Introduction
In response to endogenous and exogenous stimuli, multicellular organisms develop highly co-ordinated responses. An essential element in this communication system involves G-protein-coupled receptors (GPCRs). This ubiquitous class of membrane proteins arose early during evolution and has developed into one of the largest protein superfamilies known in nature. GPCRs have been identified in a variety of organisms, varying from yeast and Dictostelium to mammals. Several hundreds of distinct GPCRs couple extracellular stimuli as diverse as light, odours, monoamines, glycohormones and ions to intracellular signals. As such, GPCRs are of fundamental importance for cell-to-cell communication. Consequently, these proteins are the target of approximately 60% of the currently available medication against human diseases. Moreover, a large number of additional GPCRs is still expected to be discovered in genome projects. Consequently, structural studies with GPCRs have so far mainly been performed with rhodopsin. Recently, preparation of two-dimensional crystals of rhodopsin was further improved and analysis of images from cryo-microscopy produced a medium-resolution (7.5 Å) 3D structure of the membrane domain, which agrees with seven transmembrane segments with a high degree of α-helical character [3]. Studies using Fourier-transform infrared (FTIR) spectroscopy support a large content of α-helical structure that extends into some helical loops, and indicate considerable β-structure in the intracellular domain [4]. NMR analysis of soluble peptides representing intracellular loops also suggests a high proportion of β-structure and has generated a model for the intracellular domain of rhodopsin [5].

Visual pigments: photon-counting receptors
Visual pigments comprise a subfamily of GPCRs that accounts for the light sensitivity of the photoreceptor cells in the vertebrate (rods and cones) and invertebrate retina, which is essential for the process of vision [6]. Members of this family are probably also involved in the mechanisms regulating photoperiodic processes [7–9]. In their evolution towards a photosensory function, the visual pigments have developed some unique features among the GPCRs [6]. They contain a photosensitive ligand (11-cis-retinal), that is covalently bound to the protein moiety (opsin) through a protonated Schiff base linkage with a membrane-domain-embedded lysine side chain. The retinylidene ligand is responsible for the visible absorbance band of these receptors (e.g. sufficient amounts of purified material. This is largely due to the facts that tissue expression of GPCRs is very low (0.01–0.2 pmol/mg of membrane protein) and that purification is usually accompanied by heavy losses due to their low stability in detergent solution. Only the light-sensitive GPCR rhodopsin is expressed at relatively high levels in retinal photoreceptor membranes, which allows the isolation of reasonable amounts of purified protein. Consequently, structural studies with GPCRs have so far mainly been performed with rhodopsin. Recently, preparation of two-dimensional crystals of rhodopsin was further improved and analysis of images from cryo-microscopy produced a medium-resolution (7.5 Å) 3D structure of the membrane domain, which agrees with seven transmembrane segments with a high degree of α-helical character [3].

Abbreviations used: FTIR, Fourier-transform infrared; ss-MAS, solid-state magic-angle spinning; pi, post infection; GPCR, G-protein-coupled receptor; IMAC, immobilized-metal affinity chromatography; DoM, β-l-dodecyl maltoside; 3D, three-dimensional; MOL, multiplicity of infection.

'To whom correspondence should be addressed.
As an inverse agonist, it reduces the basal activity of the apoprotein opsin to such a low level that it allows the rod photoreceptor to operate under very dim light intensities, basically functioning as a photon counter \([10]\). Absorption of a light photon triggers an ultrafast (within \(200\text{ fs} \) \([11]\)) conformational change in the ligand (isomerization, \(11\text{-cis} \rightarrow \text{all-trans}\)), which converts it into a full agonist, resulting in rapid high-level activation of the receptor (within several ms) to a \(\approx 10^6\)-fold increase in activity over the dark state \([6]\). A single light-pulse can lead to synchronous activation of a sufficient number of receptors to detect the accompanying conformational steps in the protein.

Symbiosis between opsin and chromophore has pushed the efficiency of the photochemical reaction (\(\text{cis} \rightarrow \text{trans isomerization}\)) close to the limit. A nearly complete stereospecificity, femtosecond kinetics and a quantum yield of 0.67 are far beyond the values presently achieved in model systems \([12]\). Subsequent discrete conformational steps leading to the active receptor (metarhodopsin II) have been identified by their individual spectral properties and can be isolated thermally by cryospectroscopy \([13]\) or kinetically by picosecond spectroscopy \([12]\). The spectral intermediates (batho-, lumi-, metarhodopsin I and metarhodopsin II) have been shown to indeed represent discrete structural intermediates by vibrational spectroscopy \([11,14]\).

