH: 0.01 microIU/ml (N: 0.2-4.2); and TSH receptor antibody: 2 IU/ml (N: 0-10). A heterozygous missense mutation in exon 10 of the TSHr gene (C1454T) resulting in the substitution of valine for alanine at codon 485 (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. Our data 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, 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).

1.3. Ligand-receptor interactions

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

On the basis of the 3D model of ChemR23, a number of amino acids potentially involved in the binding site of the chemerin-9 nonapeptide were identified. These residues were mutated to alanine, the mutants were expressed in CHO-K1 cells, and they were tested for their binding and functional properties. Positions R5.42 and Y6.51 were identified as important for the binding of the peptide. Arg5.42 was identified as a partner for the carboxyl group of the chemerin-9 nonapeptide. We are now focusing on the binding site of the cystatin-like domain of chemerin.

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

Neuropeptides related to vertebrate tachykinins have been identified in *Drosophila* and are referred to as drosotachykinins, or DTKs. 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. Whereas DTKR was shown to be activated by DTKs (Birse et al., 2006; Poels et al., 2007) 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 now 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 (FXAXRa instead of the consensus sequence FXGXRa). Our findings suggest that NKD and DTKR provide substrates for two functionally and spatially separated peptide signaling systems (Poels et al., 2009).

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

Selective opioid receptor antagonists can be used as pharmacological tools to distinguish the activity of different types of opioid receptors. In a collaboration of P2 with prof. Janecka (University of Lodz, Poland), amino acid substituted opioid receptor ligands are being developed and their corresponding functional properties (agonism, antagonism) tested. We therefore use a screening system that is based on the co-expression of the Ca²⁺-sensitive photoprotein 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 two novel, potent and selective μ -opioid receptor antagonists. The effectiveness of selected antagonists will be further tested by means of an *in vivo* assay 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-1 β /CCL4), by grafting into it the key N-terminal pharmacophore region from PSC-RANTES. We were able to identify MIP-1 β /CCL4 analogues that retain the receptor binding profile of MIP-1 β /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).

WORKPACKAGE 2. DIMERIZATION OF GPCRS

2.1. Chemokine receptors (P1)

CCR2/CCR5, CCR2/CXCR4 and CCR5/CXCR4 heterodimers. We reported previously the existence of negative binding cooperativity between the subunits of CCR2/CCR5 and CCR2/CXCR4 heterodimers (El-Asmar et al., 2004, Springael et al., 2006, Sohy et al., 2007). We extended these observations to heterodimers formed by CXCR4 and CCR5 demonstrating that specific agonists and antagonists of one receptor can compete allosterically for the binding of a specific tracer of the other when the two receptors are co-expressed. CCR2, CCR5 and CXCR4 form thus homodimers as well as heterodimers 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. Importantly, we also showed that specific antagonists of one receptor inhibit the binding of chemokines to the other receptor as a consequence of their heterodimerization, both in recombinant cell lines and primary leukocytes. This resulted in a significant functional cross-inhibition in terms of calcium mobilization and chemotaxis. 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 SDF-1 α . 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 heterodimerization, with important implications on the activities of chemokine receptor antagonists *in vivo*. We have started to study a larger range of antagonists in order to test whether trans-inhibition is a property shared by all antagonists of these receptors or restricted to some molecules only. 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. Expression of CCR7 had no major effect on the EC50 values of the dose-response curves but decreased drastically the maximal response. This decrease of response was however 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 MCP-1 and SDF-1 β binding by MIP-1 β is specifically abrogated in leukocytes derived from a CCR5 Δ 32/ Δ 32 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 SDF-1 α . 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 mSDF-1 α binding on BMDC and that this cross-inhibitory effect is specifically lost in cells generated from ChemR23 KO mice. We are currently testing the relevance of other heterodimers on primary leukocytes that coexpressed endogenously receptors of interest.

2.2. ChemR23 and other receptors (P1)

Heterodimerization of ChemR23 with CXCR4 and CCR7. 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. These results support the view that negative binding cooperativity takes also place across receptors that bind ligands structurally unrelated. We also showed on mouse BMDC expressing endogenously ChemR23 and CXCR4, that chemerin competed for SDF-1 α 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.

WORKPACKAGE 3. SIGNALING CASCADES ACTIVATED BY GPCRS

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

3.1. 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-1 β . 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 $\frac{1}{2}$ 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. 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 (Proost et al., 2008).

3.2. 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 extracellular signal-regulated kinase 1/2 phosphorylation and calcium mobilization was significantly enhanced by CXCL8 in monocytes, 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).

3.3. 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 analysed on a genome-wide scale resulting from defective GPCR signaling. As a pilot experiment, we conducted microarray hybridization experiments utilizing mutant *Drosophila* strains, such as *ricketts* (i.e. expressing non-functional DLGR2 receptors) and *pupal* (i.e. with defective bursicon production), to examine molecular events that underlie the processes of cuticle sclerotization, melanization and wing spreading behaviour i.e. interrelated processes that previously have been shown to be initiated by the neurohormone bursicon (the ligand for DLGR2). Much effort has been put in experimental design and data analysis. 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 would like to apply a similar approach to decipher signalling pathways initiated by other insect GPCR/ligand couples.

3.4. Glucose-sensing receptor (P3)

In yeast the cAMP Protein kinase A (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 indispensable for the proper response of the yeast cellular machinery to the nutrient conditions. Yeast cells (*Saccharomyces cerevisiae*) contain a glucose/sucrose sensitive GPCR Gpr1, that detects its extracellular ligand and activates adenylylate cyclase (Cyr1) through the G α 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), adenylylate cyclase gets 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 activity). We showed that GTP-bound Gpa2 and both GTP- and GDP-bound Ras proteins are able to bind to adenylylate cyclase independently. As expected from this, in both states Ras proteins are able to activate Cyr1

even in the absence of Gpa2. In contrast, Gpa2 was unable (in both states) to activate Cyr1 in the absence of Ras. Moreover, we clearly demonstrated *in vitro* and *in vivo* that both Gpa2 and Ras proteins are required for Cyr1 to become activated to its full potential.

The intracellular concentration of cAMP has to be strictly controlled. PKA activates the phosphodiesterases Pde1 and Pde2, which break down cAMP to AMP. On the other hand, PKA (activated by cAMP) 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 mechanisms: absence of downregulation of Cyr1 activity (feedback inhibition) and absence of activation of the Pde enzymes.

Recently, we revealed that the protein phosphatases PP2A and PP1 are both under direct control of glucose sensing. We showed 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).

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. In mammalian cells, the protein kinase PDK1 controls a myriad of processes through substrate phosphorylation.

WORKPACKAGE 4. FUNCTIONAL CHARACTERIZATION OF RECEPTORS IN PHYSIOLOGICAL PROCESSES

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

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

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

Chemerin was evaluated for its role in the recruitment of pDC in psoriasis skin. Prepsoriatic skin adjacent to active lesions and early lesions were characterized by a strong expression of chemerin in the dermis and by the presence of CD15⁺ neutrophils and CD123⁺/BDCA-2⁺/ChemR23⁺ pDC. Conversely, skin from chronic plaques showed low chemerin expression, segregation of neutrophils to epidermal microabscesses, and few pDC in the dermis. Chemerin expression was localized mainly in fibroblasts, mast cells, and endothelial cells. Fibroblasts cultured from skin of psoriatic lesions expressed higher levels of chemerin messenger RNA and protein than fibroblasts from uninvolved psoriatic skin or healthy donors and promoted pDC migration in vitro in a chemerin-dependent manner. Therefore, chemerin expression specifically marks the early phases of evolving skin psoriatic lesions and is temporally strictly associated with pDC. These results support a role for the chemerin/ChemR23 axis in the early phases of psoriasis development (Albanesi et al. 2009).

