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Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification

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Abstract. G-protein-coupled receptors (GPCRs) are of prime importance for cell signal transduction mechanisms and are the target of many current and potential drugs. However, structural data on these membrane proteins is still scarce because of their low natural abundance and the low efficiency of most of the expression systems currently available. This review presents the most important expression systems currently employed for heterologous expression of GPCRs; *Escherichia coli*, yeast, insect cells and mammalian cells. After briefly recalling the specificity, advantages and limitations of each system, particular emphasis is put on the quantitative comparison of these expression systems in terms of overall expression yield, and on the influence of various factors (primary sequence, origin, cell type, N- and C-terminal tags) on the results.

Key words. GPCRs; heterologous expression; membrane proteins; receptor; *Pichia pastoris*; NMR.

Introduction

Very highly conserved through evolution and thereby expressed in nearly all organisms ranging from yeast to human beings; encoded by almost 1% of the human genome; involved in a wide spectrum of hereditary and somatic disorders and diseases from cancer to infertility; targets of more than 50% of the drugs nowadays used in therapeutics [1]; directly responsible for communication at the cellular level by their ability to be activated by a large number of very different extracellular signals such as photons, ions, lipids, peptides, nucleosides, nucleotides, hormones and neurotransmitters. Here are some of the main characteristics that describe and underline the fundamental roles of the superfamily of G-protein-coupled receptors (GPCRs). These membrane proteins are characterised by the presence of a common hydrophobic core composed of seven transmembrane spanning domains, and mediate external stimuli through a strong coupling with endogenous heterotrimeric G proteins [2, 3]. Because of their central role in biological systems, detailed understanding of the process that controls the interaction of GPCRs with their ligands and associated G proteins is very challenging. This understanding would be greatly facilitated by high-resolution three-dimensional (3D) structures of these receptors. However, the only high-resolution structure of a GPCR currently available is that of bovine rhodopsin, which was recently determined by X-ray crystallography at 2.8-Å resolution [4]. This lack of structural data mainly originates in the membranous nature of these proteins and in their very low natural abundance. The comparison of the number of high-resolution 3D structures [obtained either by nuclear magnetic resonance (NMR) or X-ray crystallography] available for soluble (> 10,000) and membrane proteins (⊕30) in the Protein Data Bank clearly reflects the difficulties encountered with membrane proteins. The presence of a specific lipid environment essential to maintain functional conformation and their high molecular weight considerably complicate the determination of their 3D structures. Another hindrance to determining the structural biology of GPCRs is their intrinsic dynamic behaviour, which is characteristic of α-helical proteins and directly linked to their activity of signal transduction through biological membranes. Finally, the two major techniques available to obtain high-resolution

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3D structures (NMR and X-ray crystallography) require high amounts of purified proteins (milligrams). Most GPCRs are naturally poorly expressed, and this renders impractical their direct purification from natural sources. Thus, their heterologous overexpression is an unavoidable step, and it is essential to develop highly efficient expression strategies. The objective of this article is to present and compare heterologous expression levels of GPCRs in the four main expression systems currently employed: *Escherichia coli*, yeasts, insects and mammalian cells.

In the context of structural biology experiments, the first objective is to be able to obtain high quantities of pure protein. In the literature, expression levels are given either in 'sites per cell' or in 'picomoles of receptor per milligram of membrane proteins', so that direct comparison is often difficult. A rapid conversion can be made using the approximate conversion rates proposed by Schertler [5] and Grisshammer and Tate [6] and summarized in table 1. The conversion factors presented in table 1 highlight that the real expression level efficiency is a combination of parameters such as the number of sites per cell, cell size and cell density. When expressed in picomoles of receptor per milligram of membrane protein the requirements for obtaining 1 mg of GPCR in 5 l of culture are respectively 13, 5 and 26 for *E. coli*, yeast and superior eucaryotic cells (insect or mammalian cells; these minimal expression levels have been calculated considering an average GPCR molecular weight of 45 kDa). Whenever the reported expression level was equal or superior to the minimal expression level previously defined, an 'overexpression factor' (O.F.) has been calculated (tables 2–5) and corresponds to the ratio between the reported expression level and what is required to produce 1 mg in 5 l of culture medium. For instance, in the case of the expression of the β-adrenergic receptor in *Saccharomyces cerevisiae* [7], the expression level is 115 pmol per milligram of membrane protein which corresponds to an O.F. of 23 (115/5).

Several very complete reviews are already available concerning the main advantages and drawbacks of all these expression systems and their use in expressing membrane proteins [5, 6, 8–13]. The present review is thus mainly focused on describing the results so far obtained, with only a brief recall of the specific characteristics of each system.

E. coli

Main characteristics of *E. coli*

The major advantages of this expression system are (i) low cost (ii) homogeneity of the recombinant proteins (no posttranslational modifications) and (iii) short generation time (20 min) and short delay of expression [6]. This system offers the possibility to rapidly test a huge number of different genetic coding sequences [14, 15] and to optimise expression levels before undertaking the purification [15]. These characteristics render *E. coli* very appropriate to the constraints of structural biology. However, this system is not the best for functional studies of the receptor in vivo (signal transduction mechanism), since this bacteria does not contain any endogenous G protein. In particular, the lack of endogenous G protein can give rise to the absence of high-affinity binding sites for agonists [16]. However, the addition of purified G proteins to *E. coli* membranes expressing the $5HT_{1A}$ receptor enabled restoration of the high-affinity binding properties [17]. Also, coexpression of G proteins and opioid receptors by gene fusion reveals high-affinity binding sites [16]. In some cases, the *E. coli* expression system is disadvantageous because the functionality of some GPCRs has been shown to depend on the presence of posttranslational modifications. For instance, it has been shown that suppression of a glycosylation site located in the N-terminus of rhodopsin perturbs the functional folding of this protein [18]. In the same way, phosphorylation of rhodopsin increases its proportion in the MII state, which activates G proteins [19]. Finally, the lipid composition of *E. coli* membranes is very different from that of eucaryotic cells, and this can greatly affect the binding properties of recombinant receptors. Indeed, certain receptors such as the oxytocin [20], the transferrin [21] and human μ-opioid receptors [22, 23] strongly depend on the lipid environment of the protein. In addition, the reductive environment of the bacterial cytoplasm can considerably slow down the production of functional membrane proteins, particularly if the correct folding of the receptor in an active conformation requires formation of disulfide bridges. Fusion of *E. coli* periplasmic membrane proteins such as the maltose binding protein (MBP) to the GPCR of interest can overcome this problem by addressing the recombinant receptor in the periplasmic oxidative environment [24, 25]. *E. coli* can also be used for production of the heterologous protein in inclusion bodies [26]. These structures mainly contain the exogenous protein of interest and are very easy to purify, and this strategy can be very attractive. Moreover, the accumulation in these inclusion bodies of recombinant proteins protects them from proteolytic degradation by cytosolic, membranous and periplasmic proteases [27]. However, despite the fact that certain factors such as temperature modulation can favour the appearance of inclusion bodies, their formation is hard to control. Moreover, proteins accumulated, in these structures are usually inactive, and functional refolding after purification is a required nontrivial step [26, 28]. This approach has already been employed for several soluble proteins, but much more rarely for membrane proteins [26, 28–31].

Table 1. Conversion factors required for the comparison of expression levels in *E. coli*, yeast, insect cells and mammalian cells.