It is obvious that visual pigments have become a paradigm for studies of the molecular mechanism of a GPCR. Ideally, a high-resolution 3D structure would serve as a basis for such studies. However, 3D crystals suitable for high-resolution X-ray diffraction analysis have not yet been produced for any GPCR. The available two-dimensional crystals of rhodopsin currently only suffice for a medium-resolution 3D structure of the membrane domain \([3]\). In spite of the progress in molecular modelling, the available models based on this medium-resolution structure still differ considerably (e.g. \([15,16]\)) and reliable high-resolution structural information cannot be extracted from such structures, yet.

Recently, alternative experimental approaches to extracting structural data from supramolecular membrane systems have been developed. FTIR spectroscopy in the difference mode can monitor changes at the molecular level (changes in absolute structure, in interaction pattern or in microenvironment) by the corresponding change in vibrational frequency and/or intensity \([14]\). In complex systems, identification or assignment to specific chemical groups usually requires site-specific mutagenesis or, preferentially, stable-isotope labelling \([17,18]\). Novel developments in the field of solid-state magic-angle spinning (ss-MAS) NMR spectroscopy now allow the derivation of highly precise structural constraints from supramolecular systems. Isotopic chemical shifts and corresponding tensor elements in combination with relaxation properties can provide detailed information on chemical and electronic characteristics of a specific atom \([19]\). Distances between atoms of up to 1 nm can be measured with high accuracy (\(\pm 0.01\text{ nm}\)) using one-dimensional rotational resonance NMR \([20,21]\). Torsional angles in bonds between adjacent atoms can be addressed by double-quantum heteronuclear local-field NMR \([22]\). The latter approach is also very appropriate for measuring short distances (0.1–0.2 nm).

Such structural studies require relatively large amounts of purified protein (FTIR, 0.2–0.5 mg; ss-MAS NMR, 10–30 mg). In addition, stable-isotope labelling is usually very helpful in FTIR studies for the purpose of identification \((^{13}\text{C}, ^{1}\text{H}, ^{15}\text{N}, ^{18}\text{O}, ^{34}\text{S})\), and absolutely essential for NMR studies \((^{13}\text{C}, ^{2}\text{H}, ^{15}\text{N})\). Consequently, we have invested considerable efforts in developing a functional expression system for visual pigments that would allow appropriate levels of stable-isotope incorporation as well as scale-up to production levels of tens of milligrams of purified protein per batch.

**Functional expression and scale-up**

With the exceptional progress in molecular biology it is now possible to overexpress various GPCRs in a variety of host systems. Expression levels that are 100–1000-fold higher than those available in native tissue can be obtained. As to visual pigments, early studies were primarily performed with the relatively stable bovine rod visual pigment rhodopsin. More 'simple' systems like *Escherichia coli* or the yeast strains *Saccharomyces* or *Pichia* produced either not much recombinant protein at all \((E.\ coli)\) or reasonable amounts of total protein, but very low levels of functional receptor \((4–8\%\text{ in yeast})\) \([23,24]\). Mamalian cell lines (COS, HEK293) generate satisfactory yields \((>10^6\text{ copies/cell})\) of functional protein \([25,26]\), but stable-isotope labelling and culture scale-up is not easily achieved, even if one

\(\lambda_{\text{max}} = 498\text{ nm for bovine rhodopsin},\) and is called the chromophore. This ligand actually functions as an inverse agonist. It reduces the basal activity of the apoprotein opsin to such a low level that it allows the rod photoreceptor to operate under very dim light intensities, basically functioning as a photon counter \([10]\). Absorption of a light photon triggers an ultrafast (within \(200\text{ fs}\) \([11]\)) conformational change in the ligand (isomerization, \(11\text{-cis} \rightarrow \text{all-trans}\)), which converts it into a full agonist, resulting in rapid high-level activation of the receptor (within several ms) to a \(\approx 10^6\)-fold increase in activity over the dark state \([6]\). A single light-pulse can lead to synchronous activation of a sufficient number of receptors to detect the accompanying conformational steps in the protein.