To determine in vivo the physiological role of chemerin, we investigated whether chemerin is able to regulate inflammation in a mouse acute lung injury model. As chemerin nonapeptides are known to be rapidly degraded by proteases, we used whole chemerin to investigate the consequences of ChemR23 activation over a time course experiment of 72 hours. WT mice were injected trans-orally with LPS alone (1 µg) or in combination with a single dose of recombinant chemerin (5 µg), and neutrophils were counted in the bronchoalveolar lavage (BAL) after 6, 12, 24 and 72h. LPS induced a strong inflammatory reaction in the lung, with a maximum influx of neutrophils reached 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 level in BAL fluids peaked between 12 and 24h, we investigated the role of chemerin in WT and ChemR23 KO 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 KO mice, as compared to WT mice, with an increase of neutrophils (50%) and macrophage (94%) content in the BAL fluid. Moreover, 18h after the LPS + chemerin challenge in WT mice, neutrophils were decreased significantly by up to 70% as compared to mice receiving LPS only. ChemR23 KO mice did not respond to chemerin and displayed similarly high levels of neutrophils. In LPS-treated WT mice, chemerin also increased macrophage recruitment. This effect is attributed to the intrinsic chemotactic activity of chemerin on mononuclear cells. Mice deficient for ChemR23 exhibited similar macrophage levels in response to LPS, whether they received or not chemerin. We also measured a set of inflammatory mediators (KC/CXCL1, IL-6, TNF- α and IL-1 β) in BAL fluids as hallmarks of the lung inflammatory response, in WT and KO mice. PBS and chemerin alone did not promote an increase in the cytokine levels over baseline, while LPS treatment resulted as expected in the overexpression of these mediators. 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-1 β , 73% for TNF- α). In ChemR23 KO 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, which is strictly dependent on ChemR23.

The work regarding the proteolytic events that control negatively chemerin activity were finalized and published (Guillabert et al. 2008). We demonstrated that neutrophil-derived proteinase 3 (PR3) and mast cell chymase are involved in the generation of specific and stable inactive chemerin variants. PR3 specifically converts prochemerin into an inactive chemerin form lacking the last eight carboxy-terminal amino-acids. PR3 has no effect on bioactive chemerin. Mast cell chymase abolishes chemerin activity by the removal of three additional amino acids from its C-terminus. Mast cell chymase does not use prochemerin as a substrate.

4.2 Human and mouse FPRL2 (P1, P4 and P5)

We have finalized and published our study regarding the distribution of FPRL2 in leukocytes by Q-PCR and flow cytometry (Devosse et al. in press). We have completed the characterization of

new cellular targets of the FPRL2 ligand (F2L), namely macrophages, plasmacytoid DCs and eosinophils. To this end, we investigated the biological activity of F2L on monocyte-derived macrophages and native pDCs. In microchemotaxis Boyden's chambers, F2L promoted chemotaxis of macrophages peaking for concentrations of 1 to 10 nM. Chemotaxis of pDCs was also promoted by hF2L with a peak at 10 nM. The migration indexes obtained with F2L for macrophages and pDCs were comparable to those of respectively fMLF (10 nM) and SDF-1 (10 nM), used as a positive controls. These data demonstrate the physiological role of F2L on human macrophages and pDCs recruitment, through its action on FPRL2.

We have identified previously the mouse receptor Fpr2, encoded by Fpr-rs2, as a partial functional homologue of human FPRL2, and demonstrated that mouse neutrophils expressing Fpr2 responded to mouse F2L (mF2L) in a chemotaxis assay. With the aim of comparing mouse Fpr2 expression with that of human FPRL2, we tested the presence of Fpr2 transcripts in various mouse leukocyte populations, using GAPDH as a housekeeping control gene. RT-PCR showed expression of Fpr-rs2 on mouse bone marrow-derived DCs and peritoneal macrophages. Activation of DCs with LPS induced up-regulation of Fpr2 transcript levels. In order to test the physiological relevance of F2L activity on Fpr2, we attempted to identify the production of endogenous mouse F2L (mF2L) *in vivo*. By analogy with the initial identification of hF2L in human, we fractionated extracts from mouse spleen by reverse phase HPLC, and tested the activity of the fractions on a cell line coexpressing human FPRL2, apoaequorin and G α_{16} . For testing whether the peptide is generated in inflammatory conditions, we used untreated mice, but also mice receiving a sublethal dose of LPS (600 μ g in 200 μ l PBS *i.p.*). Starting from about 0.5 g of spleen tissue, a biological activity was purified through three successive HPLC steps. This activity was much stronger in LPS-stimulated conditions. The partially purified material had no activity on control cell lines. The elution behaviour of the bioactivity was identical to that of human F2L tested in the same conditions. This is consistent with the conservation of the peptide between human and mouse which differ only by one amino acid (conservative K/R replacement at position 6). Mass spectrometry analysis of the partially purified bioactive fraction resulting from the third HPLC column revealed a mass corresponding to the predicted sequence for the mouse F2L peptide. We have therefore detected in mouse spleen an activity specific for FPRL2, which is increased in inflammatory conditions and which behaves as F2L on several HPLC columns.

The ability of mouse F2L (mF2L) to promote chemotaxis of neutrophils through Fpr2 was demonstrated previously. We tested further the capacity of mF2L to induce recruitment of DCs and macrophages, which also express the receptor. mF2L was tested in 48-well microchemotaxis chambers at concentrations ranging from 100 nM to 30 μ M. The peptide promoted chemotaxis of mouse monocyte-derived DCs and peritoneal macrophages with a maximum around 10 μ M, with an efficacy similar to that CCL5/RANTES (50 nM) and fMLF (1 μ M) used as positive control respectively for DCs and macrophages.

We also investigated the chemoattractant activity of mF2L *in vivo*. In an air pouch assay, the cells recruited six hours after injection of 200 nM mF2L (or buffer) were counted. In three independent experiments, a significantly larger number of cells was collected in mF2L-injected pouches than in controls receiving PBS. The cells were labelled with CD11b-PE, CD11c-FITC and GR-1-PerCp to identify specific leukocyte populations. Neutrophils, DCs and macrophages were detected, the last two populations being significantly over-represented in the mF2L-injected pouches.

4.3. Glucocorticoid-induced receptor (P1)

The glucocorticoid-induced receptor (GIR/GPR83). Glucocorticoid-induced receptor (GIR or GPR83) is a receptor 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.