Cell type	Average cell density	Conversion of the expression levels	Minimal expression levels necessary to obtain 1 mg of GPCR in 5 l of culture	
	(Cells/ml)	1 pmol/mg = **X** sites/cell, with **X** equal to:	Sites/cell	pmol/mg
E. coli	1×10^9	200	2.6×10^3	13
Yeast	1×10^8	5000	2.5×10^4	5
Insect ≅ mammalian cells	1×10^6	10^5	2.6×10^6	26

Minimal expression levels for the production of 1 mg of GPCR in 5 l of culture. On the basis of the values described by Schertler [5] and Grisshammer and Tate [6], the expression level necessary to obtain 1 mg of receptor in 5 l of culture was calculated for each expression system. An average molecular weight of 45 kDa for a GPCR is assumed. It should be noted that certain methylotrophic yeasts, such as *P. pastoris*, can reach higher cell densities ($1-3 \times 10^9$ cells/ml) so that the expression level required to produce 1 mg of recombinant receptor in 5 l of culture medium may be lower than indicated here for yeast in general.

Table 2. GPCRs expression in *Escherichia coli.*

Receptor type	Origin	Promoter	Fusion protein	Function	Receptor localisation	Expression level	Tags	Overexpression factor	Ref.
β_2-Adrenergic	human	lac	β-gal	+	M	0.4 pmol/mg	none	–	[32]
	human	T7	–	+	M	0.45 pmol/mg	none	–	[36]
	human	ptac12	LamB	+	M	220 sites/cell	none	–	[113]
	human	ptac12	LamB	+	M	0.5 pmol/mg	none	–	[34]
	human	ptac12	LamB	+	M	220 sites /cell	none	–	[114]
	human	lac	PhoA	ND	M	ND	c-myc Ct	–	[115]
	human	ptac	MBP	+	M	6 pmol/mg	6-his Ct	–	[33]
	human	ptac12	LamB	+	M	ND	none	–	[116]
β_1-Adrenergic	human	ptac12	LamB	+	M	ND	none	–	[116]
	human	ptac12	LamB	+	M	50 sites/cell	none	–	[113]
	human	ptac12	LamB	+	M	1 pmol/mg	none	–	[34]
	turkey	ptac12	LamB	+	M	0.33 pmol/mg	none	–	[34]
Serotonin $5HT_{1A}$	human	MalE	MBP	+	M	120 sites/cell	none	–	[17]
Endothelin ETB	human	ptac	–	+	M	41 sites/cell	none	–	[117]
Neurotensin	**rat**	**ptac**	**MBP**	**+**	**M**	**15 pmol/mg**	**c-myc/5-his Ct**	**1.15**	**[14]**
Neurotensin	**rat**	**T5**	*****	**+**	**ND**	**4–16 pmol/mg**	**c-myc/5-his Ct**	**1.23**	**[118]**
Neurotensin	**rat**	**ptac**	**MBP**	**+**	**M**	**15 pmol/mg**	**Bio Ct**	**1.15**	**[15]**
Neurotensin NK_2	rat	ptac	MBP	+	M	7.2 pmol/mg	c-myc and 5-his Ct	–	[37]
Adenosine A_1	human	lac	PhoA	+	M	0.4 pmol/mg	c-myc Nt	–	[115]
Adenosine A_1	human	MBP	MBP	+	M	0.4 pmol/mg	none	–	[119]
Neuropeptide Y_1	human	ptac	MBP	+	M	3.5 pmol/mg	FLAG and 6-his Ct	–	[120] [121]
Olfactive OR5		T7	GST	–	IB	ND	6-his Ct	ND	[26]
Adenosine A2a	**human**	**ptac**	**MBP**	**+**	**M**	**10–20 nmol/l**	**10-his and Flag Ct**	**4.5**	**[122]**
Vasopressin V2	human	Trc	PhoA/-gal	–	M	ND	none	ND	[123]
Opioid μ, δ, κ-	human	ptac	MBP	+	M	0.5 pmol/mg	none	–	[124]
Opioid μ-	human	ptac	MBP and $G_{\alpha o1}$ or $G_{\alpha i2}$ in Ct	+	M	⊕0.5 pmol/mg	none	–	[16]
M2 mACh	human	lac	MBP	+	M	6 pmol/mg	none	–	[125]
TSH	**human**	**T5**	**ND**	**ND**	**ND**	**2.5 mg/l**	**6-his Ct**	**12**	[126]

MBP or MalE: maltose binding protein, inner membrane protein from *E. coli*; LamB: LamB protein, outer membrane protein from *E. coli*; β-gal: β-galactosidase, cytosolic protein from *E. coli*; PhoA: alcaline phosphatase, protein located in the bacterial cytoplasm; GST: glutathione S-transferase of *Schistosoma japonicum*; aa: amino acid; ND: not determined. All promoters are IPTG inducible, Nt: N-terminus; Ct: C-terminus; 5-his, 6-his, 10-his: penta-, hexa- and deca- histidines tags, respectively; Bio: Bio tag; M: membrane; IB: inclusion bodies. TSH: thyroid-stimulating hormone; mACh: muscarinic cholinergic; the 'overexpression factor' (boldface characters) represents the ratio between the expression level and the minimum required to obtain 1 mg (or more) of recombinant protein in 5 l of culture media (minimum expression level determined in *E. coli*, 13 pmol/mg of membrane protein, see table 1 and text).
* Six different membrane proteins from phage.

Table 3. Expression of GPCRs in yeast.

Receptor	Origin	Yeast	Promoter	Signal/fusion peptide	Expression level	Ligand binding	G-protein coupling	Tags	Overexpression factor	Ref.
β_2-Adrenergic	**human**	***S. cerevisiae***	**GAL1**	**Ste2**	**115 pmol/mg**	**+**	**+**	**none**	**23**	**[7]**
	human	***S. cerevisiae***	**GAL1**	**Ste2**	**36 pmol/mg**	**+**	**ND**	**none**	**7**	**[47]**
	human	***P. pastoris***	**AOX1**	**ppαMF**	**25 pmol/mg**	**+**	**ND**	**Flag Nt Bio Ct**	**5**	**[66]**
	human	***Sz. pombe***	**ADH**	**Ste2**	**7.5 pmol/mg**	**+**	**ND**	**none**	**1**	**[55]**
α_2-C_2-Adrenergic	**human**	***S. cerevisiae***	**GAL1**	**–**	**7–70 pmol/mg**	**+**	**ND**	**Histag Nt**	**1.5–14**	**[47]**
Serotonin $5HT_{5A}$	**mouse**	***P. pastoris***	**AOX1**	**ppαMF, Pho1**	**22 pmol/mg**	**+**	**ND**	**c-myc Nt**	**4**	**[63]**
	mouse	***S. cerevisiae***	**PRB1**	**Bm**	**16 pmol/mg**	**+**	**ND**	**c-myc Nt**	**3**	**[64]**
	mouse	***P. pastoris***	**AOX1**	**ppαMF**	**40 pmol/mg**	**+**	**ND**	**Flag Nt/ Bio Ct**	**8**	**[65]**
Endothelin ETB	**human**	***P. pastoris***	**AOX1**	**ppαMF**	**30–60 pmol/mg**	**+**	**ND**	**Flag Nt Bio Nt**	**6 12**	**[127]**
Neurokinin NK2	human	*Sz. Pombe*	NMT1	Pho1	1.16 pmol/mg	+	–	none	–	[54]
Adenosine A2a	rat	*S. cerevisiae*	ADH1	–	0.45 pmol/mg	+	+	none	–	[208]
μ-Opioid	human	*P. pastoris*	AOX1	ppαMF	0.4 pmol/mg	+	–	none	–	[62]
μ-Opioid	human	*P. pastoris*	AOX1	ppαMF	4 pmol/mg	+	–	GFP Nt, c-myc, 6 his Ct	0.8	[68]
μ-opioid	**human**	***P. pastoris***	**AOX1**	**ppαMF**	**100 pmol/mg**	**–**	**–**	**GFP Nt, c-myc, 6 his Ct**	**20**	**[69]**
μ-Opioid	human	*S. cerevisiae*	GRAP1	17 aa Ste2	0.4 pmol/mg	+	–	none	–	[67]
m_1-Muscarinic	human	*S. cerevisiae*	ADH	–	0.02 pmol/mg	+	ND	–	–	[44]
m_5-Muscarinic	rat	*S. cerevisiae*	MF	ppαMF	0.13 pmol/mg	+	–	–	–	[128]
D_{2S}-Dopaminergic	human	*S. cerevisiae*	PMA1	Ste2	1–2 pmol/mg	+	ND	–	–	[52]
D_{2S}-Dopaminergic	human	*S. cerevisiae*	GAL10	Ste2	1–2 pmol/mg	+	ND	–	–	[45]
D_{2S}-Dopaminergic	**human**	***Sz. pombe***	**NMT1**	**Ste2**	**14 pmol/mg**	**+**	**ND**	**–**	**3**	**[52]**
D_{1A}-Dopaminergic	human	*S. cerevisiae*	GAP	Ste2	0.13 pmol/mg	+	ND	Flag+his Ct	–	[129]
D_2-Dopaminergic	human	*Sz. Pombe*	NMT1	–	1 pmol/mg	+	–	none	–	[53]
Somatostatin SSTR2	rat	*S. cerevisiae*	GAL1/10	–	0.2 pmol/mg	+	+	none	–	[40]
Rhodopsin	**bovine**	***S. cerevisiae***	**GAL1**	**–**	**2 mg/10^{10} cells**	**+**	**–**	**none**	**100**	**[71]**
Rhodopsin	**bovine**	***P. pastoris***	**AOX1**	**Pho1**	**0.3 mg/l**	**+**	**–**	**none**	**1.5**	**[70]**
GHRH	human	*S. cerevisiae*	GAL1/10	–	ND	+	+	none	ND	[130]
LysoPA Edg2/ Vzg1	human	*S. cerevisiae*	GAL	–	ND	+	+	none	ND	[131]
Ste2	**S. cer.**	***S. cerevisiae***	**GAP**	**–**	**350 pmol/mg**	**+**	**+**	**Flag+his Ct**	**70**	**[72]**