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turns to stably transformed cell lines [27,28]. We experienced good expression levels in the eukaryotic recombinant baculovirus expression system in combination with the insect SF9 cell line [29]. This cell line is easily adapted to suspension culture as well as to serum-free culture conditions. We have therefore optimized the baculovirus system towards batch production of rhodopsin (20-40 mg of receptor/batch) with respect to the following parameters: (i) baculovirus promoter to drive opsin expression, (ii) serum-free culture medium, and (iii) multiplicity of infection (MOI) and incubation time.

Suitable expression levels (> 5 × 10⁴ copies/cell) were only obtained with the very strong very late-phase p10 and polyhedrin promoters [30], which are switched on between 12 and 18 h after infection of the cell. The earlier, much weaker basic protein promoter did not generate detectable levels of functional opsin. As most baculovirus expression vectors utilize the polyhedrin promoter, this has become our promoter of choice. Despite the ‘late’ activity of this promoter, we always observe a high percentage of functional protein (60–80%), total receptor protein assayed by competitive ELISA; functional fraction by ligand-binding [30]), even at very high expression levels of total receptor ([30–40] × 10⁶ copies/cell).

Functional receptor is properly modified (N-glycosylation at two sites; thiopeptide at two sites), while non-functional protein is incorrectly folded and/or not glycosylated [31]. Only at the occasional unusually high expression levels exceeding 40 × 10⁶ copies/cell do relative functional levels begin to drop, suggesting that at those levels the translocation, folding and/or modification machinery of the cell becomes overloaded [30,31].

Stable-isotope labelling requires serum-free culture conditions. We screened several commercially available media in suspension culture (100-ml spinner bottles) as to cell growth, maximal cell density and recombinant rhodopsin production. Best results were obtained with InsectXPress, a protein-free medium from BioWhittaker, Walkersville, MD, U.S.A. (doubling time ≈ 22 h; cell densities up to 10 × 10⁶/ml; 20–25 pmol of functional rhodopsin per mg of cellular protein; ≈ 15 × 10⁴ copies/cell) that actually performed better in suspension culture than our serum-supplemented TNMFH medium [30] (≈ 18 h; up to 5 × 10⁶/ml; 15–20 pmol/mg of protein). All protein-free insect-cell media still require some yeast- or meat-derived supplement to sustain cell growth. This contributes peptides from which the cells also derive free amino acids, thereby 'diluting' added stable-isotope-labelled amino acids with unlabelled ones. Nevertheless, upon supplementing InsectXPress medium deficient in either lysine or tyrosine with 4 mM labelled lysine or 2 mM labelled tyrosine, label incorporation levels of 57 ± 5% for lysine (n = 4) and of 74 ± 4% for tyrosine (n = 3) were achieved, amply sufficient for FTIR and NMR analyses [18,32]. Label incorporation was checked, after proteolytic or acid hydrolysis and proper derivatization, by GC-MS analysis of the mass distribution of all amino acids (C. H. W. Klaassen, unpublished work). Scrambling of label from lysine (15N, 13C) or tyrosine (1H, 13C) into other amino acids was not detectable. This serves to show that this system can be used for protein labelling with essential amino acids as well as with non-essential amino acids with slow metabolic turnover. For amino acids with a high metabolic flux like Glu, Gln or Ala, alternative procedures are being investigated.

For infection with recombinant baculovirus a high MOI is usually employed (5–10) with cells in logarithmic growth. Large volumes of high-titre virus suspension (≈ 200 ml of ≈ 10⁶ p.f.u./ml) would then be required to infect a 10-litre bioreactor. Therefore we investigated the effect of lower MOIs. Earlier we observed quite similar expression levels for MOIs between 1 and 10 [30]. Further reduction of the MOI actually results in an increase in total expression level if longer incubation times can be tolerated (Figure 1, left panel). For the lowest MOI tested (0.01), detectable opsin expression starts with several days' delay, not surprisingly, but the total level surpasses that of MOI 0.1 and 1 by 5 days post infection (p.i.) and does not fully reach its maximal level at 6 days p.i. Since the expression level per cell only shows slight MOI-dependency [33], this net increase must be due to the higher final cell densities attained at very low MOI [33], where the cells can still go through another division before the infection really spreads. These conditions could be transferred to a 10-litre bioreactor culture with identical results (Figure 1, right panel). In this way, we are able to produce 30–40 mg of functional rhodopsin in a single 10-litre bioreactor run.