GIR is expressed in cholinergic interneurons of the striatum. In our targeting vector, part of the GIR coding region was replaced by a tau-lacZ fusion gene (placed under control of the natural GIR promoter) and selection cassettes. The tau-lacZ fusion protein was visualised by X-Gal

staining of brain slice in heterozygous animals. We observed a strong cellular staining in olfactory bulb, olfactory tubercle, nucleus of the olfactory tract, piriform cortex and scattered cells in dorsal striatum. Few cells were also stained in the thalamus, hypothalamus and CA3 region of hippocampus. In all cases, the neuronal processes were also stained. In the olfactory bulb, the localisation and shape of processes indicated the peri-glomerular identity for the GIR expressing-neurons. In the dorsal striatum, the size of the cell bodies and the arborisation suggested that GIR was expressed in large aspiny cholinergic interneurons. Co-immunostaining revealed 89% colocalization of GIR with choline acetyl-transferase (CHAT), a marker of cholinergic neurons. However cholinergic interneurons are also located in the ventral striatum where very few GIR-positive neurons were found, indicating the existence of a subclass of cholinergic interneurons in the dorsal striatum expressing GIR. Few other GIR-positive neurons did not colocalise with CHAT and had a distinctive elongated cell shape, with a single process.

GIR invalidation decreases motor learning ability. Cholinergic interneurons of the striatum have been shown to be involved in motor learning. We assessed the locomotor ability of GIR knock-out mice in four tests probing striatal and/or cerebellar function. In the rota-rod test, designed to assess motor coordination, knock-out mice displayed a reduced aptitude for coordinated movements and did not manage to learn the test even after 5 days of conditioning. Similarly, knock-out mice were inefficient compared to wild type in the strength-grip test, a test that involves muscular strength and coordination. The runway test and the foot print test are designed to explore cerebellar deficiencies. Wild type and knock-out mice displayed similar abilities in both tests. In addition to motor control, striatum also participates to motivational control and rewarding effects of drug of abuse. Dopaminergic activity in nucleus accumbens is primarily implicated in the rewarding properties of drugs such as cocaine. We assessed the rewarding effects of cocaine in the conditioned place preference test but did not observed differences between wild type and knock-out mice.

GIR invalidation affects the activity of the striatal network. Dopaminergic and cholinergic afferences modulate the GABAergic medium spiny neurons (MSNs). MSNs are commonly divided in two major subsets on the basis of their axonal projections and on the expression of neuropeptides and dopamine receptor subtypes. Striatonigral MSNs co-express D1 dopaminergic receptors, substance P and dynorphin, while striatopallidal MSNs co-express D2 dopaminergic receptors, A2a adrenergic receptors and enkephalin. Changes in striatal network activity are typically correlated with the expression level of these neuropeptides. Quantitative in-situ hybridization showed that enkephalin transcripts are slightly decreased while substance P and dynorphin transcripts were significantly increased in the caudate putamen of knock-out mice. In situ binding suggested that D1 and D2 receptors were not significantly modified in the striatum or cortex of GIR knock-out mice.

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. Scopolamine, an M2 receptor antagonist that suppresses the autocrine negative feedback of acetylcholine, increased acetylcholine secretion in both genotypes, but knock-out mice returned faster to a lower basal level. 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.

GIR invalidation affects the stress axis. Glucocorticoids and forskolin were initially described to induce GIR expression in a thymoma cell line. In the brain however, GIR expression is decreased by glucocorticoids in hypothalamus, suggesting a possible role in the regulation of stress. 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).

Motor control in stressful conditions. We monitored the spontaneous locomotor activity in actimetry boxes. On day 1, wild type and knock-out mice displayed similar locomotor activities.

After habituation and learning (day 2 and 3), knock-out mice displayed a lower activity for both the horizontal and vertical components. In the open field test, which is more stressful, GIR knock-out mice displayed a significantly higher locomotor activity during the three days of the test and spent more time in the central area. These results suggest an anxiolytic-like phenotype. Mice were tested in behavioral tests probing more specifically anxiety, namely the light and dark box and the elevated plus maze. Although the knockout animals displayed higher locomotor activity in these arenas, the parameters reflecting anxiety were not significantly different between genotypes. We assessed the effect of the D1 agonist SKF38393 and the M2 antagonist scopolamine on the locomotory activity of wild type and knock-out mice in the open-field test. Both drugs resulted in a similar biphasic effect on GIR knockout mice. At low doses, they did not affect wild type mice but decreased the locomotor activity of knockout mice to the same level as wild type animals. At higher doses, they increased in parallel the activity in both genotypes. Finally, we also tested knock-out mice in tests commonly used for probing antidepressant compounds. In the tail suspension test and the forced swimming test, knock-out mice displayed a depressive-like behaviour: they spent significantly more time immobile than wild type mice. In both tests, the injection of fluoxetine, an anti-depressant compound, reduced the time spent immobile for wild type and knock-out mice.

4.4. Other knock out models

4.4.1. *In vivo* function of the adenosine A2A receptor

In collaboration with various groups, we have pursued the characterization of a knockout model for the adenosine A2a receptor. The A2A adenosine receptor is involved in the regulation of addiction induced by different drugs of abuse. The specific role of A2A receptors in the behavioural and neurochemical responses to morphine associated with its motivational properties were tested. The acute administration of morphine induced a similar enhancement of locomotor activity and antinociceptive responses in both A2A knockout and control mice. However, the rewarding effects induced by morphine were completely blocked in A2A KO mice. Also, naloxone did not induce place aversion in animals lacking A2A. The results demonstrate that the rewarding and aversive effects associated with morphine abstinence were abolished in A2A KO mice, supporting a differential role of the A2A adenosine receptor in the somatic and motivational effects of morphine addiction. This provides evidence for the role of A2A receptors as general modulators of the motivational properties of drugs of abuse (Castañé et al. 2008).

Long-term caffeine intake has been reported to decrease the susceptibility to convulsants in mice. We investigated the occurrence of seizures following long-term oral administration of caffeine in adenosine A2A receptor knockout and control mice. Clonic seizures induced by acute pentylentetrazol (PTZ) were significantly attenuated in KO mice and also reduced by a 14-day caffeine treatment in WT mice. We showed also a protecting effect of a 21-day caffeine treatment in WT mice against kindled seizures induced by PTZ in an increasing dose schedule. The protective effects against PTZ-induced seizures occurring when the adenosine A2A receptor is absent or chronically blocked by caffeine is likely due to a decreased neuronal excitability in these conditions (El Yacoubi et al. 2008).

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 and the expression of microglia and astrocytes in the spinal cord after sciatic nerve injury was evaluated. Partial ligation of the sciatic nerve was performed in A2A knockout mice and wild-type littermates. The development of mechanical and thermal allodynia, as well as thermal hyperalgesia was evaluated by using the von Frey filament model, the cold-plate test and the plantar test, respectively. In wild-type animals, sciatic nerve injury led to a neuropathic pain syndrome that was revealed in these three nociceptive behavioural tests. However, a significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia were observed in A2AR deficient mice. Taken together, these results demonstrate the involvement of A2ARs in the control of neuropathic pain (Bura et al. 2008).

Adenosine triphosphate has previously been shown to induce semi-mature human monocyte-derived dendritic cells (DC) through the P2Y11 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 P2Y₁₁ receptor in mouse, the effects of adenine nucleotides on mouse DCs are mediated by their degradation product, adenosine, acting on the A_{2B} receptor (Ben Addi et al. 2008). 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).

4.4.2. *In vivo* function of the CB1 cannabinoid receptor

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 3,4-methylenedioxymethamphetamine (MDMA), a popular recreational drug. 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 and increased dopamine extracellular levels in the nucleus accumbens in both genotypes. However, CB1 knockout mice failed to self-administer MDMA at any of the doses used. 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 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. The endocannabinoid system is involved in neuroprotection at both cellular and emotional levels. 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. No differences were found in the levels of other neurotrophic factors, NGF and NT-3, or the activity of the BDNF receptor and transcription factor CREB. These results suggest that the lack of CB1 receptor results in an enhanced response to stress and deficiency in neuronal plasticity by decreasing BDNF levels in the hippocampus, leading to impairment in the responses to emotional disturbances (Aso et al. 2008).