ppαMF: α-mating factor prepropeptide; AOX1: promoter of the alcohol oxydase 1 gene; Pho1: acid phosphatase signal peptide; Bm: Bacillus macerans signal peptide; GAP: promoter of the glyceraldehyde-3-phosphate dehydrogenase gene; NMT1; 'no message in thiamine': promoter inhibited in the presence of thiamine; PMA1: promoter of the plasma membrane ATPase; PRB1: promoter of the endopeptidase B gene; Ste2: receptor of the α-factor; ND: not determined; Flag: Flag tag, 8 amino acid sequence: DYKDDDDK; Bio: biotinylation domain of the transcarboxylase from *Propionibacterium shermanii* [132]; GHRH: growth hormone releasing hormone; GAL: galactose inducible promotor; lysoPA: lysophosphatidic acid. Boldface characters correspond to the GPCRs for which the overexpression factor is > 1.

Table 4. GPCR expression in insect cells.

Receptor	Origin	Cell type	Expression level	Function	G Protein coupling	Tags	Overexpression factor	Ref.
β_2-Adrenergic	**hamster**	**Sf_9**	**30 pmol/mg**	**+**	**ND**	**none**	**1.25**	**[133]**
	human	Sf_9	15 pmol/mg	+	ND	none	–	[84]
	turkey	Sf_9	5 pmol/mg	+	+	none	–	[134]
	human	**Sf_9**	**10–25 pmol/mg**	**+**	**+**	**c-myc**	**1**	**[89]**
	human	**Sf_9**	**40 pmol/mg**	**+**	**+**	**none**	**1.7**	**[135]**
	human	Sf_9	5–20 pmol/mg	+	ND	KT3	–	[207]
	human	**Sf_9**	**24 pmol/mg**	**+**	**+**	**c-myc Nt**	**1**	**[137]**
	human	Sf_9	2.5 pmol/mg	+	+	FlagNt/6-his Ct	–	[138]

Table 4. (continued).

Receptor	Origin	Cell type	Expression level	Function	G Protein coupling	Tags	Overexpression factor	Ref.
β_2-Adrenergic	avian	Sf_9	20 pmol/mg	+	+	none	–	[139]
α_{2C}-Adrenergic	human	Sf_9	1.4 pmol/mg	+	+	none	–	[140]
Serotonin 1B	human	Sf_9	1 pmol/mg	+	+	c-myc Nt	–	[141]
Serotonin $5HT_1$	human	Sf_9	1 to 5 pmol /mg	+	+	none	–	[142]
Serotonin $5HT_{1A}$	human	Sf_9	0.15 pmol/mg	+	+	none	–	[79]
Serotonin $5HT_{1A}$	**human**	**Sf_9**	**5–34 pmol/mg**	**+**	**–**	**none**	**1.4**	**[143]**
Serotonin $5HT_{4A}$	mouse	Sf_9	ND	+	ND	none	ND	[144]
Serotonin $5HT_{2C}$	rat	Sf_9	1.106 sites /cell	+	+	none	–	[145]
Neurokinin NK_2	human	Sf_{21}	0.82 pmol/mg	+	+	none	–	[146]
Neurokinin NK_1	rat	Sf_9	1–2 pmol/mg	+	+	none	–	[136]
Neurokinin NK_1	**human**	**Sf_9**	**80 pmol/mg**	**+**	**+**	**none**	**3.33**	**[147]**
δ-Opioid	mouse	$high_5$	1.4 pmol/mg	+	+	none	–	[148]
μ, δ, κ-Opioid	human	Sf_9/$high_5$	1–2 pmol/mg	+	+	none	–	[149]
μ, δ, κ-Opioid	human	Sf_9	κ: 5×10^4 sites/cell	+	–	none	–	[150]
			δ: 10^5 sites/cell	+	–	none	–	
			μ: 5×10^5 sites/cell	+	–	none	–	
			and 10^5 sites/cell	+	–	6-his Nt	–	
μ-Opioid	human	Sf_9	6–7.10^5 sites/cell	+	–	6-his Ct	–	[151]
μ-Opioid	**human**	**$high_5$**	**5×10^6 sites/cell**	**+**	**–**	**none**	**2**	**[151]**
μ-Opioid	human	Sf_9	9 pmol/mg	+	–	6-his Ct	–	[152]
m_3-Muscarinic	rat	Sf_9	12–18 pmol/mg	+	ND	none	–	[153]
m_1-Muscarinic	human	Sf_9	5 pmol/mg	+	–	none	–	[134]
m_2-Muscarinic	**human**	**Sf_9**	**30 pmol/mg**	**+**	**–**	**none**	**1.25**	**[134]**
m_5-Muscarinic	rat	Sf_9	ND	+	+	none	ND	[154]
m_1-Muscarinic	human	Sf_9	0.6 pmol/mg	+	ND	none	–	[81]
m_2-Muscarinic	human		4 pmol/mg	+	ND	none	–	[81]
m_5-Muscarinic	human		0.8 pmol/mg	+	ND	none	–	[81]
m_3-Muscarinic	rat		16 pmol/mg	+	ND	none	–	[81]
m_4-Muscarinic	rat		2.5 pmol/mg	+	ND	none	–	[81]
m_3-Muscarinic	rat	Sf_9	3.3 pmol/mg	+	ND	none	–	[85]
m_2-Muscarinic	**human**	**Sf_9**	**36 pmol/mg**	**+**	**ND**	**6-his Ct**	**1.5**	**[155]**
D_4-Dopaminergic	human	Sf_9	5 pmol/mg	+	+	none	–	[156]
D_2-Dopaminergic	human	$Sf_{9,21}$/Tn_5	5–8 pmol/mg	+	+	none	–	[157]
D_1-Dopaminergic	**human**	**Sf_9**	**33 pmol/ mg**	**+**	**+**	**c-myc Nt**	**1.4**	**[91]**
D_{2L}-Dopaminergic	human	Sf_9	2.6 pmol/mg	+	+	none	–	[91]
$D_{2S,L}$-Dopaminergic	rat	Sf_9	5–8 pmol/mg	+	+	none	–	[87]
D_{2S}-Dopaminergic	human	Sf_9/Tn	6 and 10 pmol/mg	+	ND	c-mycNt/6-his C	–	[158]
D_{2S}-Dopaminergic	human	Sf_9	10–19 pmol/mg	+	+	6-his Ct	–	[83]
LH/GH		Sf_9	4.5×10^3 sites/cell	+	+	none	–	[159]
FSH	human	Sf_9	7×10^3 sites/cell	+	ND	none	–	[160]
Cannabinoids	rat	Sf_9	3.2 pmol/mg	+	ND	none	–	[161]
N-formyl-peptide	**human**	**Sf_9**	**27 pmol/mg**	**+**	**–**	**none**	**1**	**[162]**
Glutamate RI	rat	Sf_9	1.5 pmol/mg	+	ND	none	–	[163]
Glutamate R2,4	**ND**	**Sf_{21}**	**15–30 pmol/mg**	**+**	**ND**	**none**	**1.2**	**[164]**
Glutamate R2	rat	Sf_9	2.5×10^5 sites/cell	+	ND	none	–	[165]
Oxytocin	human	Sf_9	1.66 pmol/mg	+	ND	c-myc Nt	–	[20]
Olfactive Olp4	rat	Sf_9	ND	ND	ND	6-his Ct	ND	[166]
Olfactive OR17-4	human	Sf_9	ND	ND	ND	6-his Ct/Flag Nt	ND	[166]
Rhodopsin	**bovine**	**Sf_9**	**4 mg/l**	**+**	**+**	**6-his Ct**	**20**	**[167]**
CXCR4	mouse	Sf_9	ND	+	+	HA Nt	ND	[168]
Substance P	rat	Sf_9	26 pmol/mg	+	+	KT3 Ct		[169]
Substance P	human	Sf_9	ND	+	ND	none	ND	[170]
Histamine H2	rat	Sf_9	6 pmol/mg	+	+	6-his Ct	–	[171]
GRP	murine	Sf_9	6 pmol/mg	+	+	none	–	[172]
PACAP	**human**	**Sf_9**	**80 pmol/mg**	**+**	**–**	**none**	**3**	**[173]**