Large differences in the percentage of functional protein (5–80%) are reported in the literature upon expression of GPCRs with recombinant baculovirus, sometimes with quite discrepant fig-
ures for the same receptor [34]. We have tested several receptors under our optimized expression conditions. We found comparable total recombinant protein expression levels \( (10-20) \times 10^6 \) copies/cell, but again a large variety in functionality (10-80 %), indicating that specific protein parameters determine this variability (Table 1). Receptors showing lower levels of functional expression tend to be retained in intracellular compartments, probably the endoplasmic reticulum in particular. This suggests that protein modification and folding in the endoplasmic reticulum are rate-determining under these overexpression conditions, and that enhancing the endoplasmic reticulum capacity of the insect cell for protein folding and quality control may give significant improvement [35]. Indeed, we usually find higher levels of functional protein in bioreactor culture, where conditions are carefully monitored and cellular vitality is optimally preserved, as compared with spinner culture (Table 1).

**Purification and return to the roots**

A crucial element in receptor purification is the

---

**Figure 1**

**Effect of MOI on production of functional recombinant rhodopsin in the baculovirus expression system**

Cultures were infected in their mid-logarithmic growth phase with a recombinant baculovirus expressing His-tagged rhodopsin [41] using the MOIs indicated. The volumetric yield of rhodopsin (nmol/litre of culture) was determined up to 6 days p.i. Results are given for representative experiments in 100 ml spinner (left panel) and 10-litre bioreactor (right panel) cultures. Note the significantly earlier onset and higher level for MOI = 0.01 in the bioreactor. This probably reflects the superior growth conditions of a carefully controlled environment.

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**Table 1**

**Baculovirus expression of GPCR classes**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Spinner</th>
<th>Bioreactor</th>
<th>Intracellular localization†</th>
<th>Detergent solubilization‡</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodopsin</td>
<td>60–80</td>
<td>≥ 70</td>
<td>Mainly PM</td>
<td>CHAPS or DoM</td>
<td>[30,33]</td>
</tr>
<tr>
<td>Green cone pigment</td>
<td>30–60</td>
<td>≥ 60</td>
<td>PM+ER</td>
<td>CHAPS+glycerol+lipids</td>
<td>[48]</td>
</tr>
<tr>
<td>Red cone pigment</td>
<td>20–40</td>
<td>Not determined</td>
<td>Mainly ER</td>
<td>CHAPS+glycerol+lipids</td>
<td>[48]</td>
</tr>
<tr>
<td>Histamine H2</td>
<td>10–20</td>
<td>Not determined</td>
<td>Mainly ER</td>
<td>DoM+salts+antagonist</td>
<td>[43]</td>
</tr>
</tbody>
</table>

* Estimated by ELISA or immunoblot (total recombinant protein) and ligand binding (functional part).
† Determined by immunocytochemistry.
‡ Optimal conditions for solubilization and stabilization.

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choice of detergent and solubilization conditions. Generally, solubilization into detergent micelles strongly destabilizes membrane proteins. Functionality of the receptor needs to be sufficiently preserved to survive purification and reconstitution. In our experience these conditions can be quite receptor-specific. In the literature too a large variety of solubilization conditions are recommended for different GPCRs [34]. Nevertheless, our preliminary results with several receptors suggest that initial attempts are best made with CHAPS or β-1-dodecylmaltoside (DoM) in combination with specific additives to improve solubilization and/or stabilization of the protein (Table 1). For instance, solubilization of the histamine receptor required a ‘salty’ environment. Cone visual pigments could only be sufficiently stabilized to survive purification by including glycerol as well as lipids [36]. Inclusion of a suitable ligand (antagonist or reverse agonist) can also make an important contribution to receptor stability. This is already obvious from the longer half-life of rhodopsin relative to the apoprotein opsin, by several orders of magnitude, in every detergent examined [37].

In view of this stability issue, a rapid, preferentially single-step, purification procedure should be selected involving column matrices offering a high binding capacity and high flow rates. This requires a highly selective interaction principle and in fact restricts the options to affinity procedures. For example, the use of immobilized ligand with eventual elution with (another) free ligand is very selective. A disadvantage is, however, that such affinity matrices almost invariably have to be specially designed, prepared and tested, therefore they are quite expensive and usually only suitable for one specific receptor isotype. Another very selective option is epitope tagging, e.g. extending the receptor with a peptide sequence containing the epitope of a suitable antibody. A quite popular option is the C-terminal octapeptide of rhodopsin, for which a monoclonal antibody has become freely available (1D4 [38]). This approach allows excellent purification in a single step [25,39]. The affinity matrix has to be prepared, however, and may vary in performance, and the free epitope–peptide is required for elution. Such elements also render epitope tagging rather expensive and less attractive for purification of large batches of receptor.