The effect of WIN 55,212-2 were also investigated on excitatory postsynaptic currents (EPSCs) evoked by stimulation of Schaffer collaterals in CA1 pyramidal cells. WIN 55,212-2 reduced the amplitude of EPSCs in a dose-dependent manner. In rats and mice, this cannabinoid ligand inhibited excitatory synapses in two steps at the nM and μM concentrations. In CB1 knockout animals, of under treatment with the CB1 antagonist AM251, WIN 55,212-2 could still reduce the amplitude of EPSCs at μM but not nM concentrations. The inactive enantiomer, WIN 55,212-3, mimicked the effect of WIN 55,212-2 applied in high doses. The CB1-independent effect of WIN 55,212-2 at glutamatergic synapses was abolished by the omega-conotoxin GVIA, but not with the omega-agatoxin IVA. These data suggest 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, WIN 55,212-2 suppresses excitatory transmission by an additional mechanism independent of CB1, the blockade of N-type voltage-gated Ca²⁺ channels (Németh et al. 2008).

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 AM404 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 involve the FAAH-dependent metabolism of acetaminophen into AM404, the indirect involvement of CB1 receptors by this metabolite, and an endocannabinoid-dependent reinforcement of the serotonergic bulbospinal pathways (Mallet et al. 2008).

4.4.3. *Role of LGR5 in the developing small intestine.*

The orphan Leucine-rich repeat G protein-coupled receptor 5 (LGR5/GPR49) is a target of the Wnt signaling pathway. It has recently been identified as a reliable marker of adult stem cells (SC) in the intestine and skin. However, neither its function in the adults, nor during development have been addressed yet. We have investigated the role of LGR5 during ileal development by using LGR5 null/LacZ-NeoR knock-in mice. 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.

4.5. Purinergic receptors in human dendritic cells and macrophages (P1)

Angiogenic action of extracellular nucleotides through their action on dendritic cells. We demonstrated that P2Y₁₁ - an ATP receptor expressed on human dendritic cells (DCs) - played a determinant role in the generation of tolerogenic DCs (Marteau et al. 2005; Bles et al. 2007). Microarray experiments revealed a large and early ATP expression profile (more than 2000 genes) containing several promising target genes which were confirmed by quantitative PCR experiments. Our gene profiling experiments demonstrated 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). We also demonstrated that ATP was able to inhibit chemokine production (Horckmans et al. 2006) and to increase the production of VEGF and ligands of EGF receptor by human DCs (Bles et al. 2007). Recently, using an anti-VEGF blocking antibody, we demonstrated that the amount of VEGF secreted by DCs in response to ATP was sufficient to initiate endothelial cell proliferation in vitro and endothelial network in matrigel (Figure 1.18). Amphiregulin, epiregulin and Heparin-Binding EGF-like Growth Factor (HB-EGF) are all ligands of EGF receptor and were also regulated by ATP. EGF receptor is involved in the proliferation of tumoral endothelial cells and vascular smooth muscle cells which is determinant for vascular remodelling. We demonstrated that amphiregulin secretion by ATP-treated DCs was sufficient to initiate vascular smooth muscle cell proliferation. We also confirmed the regulation of these growth factors by ATP in mouse bone marrow-derived DCs (BMDCs). We have recently obtained promising data related to the regulation of tumor growth by injection of supernatants of ATP-treated DCs in the Lewis Lung Carcinoma in vivo model.

Extracellular nucleotides are intercellular signaling molecules involved in a wide spectrum of biological responses involving many different cell types. 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. They act through P2X (nucleotide gated cation channels) receptors and P2Y G protein coupled receptors. A lot of publications demonstrated that in vitro experiments, extracellular nucleotides control the biology of many if not all blood cells (Di Virgilio et al. 2000). With the aim to evaluate the physiological relevance of these observations, we are currently analysing the consequence of P2Y genes targeting.

The P2Y₆ 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 P2Y₆-null mice by gene targeting. The P2Y₆ 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 P2Y₆ 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 P2Y₆ KO macrophages. The endothelium-dependent relaxation of the aorta

by UDP was abolished in KO P2Y₆ mice. The contractile effect of UDP on the aorta, observed when endothelial nitric-oxide synthase is blocked, was also abolished in P2Y₆-null mice. In conclusion, we have shown that P2Y₆-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 Ca²⁺ increase in bone marrow derived and splenic dendritic cells. These effects were abolished in P2Y12^{-/-} DC and P2Y6^{-/-} 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 P2Y12^{-/-} and P2Y6^{-/-} 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 P2Y12^{-/-} mice than in wild types. Therefore, these results suggest that the P2Y12 and/or the P2Y6 receptors could be target of new types of adjuvant molecules.

4.6. 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. In our study of the *Drosophila* LGRs (DLGRs 1-4), we have recently mainly focused on the fly FSH-TSH-like receptor (DLGR1). To gain insight in the precise gene-expression pattern of this LGR, we 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 will allow 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) 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). Our results indicate that flies with severely down regulated DLGR1 transcript-levels do not develop into adults. In what stage development is arrested, is currently under investigation. In the future, we plan to investigate the function of the other orphan *Drosophila* LGRs (i.e. DLGR3 and 4) as well as other GPCRs and their corresponding ligands through *in vivo* RNA interference.

4.7. Glucose-sensing receptor (yeast and *Candida albicans*) and 'GPCR-controlled nutrient-sensing network' (P3)

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 G α 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 dispensable for the glucose-induced cAMP increase. This effect seems to be completely mediated by the Cdc25-Ras1 branch of the pathway. Further analysis of this pathway has now clearly shown that CaGpr1 and CaGpa2 are not working in a linear pathway as in *S. cerevisiae*.

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. Interestingly, three examples of such proteins are 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 transport 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.

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 of the compounds caused Gap1-dependent constitutive activation of the PKA target trehalase. We are currently investigating whether this effect correlates with defective downregulation of Gap1 activity. Preliminary data indicate that in the presence of these compounds vacuolar sorting of Gap1 is impaired in a similar way as in the absence of certain functions controlling endosomal trafficking. We are also studying whether these activating compounds cause changes in the ubiquitination pattern of Gap1. These and additional results suggest that binding of the compounds to Gap1 induces a conformational change that interferes with the vacuolar delivery of the permease, resulting in constitutive activation of PKA targets.

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 Gap1 Δ C6 and Gap1 Δ C9 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.

Using SCAM analysis (Substituted Cysteine Accessibility Method) we identified the Ser³⁸⁸ and Val³⁸⁹ residues as being exposed into the amino acid binding site and we showed that agonist action for signaling uses the same binding site as used for transport. Taken together, our data indicate that signaling requires a ligand-induced specific conformational change, which may be part of, but does not require the complete transport cycle.

Earlier it was found that Pho84 phosphate transporter also plays an essential role in stimulation PKA activity in phosphate-starved cells. Similarly as for Gap1, we screened a library of phosphate-containing compounds for the agonist effect on PKA-controlled response through Pho84. In general, we showed that presence of phosphate in the compounds was not enough to trigger the PKA-controlled response. Amongst the compounds acting as competitive inhibitors of phosphate transport, we selected two for further studies. For phosphonoacetic acid (PAA), we showed that binding of the compound to Pho84 is not enough to trigger signaling. In contrast, glycerol-3-

phosphate (G3P) acted as a non-transported agonist of the signaling function of Pho84, suggesting a conformational change upon binding. Recently, using SCAM ('Substituted Cysteine Accessibility Method') analysis, we characterized residues of transmembrane domains IV and VIII exposed into the phosphate-binding site.