PACAP: pituitary adenylate cyclase activating polypeptide; ND: not determined; HA: 'hemaglutinin epitope of the influenza virus'; FLAG: Flag epitope; FSH: follicle-stimulating hormone; LH/GH: lutropin/placental gonadotropin hormone; aa: amino acid; KT3: KT3 epitope TPPPEPET; GRP: gastrin releasing peptide.
Boldface characters correspond to the GPCRs for which the overexpression factor is > 1.

Table 5. GPCR expression in mammalian cells.

Receptor	Origin	Cell type	Expression level	Functionality	G Protein coupling	Tags	Overexpression factor	Ref.
α_{1B}-Adrenergic	ND	COS-7/SFV/CHO	4–5 pmol/mg	+	+	none	–	[174]
	ND	**BHK/SFV**	**24 pmol/mg**	**+**	**+**	**none**	**1**	[174]
α_2C_2-Adrenergic	human	CHO	1.2 pmol/mg	+	+	none	–	[175]
α_2C_2,C_4,C_{10}-Adrenergic	human	CHO	5 pmol/mg	+	+	none	–	[176]
α_2C_{10}-Adrenergic	**human**	**mouse C127**	**3–6×10⁶ sites/cell**	**+**	**+**	**none**	**1.5**	**[177]**
β_2-Adrenergic	**human**	**CHO**	**200 pmol/mg**	**+**	**+**	**none**	**8**	**[98]**
β_2-Adrenergic	hamster	CHO	6 pmol/mg	+	+	none	–	[178]
Serotonin $5HT1_B$	mouse	NIH-3T3	19 pmol/mg	+	+	none	–	[179]
Serotonin $5HT_{1E}$	**human**	**HEK-293**	**15–40 pmol/mg**	**+**	**–**	**none**	**1.6**	**[180]**
Serotonin 5HT-dro_{2A}	*Drosophila*	COS-7	15 pmol/mg	+	+	none	–	[181]
Serotonin 5HT-dro_{2B}	*Drosophila*	COS-7	9 pmol/mg	+	+	none	–	[181]
Serotonin $5HT_{1B}$	mammalian	L929	20 pmol/mg	ND	ND	none	–	[182]
Serotonin $5HT_{1B}$	mammalian	HEK-293	16 pmol/mg	ND	ND	none	–	[182]
Serotonin $5HT_{1F}$	mammalian	L929	9.5 pmol/mg	ND	ND	none	–	[182]
Serotonin $5HT_{1F}$	mammalian	HEK-293	3 pmol/mg	ND	ND	none	–	[182]
Neurokinin NK_1	**human**	**BHK/SFV**	**40 pmol/mg**	**+**	**+**	**none**	**1.5**	**[183]**
Neurokinin NK_2	human	CHO	7×10^5 sites/cell	+	ND	none	–	[184]
Adenosine A_1	human	HEK-293	4 pmol/mg	+	+	6-his Nt/ Flag Ct	–	[185]
Adenosine A_1	human	CHO	$4–7\times10^5$ sites/cell	+	+	none	–	[186]
Substance P	rat	COS	3 pmol/mg	+	+	none	–	[187]
Substance K	rat	COS	0.3 pmol/mg	+	+	none	–	[187]
Neuromedin K	rat	COS	0.16 pmol/mg	+	+	none	–	[187]
Substance K	human	NIH-3T3	9.7×10^3 sites/cell	+	+	none	–	[188]
Substance P + K	rat	CHO	11 pmol/mg	+	+	none	–	[189]
Neuromedin K	rat	CHO	7.6 pmol/mg	+	+	none	–	[189]
Neurokinin A	hamster	MEL	0.7 pmol/mg	+	+	none	–	[146]
	human	MEL	2.2 pmol/mg	+	+	none	–	[146]
μ-Opioid	rat	COS	ND	+	+	none	ND	[190]
μ-Opioid	human	COS-7	0.23 pmol/mg	+	+	none	–	[191]
$m_{1,2,3,4}$ Muscarinic	human	HEK	$1–3\times10^5$ sites/cell	+	+	none	–	[192]
m_1-Muscarinic	mouse	JEG-3	0.7–10 pmol/mg	+	+	none	–	[193]
m_4-Muscarinic	chicken	JEG-3	1.7–27 pmol/mg	+	+	none	–	[193]
Glutamate mGlu2,3	rat	CHO/SFV	0.5 pmol/mg	+	+	none	–	[194]
Glutamate R8	human	CHO/SFV	10–13 pmol/mg	+	ND	none	–	[195]
Neuropeptide Y1	**human**	**HeLa/VV**	**1 mg/l**	**+**	**ND**	**none**	**5**	**[196]**
TRH	human	CHO	$2–4\times10^4$ sites/cell	+	+	none	–	[86]
TRH	**mouse**	**adenovirus**	**2×10^6 sites/cell**	**+**	**+**	**none**	**1**	**[197]**
Glucagon	**rat**	**BHK**	**78 pmol/mg**	**+**	**+**	**none**	**3**	**[198]**
Glucagon	rat	COS-7	2×10^5 sites/cell	+	+	none	–	
Calcitonin	human	MEL	1.9×10^6 sites/cell	+	ND	none	ND	[199]
Thromboxane A2	human	COS-7	24 pmol/mg	+	+	6 his Ct	–	[200]
Endothelin (ET-1)	porcine	COS	5.7 pmol/mg	+	+	none	–	[201]
Histamine H2	**rat**	**COS-7/SFV**	**80 pmol/mg**	**+**	**+**	**none**	**3**	**[202]**
Olfactive OR 17-40	human/rat	HEK-293/SFV	ND	+	+	none	ND	[203]
Rhodopsin (synthetic)	**bovine**	**COS-1**	**6×10^6 sites/cell**	**+**	**+**	**none**	**3**	**[204]**
Rhodopsin	**bovine**	**HEK-293S**	**10 mg/ml**	**+**	**ND**	**none**	**50**	**[99]**