For large-scale purification of receptors we prefer histidine tagging, i.e. extending the receptor with a sequence of 6–10 histidine residues. The His-tag has high affinity for transition metals like Zn²⁺, Ni²⁺ and Cu²⁺ and allows purification by immobilized-metal affinity chromatography (IMAC) [40]. Suitable IMAC matrices are commercially available and exhibit a high protein-binding capacity (up to 70 nmol/ml of gel [33]). The selectivity of this approach is somewhat lower, since proteins with properly placed His residues as well as haem proteins exhibit medium affinity for such matrices. These can, however, usually be removed by proper washing procedures [33,41]. Elution is usually accomplished with high concentrations of imidazole (200–500 mM), but we prefer to use histidine that can be used at much lower concentrations (50–100 mM), which is better tolerated by solubilized receptors [36]. Depending on the stability of the receptor the purity of the eluted material varies between 40 and 90%. More detergent-resistant proteins like rhodopsin tolerate more extensive washing routines when bound to the matrix and purity levels of 80–90% can be easily achieved [33,41]. For quite unstable receptors like the cone visual pigments we restrict column residence time by minimizing washing procedures. This usually results in purity levels between 40 and 80% [36].

When evaluating solubilization conditions it is important to include binding tests to the IMAC matrix because we have observed quite receptor-specific phenomena in this respect. For instance, while CHAPS-solubilized His-tagged rhodopsin shows very high affinity for such a matrix, CHAPS-solubilized cone pigments have a very low affinity and DoM or a combination of CHAPS and DoM has to be used [33,36]. We surmise that size of the micelle, charge distribution and lipid affinity of the receptor exercise special effects. Indeed, excess lipid can seriously interfere with binding to the IMAC matrix. If lipids are required for stabilization of the receptor, they should be included after it has bound to the matrix.

In the final step, the purified receptor is reconstituted with appropriate lipids into proteoliposomes to generate a membrane matrix that is as close to native membranes as possible. This not only provides the most stable environment, but also allows proper analysis of structure and conformational changes induced by ligand binding or G-protein interaction. Since available methods for exchange of detergents for lipids are not generic and usually do not handle large batches very well, we have developed a special procedure for this reconstitution step, based on detergent inclusion by cyclodextrins, that tolerates large batches and is...
compatible with any detergent type [42]. For reconstitution a suitable lipid mixture is used. For visual pigments we add a lipid extract from bovine retina [30]. Commercially available brain, egg or soy lipid extracts are often employed for other GPCRs. Detergents are selectively extracted from the mixed detergent/lipid/receptor micelles by addition of a suitable cyclodextrin [42], which evokes spontaneous formation of vesicles with receptor incorporated into the membrane. These proteoliposomes are separated from soluble components and non-incorporated protein by centrifugation through a sucrose gradient. This procedure affords another 2- to 3-fold purification, yielding receptor preparations with a purity always exceeding 80%, usually over 95%, as long as the IMAC-purified receptor has a purity greater than 30% [36,41,42].

**Functional properties and receptor mechanism**

The proteoliposome preparations of His-tagged visual pigments obtained as described above have been characterized extensively with respect to photochemical behaviour [30,36,41,42], to structural transitions accompanying receptor activation [18,33] and to G-protein activation [33,36] and have been shown to behave identically to their native counterparts. This demonstrates that the C-terminal His-tag does not interfere in any way with the structural and functional properties of these receptors. A similar conclusion has been drawn for other GPCRs [43–46].

The combination of large-scale production and stable-isotope labelling of recombinant rhodopsin has recently afforded the first FTIR [18] and solid-state NMR [28,32] analyses of a labelled eukaryotic membrane protein. The NMR data provide the first highly precise structural details for the protein–ligand interaction in the binding site. The FTIR data provide the first evidence for the involvement of tyrosine residues in the active state of the receptor. We recently also recorded FTIR difference spectra of labelled rhodopsin for the first step in the activation cascade (rhodopsin → batho). This clearly demonstrates that a tyro-