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

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

5.1. 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).

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 glioblastoma. When GI261 murine glioma cells were infected *in vitro* with an 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).

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 levels. 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 ($rs=0.567$; $p=0.022$) and CCL21 ($rs=0.534$; $p=0.04$). It can be concluded that circulating fibrocytes may function as precursors of myofibroblasts in PVR membranes (Abu El-Asrar et al., 2008).

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

Lung inflammation and viral infection. A number of elements suggest a role of chemerin and ChemR23 in inflammatory and viral diseases of the lung. These include the expression of ChemR23 on alveolar 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 (KO), we studied models of asthma, lung fibrosis, LPS induced 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, bronchiolar and peribronchiolar eosinophilic infiltration, IL-5 and total eosinophil count in the broncho-alveolar lavage. No significant difference was observed between WT and KO mice with the exception of a higher basal level of IgE in the serum untreated KO 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.

For the LPS-induced acute lung injury model, a trans-oral instillation into the trachea of 1 µg LPS and/or 5 µg recombinant mouse chemerin was performed, and the animals were evaluated over a time course of 72 hours. At selected time points, mice were sacrificed and a bronchoalveolar 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 KO 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 KO 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-1 β), 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-1 β , 73% for TNF- α). In ChemR23 KO 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.

Such an anti-inflammatory property of chemerin is also supported by preliminary 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 RSV, known to infect ~85% of children by age 18 mo and leading to an acute bronchiolitis. ChemR23 KO mice exhibited higher susceptibility to the infection, which was characterized by a significantly higher mortality rate (40%), as a result of an excessive and inadequate inflammatory response. Indeed, preliminary data suggest that, as in the LPS model, there is recruitment of a higher number of PMNs in these mice, associated with a reduced viral clearance. The underlying mechanisms could include defective DC (especially plasmacytoid DC) and NK cell recruitment in ChemR23 KO mice. The decreased plasmacytoid DC recruitment could lead to an impaired anti-viral immunity. Indeed, plasmacytoid DCs are high producers of type I interferons, which play a crucial role in anti-viral defense by increasing the viral resistance of uninfected cells, inducing the apoptosis of infected cells, and activating NK and CD8+ cytotoxic T cells.

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.

Chemerin and ChemR23 in multiple sclerosis. We contributed to a study investigating the roles of plasmacytoid dendritic cells (pDCs) and their response to IFN- β therapy in multiple sclerosis patients. We identified pDC accumulation in white matter lesions and leptomeninges of multiple sclerosis brains and evidence for Type I IFN production by activated pDCs in the lesions. Chemerin was detected in intralésional cerebrovascular endothelial cells, and the ChemR23 was expressed on infiltrating leukocytes, including pDCs. By testing the effect of IFN- β on pDC phenotype and function in MS patients, it was shown that IFN- β modulates the immunologic functions of pDC, thus identifying pDCs as a novel target of IFN- β therapy in MS patients. As chemerin is involved in DC recruitment, these data suggest a role of the chemerin-ChemR23 system in the development of MS (Lande et al. 2008).

5.3. Prolactin-releasing peptide receptor (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 stressful conditions. This low corticosteroid 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 are now starting the experiments that will allow determining whether the inflammatory syndrome is spontaneous or induced by a specific pathogen, and will expose these mice to different models of hepatitis (D-galactosamine/LPS, concanavalin A, CCl₄ and Murine Hepatitis Virus A59). We will also test whether the inflammatory phenotype is restricted to the liver or applicable to other organs as well.

5.4. QTL analysis in animal models (P1, P5)

Modelization of influenza virus-associated diseases in the laboratory mouse were intended in order to generate models of human infections with highly pathogenic influenza viruses. These models are aimed (i) at detecting QTLs governing individual susceptibility to the severe disease on the one hand, and (ii) at screening the possible involvement of selected GPCRs by screening the KO lines made available to the project by partner P1. Thus, the main objective of this year's study was to validate 2 influenza models, one using the current H5N1 virus and another using a 1918-like H1N1 hypervirulent strain.

Mice — Eight-wk-old female FVB/J mice weighing 20-25 grams were obtained from Charles River Laboratories. Challenge studies were conducted under biosafety level 3 laboratory conditions and in BCLAS-accredited animal facilities, under the guidance of the Institutional Animal Care and Use Committees of the Veterinary Agrochemical Research Center and University of Liège. The mice were housed in microisolator cages ventilated under negative pressure with HEPA-filtered air. They were allowed free access to food and water and maintained in 12-hour light/dark cycles. Prior to each inoculation or euthanasia procedure, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (30 mg/kg).

Viruses — Two influenza A viruses of low pathogenicity for the laboratory mouse, the clade 1 avian H5N1 virus A/crested_eagle/Belgium/1/2004 (H5N1) and the porcine H1N1 virus A/swine/lowa/4/1976 (H1N1) were used in this study. Both viruses were first propagated in the allantoic cavity of 10-day-old embryonating hen's eggs at 37°C for 24 h and then adapted to the mouse by lung-to-lung passaging. At each passage, a set of mice were inoculated intranasally with 50 μ l of either allantoic fluid or lung homogenate containing influenza A virus. On day 5 post-inoculation (pi), the mice were euthanized by pentobarbital overdosing followed by exsanguination, the lungs were combined and homogenized in PBS-penicillin-streptomycin, the homogenates were centrifuged at 3,000 g for 10 min, and the supernatant was used for the next passage. The process was stopped when the mice became obviously sick on and after day 3-4 pi. This occurred after 7 (H5N1) or 31 (H1N1) passages. Lung homogenates from the last passage were homogenized and aliquoted for use in pathotyping studies, and their titers determined by standard plaque (H1N1) or median tissue culture infectious dose assays (H5N1). Inoculations of serial dilutions of each adapted virus stock were then performed in FVB/J mice and the fifty-percent mouse lethal dose (MLD50) was calculated according to the method of Reed and Muench.

Pathotyping studies — For assessment of virus-induced pathogenicity, two series of mice were inoculated intranasally with 10 MLD50 of virus by instillation of 50 μ L diluted stock. Ten mice were monitored daily for changes in body weight in order to assess virus-induced morbidity. At selected time intervals, 5 (virus titration or histopathology) or 15 mice (same + wet-to-dry-weight ratio) were overdosed with sodium pentobarbital and exsanguinated by cutting the brachial artery. Lungs and

pieces of heart, liver, spleen, kidney, brain, and adipose tissue from 5 mice were fixed in 4% neutral-buffered ice-cold paraformaldehyde, routinely processed, and embedded in paraffin for evaluation of histopathology. The whole lungs were weighed prior to fixation, which was done by perfusion through the trachea, thus excluding the luminal contents from the subsequent tissue sections. Five-micrometer sections were stained with hematoxylin and eosin (HE) or with periodic acid-Schiff (PAS) for lesion detection. For virus detection, sections were stained by an indirect immunofluorescence method and by a streptavidin-biotin complex immunoperoxidase method. Polyclonal goat (H1N1, from Abcam) or rabbit (H5N1, in-house) antisera were used as primary antibodies and alexa-fluor[®]488- or HRP-conjugated anti-goat or anti-rabbit IgGs were used, respectively, as secondary antibodies. For immunohistochemistry, peroxidase was revealed with 3-amino-9-ethyl-carbazole, resulting in a bright red precipitate, and sections were counterstained with Mayer's hematoxylin. For virus titrations, lungs from 5 mice were weighed, homogenized in 1 ml PBS, and clarified. The supernatants were used for virus titration by plaque or median tissue culture infectious dose assays. On day 6 (H5N1) or 8 (H1N1) pi, lung homogenates from 5 additional mice were lyophilized for wet and dry weights determinations.