TRH: thyrotropin teleasing hormone; CHO: Chinese hamster ovary; HEK: human embryonic kidney; BHK: baby hamster kidney; JEG-3: human choriocarcinoma cells; COS: African monkey cells; MEL: murine erythroleukemia; L929: mouse fibrosarcoma; SFV: Semliki Forest virus; synthetic: synthetic gene; W: vaccinia virus; NIH3T3: mouse fibroblasts. Boldface characters correspond to the GPCRs for which the overexpression factor is > 1.

Results obtained with *E. coli*

The most widespread strategy used to express GPCRs in *E. coli* is to fuse the receptor of interest with a bacterial protein. The different GPCRs expressed in *E. coli* and their associated expression levels are presented in table 2. The first GPCR expressed in this bacteria is the β_2-adrenergic receptor, fused in its N-terminus to the 279 initial residues of β-galactosidase, a cytosolic protein naturally expressed in *E. coli* [32]. A large number of attempts to improve the initial expression level have been performed, mainly by changing the promoter and by fusing the receptor to different partners. Fusion of the receptor to proteins belonging to the inner or to the outer membrane of *E. coli* such as the MBP [33] or LamB protein [32, 34, 35] have enable a 10-fold increase in the initial expression level of the β_2-adrenergic receptor. Nevertheless, no fusion protein has been used in the study by Breyer et al. [36] and the expression level of the 2-adrenergic receptor under the control of a strong isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible promoter (gene 10 of the T7 bacteriophage) is identical to those obtained for the same protein fused to the LamB or MBP genes [36]. Thus, the correct targeting of a GPCR to *E. coli* membrane does not strictly rely on its fusion to a membranous bacterial protein. However, fusion of the neurotensin receptor to the MBP resulted in a 40-fold increase in expression level [14]. This kind of difference suggests that the behaviour in terms of expression level is clearly receptor dependent. As shown in table 2, expression levels range from 0.2 to 16 pmol of receptor per milligram of membrane protein (equivalent to 15–450 sites per cell), and are quite low whatever the strategy employed (with the notable exception of the TSH receptor, see [126]). Moreover, within a given receptor family the results can be different even though the expression strategy employed is identical. For instance, the expression level of the β_1-adrenergic receptor is fourfold lower than that of the β_2-adrenergic receptor [113]. This clearly shows that for each GPCR, a variety of genetic constructs must be systematically employed because the results depend on the exact primary sequence [34]. This difference of behaviour could be explained by the presence of rare codons in higher quantities in certain particular sequences. For instance, the leukotriene B4 (LTB4) receptor was overexpressed in *E. coli* after an optimisation step consisting of replacing all the rare codons initially present in the sequence of the receptor [206]. Although the recombinant protein is chemically homogeneous, several populations of receptor can sometimes be observed. For instance, a population of both high- and low-affinity binding sites have been observed for the neurotensin NK_2 receptor [37]. The origin of these two populations is not clearly explained by the authors, but the hypothesis is that during the insertion of the receptor into the membrane, two different conformations could be generated [37]. Immunodectection experiments reveal high quantities of proteins where only low expression levels are observed using radiolabeled ligand-binding experiments, which again suggests the presence of different populations of receptor [14]. The size and topology of GPCRs can also influence their production. Thus, the work by Kiefer et al. shows that the presence of positively charged residues in the intracellular loop 1 (IL1), favours expression of the OR5 olfactive receptor by preventing its insertion into the bacterial membrane [26]. Indeed, if the protein is toxic for the bacteria, its insertion into the membrane (thought to increase toxicity) can limit its expression. The studies by Banères et al. [205] and by Kiefer et al. [26] are, to our knowledge, the only ones reported that describe the expression of GPCRs in inclusion bodies. Despite the fact that the expression level of the receptors produced has not been determined accurately because of lack of activity, these studies are particularly relevant because they were pursued up to the functional reconstitution step. Thus, even if the functional refolding of all GPCRs is not a guarantee, this approach is very promising.

Yeast

Main characteristics of yeast

Yeast present short generation times (2 h), grow on very simple media, and their manipulation is easy and inexpensive. Moreover, the experience developed in particular by industries in the field of fermentation facilitates scaled-up production. Numerous plasmids are available for the expression of heterologous genes, and are either maintained under an episomal form or integrated in the genome of the selected yeast. In yeast, strong promotors are known and are either constitutive or inducible, such as PGK1 (constitutive) and GAL1 (galactose inducible) in *S. cerevisiae*, or AOX1 (methanol inducible) in *Pichia pastoris*. Contrary to bacteria, yeasts possess endogenous GPCRs and G proteins [38–41], and the eucaryotic nature of these microorganisms confers on them the capacity to perform posttranslational modifications [42, 43], which are sometimes essential for protein functionality [18, 19]. Nevertheless, the composition and quantity of *N*-glycans added by yeast are different from that of mammalian cells, and this can be problematical when precise oligosaccharide structures are essential to GPCR functionality [18]. The lipid composition of yeast membranes is also different from that of mammalian cells. Thus, yeast do not synthesise cholesterol like mammalian cells, but rather ergosterol, and this difference was shown to alter the functionality of some GPCRs [23]. Finally, yeast are surrounded by a cell wall that must be disrupted in order to extract membrane and intracellular proteins.

Results obtained in yeast

The results obtained for the heterologous expression of GPCRs in yeast are presented in table 3.

S. cerevisiae

The first yeast used for the production of recombinant proteins is *S. cerevisiae*. The most frequently employed promoters are galactose inducible, such as GAL1 and GAL10. It has rapidly been concluded that the fusion of the receptor, in its N-terminus end, to a yeast signal sequence favours its insertion into the membranes. Among the most used fusion sequences is that of the α-mating factor prepropeptide (ppαMF), a sexual mating factor excreted by *S. cerevisiae*, which binds to the Ste2 receptor. The Ste2 N-terminus can also be used as a targeting sequence. The first attempts to express GPCRs in *S. cerevisiae* were those of the human β_2-adrenergic and m_1-muscarinic receptors [7, 44]. The m_1-muscarinic receptor was able to bind a variety of specific ligands, but its expression level (20 fmol/mg of membrane proteins) was extremely low [44]. On the contrary, the β_2-adrenergic receptor was very highly expressed: 115 pmol of receptor per milligram of membrane protein [7]. This result was obtained after the following handling: (i) replacement of the 5′-untranslated region and the 14 subsequent codons of the β_2-adrenergic gene by the corresponding region of the STE2 receptor gene; (ii) use of a strong promotor: GAL1 and coexpression of its transcriptional transactivator and (iii) induction of the β_2-adrenergic receptor expression in the presence of an antagonist which is likely to act as a structural stabilizer. In this study, the receptor coupling to the $G_{\alpha s}$ subunit (also coexpressed) was demonstrated [7]. However, the use of an identical strategy (*S. cerevisiae*, GAL-type promotor, STE2 fusion) for the D2S-dopaminergic receptors resulted in a lower expression level (1–2 pmol of receptor per milligram of membrane protein) [45, 46]. This shows again that each GPCR behaves in a specific way and that the transposition of an efficient strategy from one to another is never guaranteed. Moreover, the transposition of batch-optimised culture conditions to the preparative scale (fermenters) can also result in modification of the expression level. For instance, the human β_2-adrenergic receptor, expressed under the conditions described by King et al. [7] but grown in fermenter, was 'only' produced at a level of 36 pmol per milligram of membrane protein [47]. The crucial factors for this study were the use of a selective medium without glucose, the control of pH between 7.2 and 7.5, and the presence of an antagonist in the induction medium. These conditions have been applied to the human α_2-C2-adrenergic receptor fused in its C-terminus to a pentahistidine tag, but devoid of any fused sequence in its N-terminus. The production of the receptor, monitored by immunodetection using a receptor-specific antiserum and by radioactive ligand-binding experiments, has reached levels between 7 and 70 pmol of receptor per milligram of membrane protein, depending on the experiment [47].