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**Figure 2**

*Analysis of the rhodopsin to batho transition by FTIR difference spectroscopy*

FTIR difference spectroscopy was performed on membrane films of unlabelled (bold, upper curve) or ring-$^2\text{H}_2$-Tyr-labelled (bottom curve) recombinant His-tagged rhodopsin, as described [18]. Note that both spectra are identical except for two bands at 1252 and 1244 cm$^{-1}$ (inset). In the labelled receptor the negative band at 1252 cm$^{-1}$ has shifted to lower frequency, thereby cancelling the positive 1244 cm$^{-1}$ band. This identifies the 1252 band as a tyrosine-ring vibration, and demonstrates that the interaction pattern of one tyrosine side chain is altered upon batho formation, the first step in receptor activation.
sine residue already participates at this early stage in the activation mechanism (Figure 2). As this step only involves chromophore and binding-site residues, Tyr-268 is the only candidate. Such an early role for this residue agrees with site-specific mutagenesis studies showing that mutation of this residue seriously affects receptor function [47].

Conclusion

Cost-effective drug design requires detailed information on ligand–receptor interaction, preferably at atomic resolution. To acquire such a high-resolution insight into the structure and mechanism of GPCRs, hundreds of milligrams of purified receptor are required. These amounts will not only allow extensive crystallization attempts to produce high-resolution 3D crystals, but they will also allow access to alternative powerful biophysical techniques like FTIR and solid-state NMR spectroscopy that are able to extract high-resolution data from supramolecular systems.

The large-scale expression and purification protocol that we have developed for the bovine visual pigment rhodopsin is able to generate the required amounts of purified protein. Recombinant baculovirus-based expression of 30–40 mg of functional receptor in a single 10-litre bioreactor batch of SF9 cells has been reproducibly achieved and there is no limitation to further scale-up to 50- or 100-litre batches. The single-step His-tagging-based purification procedure and rapid cyclodextrin-based reconstitution approach tolerate batches containing at least 10 mg of receptor and generate membrane-bound receptor of high purity (95%) and with a very satisfactory overall recovery (40–70%). First results with other GPCRs indicate that, with some receptor-specific modifications in the solubilization step, this protocol can serve our purpose to develop a generic procedure for functional overexpression and efficient purification of these complex membrane proteins on a large scale.

This development will finally pave the way to structural and mechanistic studies on a variety of GPCRs. Such structural knowledge will be of major scientific and medical importance. It will expose the generic basis of the mechanism of receptor activation in the superfamily of GPCR proteins, as well as the individual variations in this common theme for ligand binding and G-protein activation, necessary to acquire subclass and isoform specificity. This will generate novel concepts in the field of drug design and make an important contribution to the development of the next generation of medication.

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Abbreviations used: nAChR nicotinic acetylcholine receptor; GABA, γ-aminobutyric acid.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of an extensive family of neurotransmitter-gated ion channels which also includes receptors for γ-aminobutyric acid (GABA), glycine, glutamate and 5-hydroxytryptamine (serotonin). For many years nAChRs have been the receptors for γ-aminobutyric acid (GABA), glycine, glutamate and 5-hydroxytryptamine (serotonin). For many years nAChRs have been the best-characterized member of this family, largely as a consequence of there being an abundant and readily available source of nAChR (the electric organs of fish such as the marine ray, Torpedo, and the freshwater eel, Electrophorus). Indeed, the Torpedo electric organ nAChR was the first neurotransmitter receptor to be cloned (independently by four research groups in 1982 [1-4]). It was also the first cloned neurotransmitter receptor to be expressed functionally in a heterologous expression system (in 1984, by micro-injection of cRNA into Xenopus oocytes [5] and, subsequently, by stable transfection of cultured cell lines [6]).

Since the molecular cloning of the Torpedo electric organ nAChR, a family of nAChR subunits have been identified in both vertebrate and invertebrate species. The best-characterized mammalian nicotinic receptor is the 'muscle-type' nAChR, expressed at the neuromuscular junction, which has a subunit composition of either α1β1γδ or α1β1γδ (two copies of the ligand-binding α1 subunits, co-assembled with three other 'structural' subunits). In addition to the muscle nAChR, a family of pharmacologically distinct nAChRs are expressed in the mammalian nervous system ('neuronal' nAChRs). The precise subunit composition of the various subtypes of neuronal nAChR is still being established, but strong evidence indicates that they are also pentamers, like the muscle and electric organ nAChRs. To date, eleven vertebrate neuronal nAChR subunits have been identified (α2-α9 and β2-β4). In insects