Clinical, gross pathological and virological observations — The H1N1 and H5N1 influenza A virus strains used in this study were isolated respectively from a diseased pig in the US in 1976 and from two crested eagles smuggled from Thailand in 2003. Both were non-pathogenic for FVB/J mice (MLD50 >10⁶ PFU/TCID50). After adaptation, they showed a similar pathogenic outcome in FVB/J mice, i.e. very close MLD50 values: 3 PFUs for H1N1 and 6.4 TCID50 for the H5N1 strain. This allowed a relevant comparison of their respective pathological signatures. Overall, virus-associated morbidity, body weight loss, and gross lesions caused by inoculation of 10 MLD50 were similar for both viruses, except that body condition and respiratory function deteriorated more rapidly after H5N1 inoculation. The pathological processes remained totally asymptomatic for the first 3 (H5N1) or 4 (H1N1) days and then gave rise to general signs such as gradually slower, less frequent, and more erratic spontaneous displacements and a ruffled coat. By day 4 (H5N1) or 5 (H1N1) pi, all mice became lethargic and abruptly exhibited clinical signs of respiratory disease, including respiratory distress, labored breathing, and forced expiration. Weight loss was acute and showed a biphasic profile : a 10% loss between virus inoculation and the appearance of respiratory symptoms and an additional 20% during ARDS (Fig. 1). On day 6 (H5N1) or day 8 pi (H1N1), the mice had to be euthanized because of dramatic weight loss, adoption of abdominal-type breathing, and behavioral changes associated with CNS disturbances. Autopsies performed on the last day of the disease consistently showed the dark purplish bulky noncrepitant lungs compatible with a diagnosis of massive pulmonary congestion and hemorrhagic edema. The process had not caused any significant change in lung dry weight, but the final wet/dry weight ratio of the lungs of H5N1-infected mice was significantly higher ($p < 0.01$) than that of the H1N1-infected mice. No obvious gross lesions were observed in the hearts, livers, spleens, kidneys, brains, or perivisceral fat.

Histopathological observations — An exhaustive list of the histopathological lesions caused by the two viruses was made available. Some changes in lung morphology were identical for both viruses. Firstly, a clear topographical extension of the lesions was perceptible between the first and the last day of infection, with centrifugal spreading from the terminal bronchioles or the alveoli adjacent to the airways. Qualitatively, all of the alterations characterizing the exsudative phase of the histopathological condition termed *diffuse alveolar damage* were identifiable, with intense congestion of the alveolar capillaries, marginated intra-capillary neutrophils, necrosis of the alveolar epithelium, interstitial and alveolar edema, hyaline membranes, and invasion of the alveoli by (mostly) mononucleate cells, principally macrophages. On the other hand, we observed neither cuboidalization of the alveoli (hyperplasia of type II pneumocytes), nor hyperplasia or squamous metaplasia of the airway epithelia. This is indicative of extremely rapid disease progression and/or of nearly complete elimination of type II pneumocytes. Despite these similarities, when sections of lung-tissue samples taken on the last day from the H1N1- and H5N1-infected mice were pooled, it was easy for an examiner unaware of which infection he was looking at to distinguish one from the other. The criteria for attributing the lung lesions to the H1N1 strain were: (1) earlier and much more extensive degeneration, necrosis, and desquamation of the airway epithelium, (2) a much higher cell density of the peribronchial, peribronchiolar, interstitial, and intra-alveolar infiltrates, (3) the presence of dense cuffs of mononucleate cells around the arterioles, (4) less extensive alveolar

edemas, and (5) the rarity of alveolar hemorrhage. Conversely, the lesions caused by the H5N1 strain were distinguishable by the extent of the alveolar edemas, the very low cell density of inflammatory infiltrates, the high number of alveolar hemorrhage foci, and the unusual appearance of the pulmonary arterioles, which seemed to have been dissected from the surrounding tissues because of the magnitude of the perivascular edema. On the other hand, no arteriole showed any cuff of infiltrated mononucleate cells. Some blood-vessel walls also showed hemorrhage inside the muscle layer. No other organ examined was found to carry any histopathological lesions except, remarkably, the liver in H5N1-infected mice. These livers displayed multifocal necrosis, with necrotic foci consisting of aggregates of hypereosinophilic pyknotic and caryorhectic hepatocytes admixed with a few neutrophils and lymphocytes. Such foci were also seen in the spleen of some animals. Strikingly, numerous PAS-positive islets were detected throughout the livers of H5N1-infected animals, each overlapping with a necrotic focus. Patterns of centrilobular hydropic and granular (day 3 pi), centrilobular (day 5 pi), and panlobular (day 7 pi) microvesicular fatty degeneration were also observed in the livers of all H5N1-infected animals. In their renal medulla, interstitial hemorrhages were seen.

Detection of viruses in tissues — The results of both indirect immunofluorescence and immunohistochemistry were very homogeneous for animals infected with the same strain. Overall, they showed that the H1N1 strain remained strictly confined to the lungs, whereas the H5N1 virus spread to the liver, kidneys, spleen, brain, and perivisceral fat. The H1N1 virus was detectable in the epithelium of a few bronchi and bronchioles on day 3 pi. By day 5, the stain was more conspicuous and it appeared also in the alveolar epithelium of the areas adjacent to the airways. By day 7 pi, the virus was detectable in the epithelia of all bronchi and bronchioles and in the alveolar epithelium in extensive areas of the lungs. In the alveolar structures, immunofluorescence staining revealed the virus in type I and type II pneumocytes and in alveolar macrophages. In the case of H5N1 infection, the virus was detectable from day 3 in some bronchiolar epithelial cells, some type 2 pneumocytes, some interstitial/alveolar macrophages, and some endothelial cells in the vicinity of the positive bronchioles. In contrast, no non-respiratory organ examined showed any positive cells. By day 5, staining of the airway epithelium was still discrete and limited, whereas the alveolar epithelium showed more pronounced staining diffusely distributed throughout the lung. In the liver, multiple nests of positive hepatocytes were detectable, corresponding exactly with the above-mentioned necrotic foci. A few renal tubular epithelial cells were also positive. On day 7 pi, the alveolar epithelium was still diffusely stained but much more pronounced than on day 5 pi. For the first time, staining of the bronchiolar epithelium was also visible, but not all bronchioles -far from all, in fact- showed this staining. The appearance of the kidneys and liver was the same as on day 5, with more conspicuous staining. For the first time, virus-positive glial cells, splenic macrophages, and peritoneal adipocytes were also detected. Type II pneumocytes and alveolar macrophages were more often positive than type I pneumocytes. The evolution of pulmonary virus titers was followed too.