Schizosaccharomyces pombe

Initially used to express bacteriorhodopsin [48–50], the yeast *Sz. pombe* has been successfully employed for GPCR expression. The strong *nmt1* (no message in thiamine) promoter, which is repressed by thiamine was generally used for expression [51]. The first GPCR expressed in *Sz. pombe* was the short form of the D_2-dopaminergic receptor (D_{2S}), placed under the control of the *nmt1* promoter and fused, in its N-terminus, to part of the Ste2 receptor [52]. In terms of selective ligand affinities, the recombinant receptor presented a pharmacological profile, typical of the D_{2S}-dopaminergic protein, but the low affinities detected suggested a lack of coupling with endogenous yeast G proteins. The expression level of this receptor could be increased sevenfold from *S. cerevisiae* to *Sz. pombe*, using a strong promoter in both cases: GAL1 and *NMT1,* respectively. The expression of the 'long' form of the D_2-dopaminergic receptor (D_{2L}), has been realised under the control of the *NMT1* promoter. The expression level was 1 pmol per milligram of membrane protein with a pharmacological profile typical of a D_2-dopaminergic receptor lacking coupling with the endogenous G proteins of *Sz. pombe* [53]. However, the neurokinin NK2 receptor was only produced at a level of 1.2 pmol per milligram of membrane protein [54], and the β_2-adrenergic at 7.5 pmol per milligram of membrane protein [55]. In the case of the NK2 receptor, the fusion sequence used was that of the signal sequence of the acid phosphatase *PhoI*, a yeast protein located at the cell surface. This sequence was essential in the case of NK2 receptor production, and no functional receptor was detected with a construct containing the receptor alone [54]. Like the ppαMF sequence, the *PhoI* sequence is supposed to favour the correct targeting to yeast membranes. For the NK2 receptor, the difference observed between the expression level detected on whole cells (430 sites per cell) and on membrane preparations (5500 sites per cell) suggests that only a small part of the protein is correctly targeted into the plasma membrane [54]. In the case of the β_2-adrenergic receptor, the alcohol dehydrogenase promoter (ADH) used was constitutive. Fusion to this GPCR of the Ste2 receptor in its N-terminus was required for expression of the protein, whether it was monitored by radiolabeled ligand binding experiments or by immunodetection [55].

Pichia pastoris

Methylotrophic yeasts such as *P. pastoris*, *Hansenula polymorpha* and *Candida boidinii* have been used for

many years as hosts for the production of recombinant proteins [56–61]. The particularity of these yeast strains resides in their ability to use methanol as a single carbon source. Therefore, the methanol metabolism is completely central in these yeasts and the key enzymes involved in methanol assimilation are highly produced by the cells and can represent up to 80% of their total protein content. Thus, the use of strongly regulated and highly inducible (by methanol) promoters that control these enzymes expression, has allowed the production of a large number of recombinant proteins. These methylotrophic yeasts are particularly efficient, as they can grow rapidly, reach high cell densities, do not give rise to hyperglycosylation phenomena and also possess extremely efficient secretory machinery [43]. As shown in table 3, all the expression experiments of GPCRs in methylotrophic yeasts have focused on the use of *P. pastoris* by placing the heterologous gene under the control of the methanol-inducible alcohol oxidase 1 promoter (AOX1), and by using different signals/proteins fused to the receptor studied. In *P. pastoris*, only vectors leading to genome integration of the expression box are available, which is a guarantee of stable expression.

The expression levels of GPCRs vary from 0.4 for the human μ-opioid receptor [62] to 40 pmol per milligram of membrane protein for the $5HT_{5A}$-serotoninergic receptor [65]. For the $5HT_{5A}$-serotoninergic receptor, this expression level is threefold superior to that previously obtained in *S. cerevisiae* [64]. Initially, two types of fusion proteins were used for expression of this receptor [65]: the signal peptide of the acid phosphatase (Pho1) of *P. pastoris*, and the prepropeptide of the α-mating factor (ppαMF) of *S. cerevisiae*. The best results obtained for the $5HT_{5A}$-serotoninergic receptor (22 pmol per milligram of membrane protein) were reached when using the ppαMF signal sequence and a particular yeast strain: SMD1163, deficient in two proteases. The selection of clones bearing several copies of the expression box inserted in the yeast's genome and the addition of ligand in the culture medium enabled an expression level of 40 pmol per milligram of membrane proteins [65]. For the human β_2-adrenergic receptor, the expression level in *P. pastoris* was 'only' of 25 pmol per milligram of membrane protein [66], in comparison with the 115 pmol per milligram of membrane protein previously reported in *S. cerevisiae* [7]. For the human μ-opioid receptor placed under the control of the AOX1 promoter and fused to the ppαMF signal sequence, the expression level of 0.4 pmol per milligram of membrane protein [62] was rigorously identical to that reported in *S. cerevisiae* [67]. In *P. pastoris*, this expression level was not increased by the use of protease(s)-deficient strains such as SMD1163 or SMD1168, and the recombinant receptor presented a pharmacological profile for various opioid ligands typical of a μ-opioid receptor lacking coupling with endogenous yeast G proteins [62]. The same result (correct pharmacological profile but lack of G protein coupling) has been observed for this receptor in *S. cerevisiae* [67]. It is however interesting that G protein coupling can be restored in vitro. In addition, comparative quantification experiments of the total (fluorescence experiments) versus the active (binding experiments) proportions of the μ-opioid receptor have shown that the majority of the receptor produced in *P. pastoris* is not detected by classical binding experiments [69]. Moreover, while the total amount of receptor produced is constant, the active proportion of this receptor can be increased fourfold by changing the induction conditions [68].

Bovine rhodopsin has also been expressed under the control of the AOX1 promotor and fused to the *PhoI* signal sequence in *P. pastoris* [70] and the reported expression level was 3.7×10^6 sites per cell (2.4×10^6 in *S. cerevisiae* [71]). Thus, here again, the efficiency of a particular cell type for the production of a given GPCR is strongly dependent on the intrinsic nature of the protein considered. The highest GPCR expression level so far reported in yeast is that of the Ste2 receptor expressed at a level of 350 pmol per milligram of membrane protein in *S. cerevisiae* [72], but in the case of homologous expression. It has been observed that the recombinant receptors may or may not be properly coupled to G proteins in yeast. This hazardous coupling phenomenon can be explained by the difference in nature between yeast and superior eucaryotic cell G proteins. Expression of GPCRs in superior eucaryotic cells therefore appears to increase this coupling efficiency.

The baculovirus/insect cells system

Main characteristics of insect cells

The *Autographa californica* baculovirus is a double-stranded DNA virus surrounded by a lipid membrane (for review see [73]). This virus is able to selectively infect different insect cell types, among which the most frequently used are those of *Spodoptera frugiperda* (cell lines Sf_9 and Sf_{21}) and of *Trichoplusia ni* (cell lines Hi_5 and MG_1). These insect cells present relatively long generation times (up to 24 h) and require complex culture media. Their eucaryotic nature guarantees their ability to perform posttranslational modifications. The general principle of this system is to place the gene of interest in a plasmid between sequences of high homology for the baculovirus genome, under the control of a strong promoter, most often polyhedrin. Produced at a level of 50% of the total protein content of the cell, polyhedrin is one of the major components of the protective matrix in which the virus is subsequently embedded and is therefore responsible for virus survival after host cell death. Cotransfection of the plasmid and viral DNA in insect

cells allows insertion of the gene of interest into the viral genome in vivo by homologous recombination. The polyhedrin promoter is a 'late' promoter, and its activation occurs long after the infection process (8–24 h post-infection). This is an advantage when the recombinant protein is toxic for the cell. The drawback, however, is that receptor production occurs only at the end of the cell's life. In other respects, this system was initially established for transient expression, which is not optimum for large-scale production. The establishment of stable cell lines under the control of either a viral or insect promoter have subsequently been developed [74–78]. These stable cell lines offer an environment unperturbed by the viral cycle, which is ideal for functional studies. But the promoters are generally weaker than that of polyhedrin.

A large number of vectors and viral linearised DNA for transient expression are commercially available, and it is possible to obtain important virus stocks in a relatively short time (month scale).

Results obtained with insect cells

Many GPCRs have been transiently expressed, but the attempts to express GPCRs in stable insect cell lines are still isolated [75–78]. The main results, presented in table 4, show that most of the time S_{f9} or S_{f21} cell lines were employed. In all cases, the GPCRs produced were able to bind radiolabeled ligands (agonists and antagonists) in a specific and saturable manner and to couple to endogenous G proteins. A detailed analysis of the results shows that the expression levels are very different from one GPCR to another: from 0.15 pmol/mg for human $5HT_{1A}$-serotoninergic [79] to 80 pmol/mg for neurokinin NK1 receptors [80, 180]. Moreover, these expression levels are highly dependent on receptor subtype: using exactly the same expression strategy, the values obtained for each of the five muscarinic receptor subtypes were comprised between 0.6 and 16 pmol/mg [81]. Less variability was observed for the three opioid receptors subtypes, which were weakly expressed by this system [75, 76, 148–151]. Some parameters, such as the presence and the position of tags can also influence the expression level of a given receptor. For instance, fusion of a hexahistidine tail to the human μ-opioid receptor N-terminus (hMOP1) diminished its expression level 10-fold, while it had no influence when fused to the C-terminus end of the receptor [151]. Positive charges brought by this polyhistidine tag could inhibit targeting of the receptor to the membrane. The addition of signal sequences such as that of prepromelittin [158] or of hemagglutinin [82] can also improve the expression level of a receptor in the plasma membrane. However, a large proportion of the GPCR can be trapped in intracellular membranous compartments such as the Golgi apparatus or the endoplasmic reticulum [83]. One major drawback of this system from the perspective of structural biology experiments is that it often gives rise to heterogeneous populations of receptors (in terms of posttranslational modifications). For instance, glycosylation heterogeneity in the human β_2-adrenergic receptor N-terminus [84] and in the rat m_3-muscarinic cholinergic receptor has been described [85]. In the case of the thyrotrophin receptor, despite high messenger RNA (mRNA) levels, no receptor molecule was revealed by binding experiments [86]. This underlines the importance of using an alternative means to detect the expressed protein, independent of radiolabeled ligand-binding experiments. Moreover, high-affinity binding sites for agonists are not always observed, probably due to an insufficient number of endogenous G proteins available to couple with the overexpressed GPCR [20, 87]. The very low abundance of cholesterol in insect cells can also explain the binding properties of certain GPCRs, as was observed for the oxytocin receptor [88]. Despite this phenomenon, a large number of studies have found functional coupling between endogenous G proteins and heterologously expressed GPCRs [89–91].

Mammalian cells

Main characteristics of mammalian cells

The main advantages of mammalian cell expression system are that (i) these cells present the environment closest to the native tissues in which GPCRs naturally occur; (ii) they are able to perform complex posttranslational modifications and (iii) the lipid composition of their membranes is close to that of the native receptor. Especially, these cells can furnish G proteins able to interact with the GPCRs produced and secondary effectors for studying signal transduction. Thus, mammalian cells appear as the most appropriate for functional studies, which require an active protein, but not in very high amounts. There are two major approaches for heterologous expression of GPCRs in mammalian cells: transient and stable expression. Transient expression is performed under non-selective conditions. In this case, cells can be transfected with the gene encoding the GPCR of interest either with a plasmid or with a recombinant virus. Several virus types can be used, such as the vaccinia virus (for review see [92–94]) and the Semliki Forest virus (for review see [95]) for instance. Even if the "transfection" step using these viruses is very efficient, GPCR expression is transient since the cells die after several days. Thus, if high quantities of material are necessary in a repetitive way, establishment of stable cell lines is highly recommended. In the case of stable expression, the cells are grown under selective conditions thanks to the use of a selection marker cotransfected with recombinant DNA. The recombinant DNA can either be integrated in the host cell genome by using an appropriate vector or non-integrated in the case of vectors capable of episomal replication. In-

serted next to the gene encoding for the dihydrofolate reductase or the glutamine synthetase, the gene of interest can then be amplified. Selection of clones bearing multicopy insertion events can then be realised using a high pressure of selection (high antibiotic concentration, for instance). Whatever the strategy employed, generation of an amplified stable cell line overexpressing recombinant GPCR is usually time-consuming (month-long delays).

Results obtained with mammalian cells

A huge number of GPCRs have been expressed in mammalian cells, but this expression system has mainly been used to study the key residues involved in binding specific ligands and the possible corresponding structure of the active site of the receptor by associating transient expression and mutagenesis experiments (for review see [96, 97]). Rare are the GPCRs produced in mammalian cells that carry special tags for a subsequent purification step since they are most often dedicated to functional studies and not to overproduction (indeed, most of the "tagged" GPCRS have been used in the context of internalisation/desensitization or dimerisation studies for instance).

Table 5 shows some of the results obtained for expression of GPCRs in different mammalian cell lines, either by stable or transient expression. Indeed, these cells have been largely used for heterologous expression of GPCRs, particularly during their respective cloning step, in the case of the study of mutant or chimeric receptors or for studying GPCR oligomerisation and internalisation processes. Thus, the results summarised in table 5 are presented as an illustration and are not exhaustive. One can easily see that the expression level is highly variable from one GPCR to another. The highest production level reported is that of the human β_2-adrenergic receptor, which reached 200 pmol per milligram of membrane protein [98] and rhodopsin with 10 mg/l in culture of HEK-293 cells. In the latter case, purification to homogeneity was reported [99]. This result is very encouraging for other receptors even if scale-up of mammalian cell culture is likely to be difficult.