Interpretation — Two influenza A viruses of different subtypes, derived from very 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 addition to having similar MLD50s, our two viruses cause clinically and virologically similar diseases, with similar clinical signs, severe weight loss, and a similar evolution of the pulmonary virus titer. In both cases the weight-loss profile was biphasic, culminating at agony with a loss of 25 (H1N1) or 22% (H5N1). Viral amplification was maximal for both viruses on days 5-6 pi, corresponding to the typical inoculation-to-peak lag of natural murine respiratory viruses (cf. our last year's report). On the other hand, our two viruses adapted in the lungs showed 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/Porto 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 our two viruses is the diffuse alveolar damage dominating both histopathological profiles. As already mentioned, the fact that we consistently observed this feature corroborates the pathological data available in the literature. Yearly 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 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) to the prepandemic H5N1 strains isolated from humans since 2003, additionally colonize, preferentially or not, the bronchiolar and alveolar epithelia, causing diffuse alveolar damage as an additional primary lesion. The same lesion has been found in experimental animals injected with a recent H5N1 strain. In summary, the overall clinical, virological, and histopathological signatures of the infections caused by the viruses studied here are quite similar and corroborate the data accumulated so far in the literature.

At first glance, these similarities suggest that the disease processes induced by all influenza virus strains, provided they are highly virulent, might involve similar physiopathological mechanics. Yet we have identified flagrant differences which makes the H1N1 infection easy to distinguish from the H5N1 infection. For the H1N1 virus, the disease becomes fatal at a point where the pulmonary edema is less intense and leaves a histopathological signature characterized by much more dense inflammatory cell infiltrates, generating cuffs around the bronchioles and blood vessels. The H1N1 virus, secondly, colonizes the epithelia of both the upper and lower airways, without any obvious preference, whereas H5N1 remains confined essentially to the alveoli and 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. We believe these differences demonstrate unambiguously that the two hyperpathogenic influenza A viruses studied here cause two different diseases. This suggests that the physiopathological data obtained when studying one hyperpathogenic strain should not be extrapolated automatically to other strains. Furthermore, it is possible that two fatal diseases caused by two different strains might require different therapeutic measures. Such 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 prepandemic 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 our H5N1 strain spreads beyond the respiratory system confirms similar observations on both humans and laboratory animals. Lastly, the A/South Carolina/1/18 [H1N1] and A/Thailand/16/2003 [H5N1] viruses have been shown to trigger different cytokine responses both in the lungs of infected mice and in primary cultures of human macrophages.

If one integrates the results gathered here with the diverse pieces of evidence reported elsewhere, it thus seems clear that fatal infections caused by different highly virulent influenza A viruses do not necessarily display the same pathogeny. To be convinced, one has only to see how easy it is to distinguish the histopathological sections typical of our two strains, in the absence of any virus labeling. These different histopathological signatures and different pathogenies 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. Furthermore, it might possibly be advantageous to tailor the therapeutic approach to the pathotype. These two models are now being implemented among

diverse mouse inbred strains to seek after strain-specific differences and in studies devoted to the comparison of the two “cytokinetic storms” which are expected to be different.

WORKPACKAGE 6. IDENTIFICATION OF NOVEL RECEPTORS AND THEIR LIGANDS

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

6.1. Chemoattractant and neuropeptide receptors (P1)

6.1.1. Functional screening assays.

With the aim of identifying the natural ligands of orphan receptors, we had established cell lines coexpressing $G_{\alpha 16}$, apoaequorin and genes encoding selected orphan G protein-coupled receptors.

In order to have a screening assay available which would not depend directly on G protein signalling, we have started to implement the TANGO® system (Invitrogen). This system is based on the recruitment by an activated receptor construct of a chimeric beta arrestin, hooked to the TEV protease. Upon binding of the construct to the activated receptor, the TEV protease cleaves a target site engineered in the C-terminal segment of the receptor of interest, upstream of a Gal4-VP16 transcription factor. The released Gal4-VP16 will then activate transcription from a sensitive promoter placed upstream of a beta lactamase reporter gene. The beta lactamase activity is measured fluorometrically. The system has two theoretical main advantages: (i) it does not require prior knowledge of the regulatory cascade controlled by the receptor; (ii) it is immune to the background of endogenous receptors present in the recipient cell. The system is currently being tested with two orphan receptors, LGR4 and LGR5.

6.1.2. Characterization of natural or surrogate ligands.

The cell lines expressing orphan receptors were used for the screening of biological activities in a library of fractions prepared from natural sources.

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. We are now optimizing a third dimension by testing different columns such as cationic exchange columns or reverse phase HPLC using C18 or C4 columns.

6.1.3. Further characterization of deorphanized receptors.

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, several poly-unsaturated fatty acids (PUFA) were reported in a patent to act on GIR, following their isolation from pig brain. Both hypotheses were tested, following the expression of human GIR (hGIR) in CHO-K1 cells also 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 cell. DHA in combination with PYY (3-36) or NPY induced a stronger activation. In the presence of DHA, the EC50 for PYY-3-36 decreased to the nanomolar range and the Emax 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. We observed the GPCR internalisation was detected either by fluorescent confocal microscopy or FACS. DHA at 10 μ M 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 internalise 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 ¹²⁵I-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 ¹²⁵I-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 unable 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.

6.2. Chemokine receptors (P4 and P1)

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, phosphorylation 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).

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

In search of a putative sugar-sensing GPCR present in the mammalian small intestine, we identified the β -adrenoreceptor with a high expression on the apical side of the enterocyte, thus seemingly monitoring the content of the small intestine. Although this receptor binds a known hormone ligand, ligand promiscuity cannot be excluded, and we hypothesize the receptor serves an alternative function as sugar-sensor in the small intestine. Preliminary functional experiments in *Xenopus* oocytes and further testings in a human HEK-293T cells, supported this idea. Moreover, we constructed an *in silico* model for sugar-induced activation of the GPCR. Interestingly this model fits our sugar-specificity data suggesting that glucose and fructose can be recognized as ligands, whereas galactose is not. Using radio-ligand binding techniques, we will assess in more detail this sugar-specificity, the specific binding of the ligand, and the effect of specifically blocking the receptor *in vivo*.

Several general experimental approaches are now available to identify new examples of transceptors. We will investigate whether other nutrients, like sulphate, vitamins and metal ions, can also activate the PKA pathway using such a transceptor system. A strong indication for this is that glucose fermenting yeast cells starved for these nutrients arrest at the start point of the cell cycle and go into stationary phase G0 in a very similar way as cells starved for nitrogen or phosphate. Moreover, in the case of sulphate, it has already been shown that re-addition of this nutrient triggers rapid activation of the PKA pathway.

6.4. Studies on insect orphan GPCRs (P2)

6.4.1. 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 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 the disposal of 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+} signaling 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 many metazoan GPCRs. 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 recently led to the identification in the lab of a fruit fly receptor for DTK-6 (*Drosophila* tachykinin-related peptide 6) and MIPs (myoinhibiting peptides). 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).

6.4.2. Exploring a novel EST database from desert locust CNS

The Desert Locust (*Schistocerca gregaria*) is a notorious agricultural pest that threatens the livelihood of millions of people and therefore is constantly monitored by the FAO (<http://www.fao.org/ag/locusts/>). At the root of this threat is an extreme form of environmentally driven phenotypic plasticity by which one genome can manifest itself in two very different kinds (phases) of animal. At low population densities, locusts usually occur in the *solitary phase*. Occasionally, however, patchy food resources and increasing population densities can force many solitary locusts into close contact. This forced crowding triggers a striking phenotypic switch 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 desert locust CNS (<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 35000 sequences) revealed that the EST database currently represents ca. 12700 unique transcripts and constitutes a rich source of novel, high quality macromolecular sequence data, including a variety of neuropeptide precursors, receptors and other components of signal transduction cascades.