Other systems

Aside from the major expression systems previously described, many other strategies have been used to express GPCRs. For example, two receptors were expressed with femtomolar expression levels in the Archea *Halobacterium salinarum*, the β_2-adrenergic receptor [101] and the yeast α-mating factor receptor [100]. A caterpillar expression system was used to express, in a nonfunctional form, the extracellular part of the luteinizing hormone (LH) receptor, a member of the glycoprotein hormone family [102]. Heterologous expression in *Xenopus* oocytes was realised, but this system was mainly used to study GPCR functional coupling since protein expression requires mRNA injection into each oocyte, which prevents large-scale production [103]. An original system was recently developed for the expression of GPCRs in fly eyes [104]. The *Drosophila melanogaster* metabotropic glutamate receptor was expressed in good yield (170 µg/g fly heads), but heterologous expression of GPCRs is still a challenge in this system. Finally, cell free expression systems have many advantages, and numerous experiments have been reported with soluble proteins [105–109]. Although no GPCR has yet been expressed, these systems might become important in the following years, in particular in the context of structural genomics projects [110, 111].

Discussion: factors influencing GPCR overexpression

Tables 2 to 5 (column 'Overexpression factor') clearly show that in general, GPCRs are only slightly overexpressed by the different heterologous systems presented above. The difficulty of all these systems in achieving overexpression of GPCRs finds its origin in the complex folding mechanism of membrane proteins, in host membrane insertion troubles, or in an inappropriate lipid environment and/or posttranslational modifications. Thus, only a better understanding of the in vivo folding mechanisms of these membrane proteins and detailed characterisation of the proteins and factors which participate in this folding process will lead to improvements in the use of these different expression systems. Today, systematic comparison of the expression of a given membrane protein in a particular system only generates an approximate and empirical guide to factors that can influence results. One should also keep in mind that negative results are often not published. Comparison of the different expression levels from one GPCR to another shows that it is difficult to predict whether a receptor will be efficiently overexpressed. Among a given expression system (for instance in insect cells), the expression level of different GPCRs can vary 100-fold. Similarly, the expression level for a given GPCR is significantly different from one system to another. Up to now, the highest expression level reached is that of the Ste2 receptor of *S. cerevisiae*, overexpressed in *S. cerevisiae* at a level of 350 pmol per milligram of membrane protein [72]. This example clearly illustrates the fact that the closer to the native environment of the receptor a system is, the higher the chance of achieving overexpression. The majority of heterologously expressed GPCRs are from murine and human origin, and this suggests that mammalian cells should present the more appropriate environment. However, and even if the data presented in table 5 are not exhaustive, mammalian cells are the least developed for high-scale production of recombinant proteins, and up to now have mainly been used for functional studies. It is therefore interesting to

notice that only a few of the GPCRs expressed in mammalian cells presented a tag enabling subsequent purification (e.g. Histags, Biotags, Flag tags). Whether they derive from mammals or insects, superior eucaryotic cells are able to perform posttranslational modifications that can be the source of heterogeneities and troubles during the purification step. In particular, N-glycosylations are very important for several proteins for their folding and/or stability.

The specific sequence of each GPCR is one of the key elements that controls its expression. Indeed, as shown in table 6, some receptors such as the β_2-adrenergic, are expressed very efficiently in almost every system, though the quantities vary from one to another. Other GPCRs, such as the human μ-opioid receptor, are expressed at low levels whatever the system used (table 6), except in *P. pastoris* (100 pmol/mg), where 1% of the expressed receptor is active.

In conclusion, it seems that the only way to optimise the overexpression of a particular GPCR is to systematically test different expression systems and strategies. Several criteria can guide the researcher's choice, and particularly the ultimate goal of the work. Whenever the goal is high-resolution 3D structural data, the objective is simple: obtaining purified receptor, chemically and structurally homogeneous, in high quantities. To compare the efficiency of the different expression systems, Grisshammer and Tate [6] have proposed a minimal production of 1 mg of recombinant protein in 5 l of culture medium. The corresponding minimal expression levels are reported in table 1 and are 13 pmol/mg of membrane protein in *E. coli*, 5 pmol/mg in yeast and 26 pmol/mg of membrane protein in superior eucaryotic cells. This rapid estimation indicates which receptors can be produced in high quantities and in which cell types. These examples appear in boldface in tables 2–5. An initial rapid analysis of the four tables clearly shows that yeast is the system in which this objective was most frequently obtained. Of the 25 GPCRs expressed in this organism (table 3), 14 were expressed at a level higher than 5 pmol/mg of membrane protein. In *E. coli*, of 25 GPCRs expressed, only 4 exceeded the minimal expression level, and the numbers were 13 out of 60 in insect cells and 10 out of 55 for mammalian cells. The results presented in table 6 clearly show that this objective was easily reached for the β_2-adrenergic receptor whatever the system used, while it was possible only in *P. pastoris* for the human μ-opioid receptor. In general, the term 'overexpression' is used to describe the production of 10–100 mg of protein per liter of culture. Indeed, for 3D crystallisation trials, several tens of milligrams of pure protein are needed, and an expression level of 1 mg in 5 l of culture media will rapidly be limiting, particularly when considering that the overall yield of the purification procedure is generally very low. In some case it is difficult to determine the expression level when the expressed GPCRs are not active in terms of ligand binding. For instance the OR5 olfactive receptor expressed in *E. coli* by Kiefer et al. [26] was expressed in inclusion bodies in an inactive form. It was then successfully purified and functionally reconstituted in proteoliposome. Thus, an initially inactive GPCR can be refolded into an active conformation, and the initial activity is not an absolute prerequisite [205, 206]. This strategy is greatly simplified if a detection test, independent of binding experiments, is available to quantify the GPCR expression level. In this regard, expression of a fusion protein between the receptor and green fluorescent protein may be of general interest [68, 69]. Progress in the in vitro re-

Table 6. Comparative heterologous expression levels of the human β_2-adrenergic and μ-opioid receptors.

Receptor	Host cell	Strain	Expression level (pmol/mg)	Expression level (sites/cell)	Expression level necessary to obtain 1 mg of receptor in 5 l of culture media		Ref.
					pmol/mg	sites/cell	
Human	*E. coli*		1	200 #	13	2.6×10^3	[36]
β_2-Adrenergic	yeast	*S. cerevisiae*	115 #	6×10^5	5	2.6×10^4	[7]
	insect cells	*Sf*$_9$	10	10^6 #	26	2.6×10^6	[84]
		Sf$_9$ stable	3–4	3.5×10^5 #	26	2.6×10^6	[77]
	mammalian cells	CHO stable	200 #	2×10^7	26	2.6×10^6	[98]
Human	*E. coli*		0.4 #	80	13	2.6×10^3	[124]
μ-Opioid	yeast	*P. pastoris*	0.4 #	2×10^3	5	2.6×10^4	[62]
		P. pastoris	100 #	5×10^5	5	2.6×10^4	[69]
		S. cerevisiae	0.4 #	2×10^3	5	2.6×10^4	[67]
	insect cells	*Sf*$_9$	9 #	10^6	26	2.6×10^6	[152]
			10	10^6 #	26	2.6×10^6	[151]
	mammalian cells	COS-7	0.23 #	2.3×10^4	26	2.6×10^6	[191]

The # symbol represents the expression levels as they were given by the authors, while the other expression levels correspond to the conversions according to the values given in table 1.

folding of GPCRs would make overexpression in inclusion bodies in *E. coli*, or in any equivalent compartment in yeast, a strategy of choice for GPCRs, as it is already the case for many soluble proteins.

Whatever the strategy employed, once the overexpression step is reached, the protein must be extracted from the chosen organism and purified. To date (apart form the noticeable exception of rhodopsin), despite the numerous reports of the heterologous expression in GPCRs, only a single recombinant one has been solubilised, purified and subsequently used in a high-resolution NMR experiment [112]. This study has allowed the determination of the 3D structure of a small peptide ligand (PACAP), interacting with its corresponding GPCRs, using the transferred nuclear Overhauser effect approach [112]. It can be foreseen that the improvements observed recently in the field, and the numerous high-throughput initiatives for GPCR overexpression, will drastically change the situation in the near future.

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