Following the recent discovery that serotonin plays a very important role in the gregarization process of solitary locusts (Anstey et al., 2009), we try to unravel its signal transduction cascade. Therefore, we are currently analyzing a putative serotonin receptor and some of its putative downstream factors such as the catalytic and regulatory subunits of protein kinase A. Knock-down of these factors by RNAi causes a significant effect on the behavioral gregarization speed of solitary locusts induced by crowding.

6.5. 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 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 also generated expression plasmids for two other *Drosophila* LGRs (DLGRs 3-4). Although no 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 will use intracellular cAMP-level measurements in an attempt to identify ligands for the remaining orphan insect LGRs.

WORKPACKAGE 7. OLFACTORY RECEPTORS AND EVOLUTION OF GPCR FAMILIES

7.1. Olfactory receptors (P1, P2)

The structure of olfactory receptors has been known for 15 years, but their functional expression is still a major problem, as mammalian ORs are poorly targeted to the cell surface in heterologous systems. Due to these limitations, only a few mammalian ORs have been characterized functionally to date. One of the most significant improvements reported over the last few years is the demonstration that the transmembrane proteins RTP1, RTP2 (both expressed specifically in olfactory neurons) and REEP1 contribute to the translocation of ORs to the plasma membrane, and promote their functional expression in mammalian cell lines (Saito et al. Cell 2004), allowing the design of more reliable functional assays. It is however our hypothesis that additional membrane or soluble proteins are required for the reconstitution of an efficient 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 the adenylate cyclase III protein, which is part of the signalling machinery proximal to the receptor. In order to purify further the protein complexes containing the receptors, it will be necessary to express a specific tagged olfactory receptor in all olfactory neurons. To this end, we 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 G γ 8-TTA), allowing the permanent expression of a given receptor in all olfactory neurons. Such expression is not achieved adequately using less sophisticated approaches, as a result of a complex control of receptor expression in olfactory neurons, involving apparently the coding sequence of the receptors themselves (Nguyen et al. Cell 131:1009-17, 2007). We now have several mice expressing the three transgenes and verified receptor expression in mice expressing OMP-TTA and TRE-F5-OR. As expected, we found that many but not all olfactory neurons do express the olfactory receptor labelled with an anti-Tag antibody. Co-labelling with anti-adenylate cyclase III antibody (ACIII) also indicated that these receptors were expressed in the cilia of olfactory neurons. We now plan to verify that triple transgenic mice indeed express the same olfactory receptor in all neurons and then prepare an OR-enriched fraction from these mice by immunoprecipitation with an anti-Tag monoclonal antibody. Once purified, we will screen by 2D-DIGE and 2D-LC the proteins over-represented in this fraction, as compared to a total membrane fraction of the same initial homogenate 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, Golf, 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.

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

We have initiated the analysis of the conservation of GPCRs of interest and their ligands across species, starting with ChemR23 and its protein ligand, chemerin, as a necessary step to study the biological functions of this new leukocyte chemoattractant system in animal model systems. Prochemerin (Ensembl ID ENSMUSG00000009281) and ChemR23 (Ensembl ID ENSMUSG000000042190) amino-acid sequences from vertebrate species were aligned with ClustalW and a dendrogram was constructed. For chemerin, we have 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.

7.3. 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 arisen 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. The general amino acid permease Gap1 in yeast has become the best characterised model system of a transporting transceptor (see section 4.7). However, the signaling mechanism is currently unknown. Using a split-ubiquitin membrane yeast two-hybrid assay, we identified the α -subunit of eukaryotic initiation factor 2 (eIF2 α) as a Gap1-interacting protein. This interaction was confirmed using co-immunoprecipitation. Other preliminary results indicate that another subunit of eIF2 may directly interact with the catalytic subunits of PKA. eIF2 is a heterotrimeric G-protein that plays a critical role in the initiation of protein synthesis. We are currently investigating whether

Gap1 could act as a guanine nucleotide exchange factor for eIF2 with the purpose of 'kick-starting' protein synthesis once amino acids become available again to nitrogen-deprived cells. At the same time eIF2 would act as a signal transducer for Gap1 activation of PKA. If this can be confirmed Gap1 would actually function as a G-protein coupled receptor for nutrient activation of the PKA pathway. This would provide a striking argument for the evolution of receptors from nutrient transporters.

2. NETWORK ORGANISATION AND MANAGEMENT

Network's organization

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

Plenary meetings of the network

A second plenary meeting was organized on November 28, 2008 on the Erasme campus of the ULB. This meeting has gathered all groups, including most senior and junior staff involved in the program, in the presence of Véronique Feys. The program of this meeting is provided as an annex (Annex 1). 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 second 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, this meeting was successful in providing all researchers in each group with a clear picture of the network activities. It has been the occasion to establish or re-establish personal relationships between the PhD students and post-docs of the various groups, leading to exchange of information, ideas and reagents.

Besides this scientific session, a coordination session of the steering committee took place at the end of the day. This meeting has also been the occasion of summarizing the network's achievements in prevision of this annual report. It was considered that the program was going well for most workpackages. No major changes in the orientation of the work or in the structure of the partnership have been considered at this stage, as most ongoing research activities were planned at the initiation of the program. The next meeting will be organised by ULB in November or December 2009, and will likely take place in Brussels.

Interactions between partners

Exchange of material and data

P1-P2.

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

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

Exchange of information and materials for the study of LGRs

One common publication has resulted from these interactions (Van Loy et al. 2008)

P1-P4.

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

- 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., 2008).

- 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)

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).

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) is working on a regular basis in the P5 laboratory for performing the infections and learning the techniques. This interaction is being pursued in 2009.

P1-EU1.

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 models of ChemR23 and CCR5 that are being used in the structure-function studies. The results of these studies will feed back the model.

P2-P3.

P2-P3. J. Vanden Broeck (P2) was member of the jury at the public PhD-defence of Patrick Van Dormael (promotor: J. Thevelein, P3): 04/07/2008

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

P2-P4.

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

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

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

P2-EU1.

Data have been exchanged between Partner 2 (KUL-Vanden Broeck) and the group of Leonardo Pardo (EU1) in collaborative work which should result in a common publication.

P4-P5

A bank of acute and convalescent bovine serums gathered by partner 5 in the context of viral pneumonias in cattle (ULg-Desmecht) has been transferred to the group of Jo Vandamme (partner 4) for starting a large screening of circulating regakine levels. These measurements are currently ongoing.

Exchange of personnel

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

Website

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

3. PUBLICATIONS

3.1. PUBLICATIONS OF EACH TEAM

3.1.1. P1

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3.2. COPUBLICATIONS

P1-P2

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P1-P4

Gouwy M, Struyf S, Noppen S, Schutyser E, Springael JY, Parmentier M, Proost P, Van Damme J (2008). Synergy between coproduced CC and CXC chemokines in monocyte chemotaxis through receptor-mediated events. *Mol Pharmacol* 74:485-495.

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P1-EU1

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Annex 1: Program of the second annual meeting of the consortium**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-Teixeira 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**

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