Understanding neurotransmitter receptors: molecular biology-based strategies

Mark Wheatley

School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

Introduction

For a cell to be responsive to a neurotransmitter, it must express an appropriate receptor protein on its surface. These receptors have to perform the two basic functions of binding the natural agonist when it is present in the extracellular milieu and generating a signal inside the cell. Although various families of receptor proteins have been described, neurotransmitter receptors can be classified as either G-protein-coupled receptors (GPCRs) or ligand-gated ion channels (LGICs), as shown in Figure 1. Receptors within each family share a characteristic architecture. GPCRs consist of one polypeptide composed of seven hydrophobic transmembrane (TM) domains connected by extracellular and intracellular loops (Figure 1c), whereas LGICs are oligomeric and possess an integral ion channel (Figure 1d). Both classes of receptor are fundamentally important to the activity of individual neurons and also underlie complex phenomena such as memory, emotion and intellect. As such, the structure and function of neurotransmitter receptors is of interest not just to basic neuroscientists but also to the pharmaceutical industry, as they represent potential targets for therapeutic intervention. Consequently, this has been an area of sustained investigation. The application of molecular biology techniques has greatly increased our understanding of neurotransmitter receptor structure and
function, and recent progress will be reviewed. Due to limitations of space, this article will focus predominantly on GPCRs.

**How many receptors?**

It might be thought reasonable that for every neurotransmitter there would be a corresponding receptor protein. Consequently, the number of receptors would be equivalent to the number of neurotransmitters. However, as long ago as 1914, Sir Henry Dale established that multiple receptors exist for the classical neurotransmitter acetylcholine. He demonstrated that physiological responses to acetylcholine could be mediated by either muscarinic receptors (activated by muscarine; blocked by atropine) or nicotinic receptors (activated by nicotine; blocked by curare). The natural agonist, acetylcholine, was non-selective and activated both receptor subtypes. This set the norm for receptor classification for the next 70 years, in that demonstrating the existence of receptor subtypes was dependent on the discovery of subtype-selective antagonists or agonists. Muscarinic acetylcholine receptors (mAChRs) were them-
selves subdivided into M₁ and M₂ receptors on the basis of different pharmacological profiles to selective ligands. In the early 1980s, this classification was extended to M₁, M₂ and M₃ receptors on the basis of differential affinity for certain antagonists, notably pirenzepine, AF-DX 116 and hexahydrosiladifenidol. Pirenzepine is selective for M₁ receptors (expressed by neuronal tissue) and is used clinically in the treatment of peptic ulcer disease by inhibiting vagally stimulated gastric acid secretion.

There are inherent problems, however, associated with the use of pharmacological profiles to define neurotransmitter receptor subtypes. For example, the discovery of very selective ligands, which are capable of revealing the subtle differences in receptor architecture, is entirely capricious, and differences in primary sequence (isoreceptors) may not necessarily generate pharmacological differences. In addition, pharmacological differences may not be due to receptor subtypes but may reflect differences in cell membrane composition or variation in post-translational modifications (glycosylation, phosphorylation or acylation). A further complication for GPCRs is that the affinity of ligands, particularly agonists, is influenced by the receptor:G-protein coupling state. Consequently, the unambiguous demonstration of receptor subtypes depends on primary sequence information. This was achieved for mAChRs in 1986, when Professor Shosaku Numa and co-workers cloned M₁ and M₂ receptors. These corresponded to the pharmacologically defined M₁ and M₂ receptors respectively [1]. At this time, cloning of neurotransmitter receptors was a major undertaking of almost Herculean proportions, as it required: (i) solubilization of active receptor; (ii) approx. 13000-fold purification of the receptor protein by affinity chromatography; (iii) generation of proteolytic fragments; (iv) purification of receptor-derived peptides; (v) peptide sequencing; (vi) synthesis of degenerate oligonucleotide probes based on the amino acid sequence data; and (vii) screening of cDNA libraries for positive clones. Once clones encoding neurotransmitter receptors became available, sequence identity could be exploited to clone closely related receptors from different tissues or species. Employing less stringent screening conditions allowed the cloning of receptor subtypes or even entirely different neurotransmitter receptors that share structural identity. For example, the first dopamine receptor cDNA was cloned from rat using the hamster β₂-adrenergic receptor as the probe. Aligning sequences of different cloned GPCRs revealed regions of sequence identity. Oligonucleotide probes corresponding to these domains were used to clone the M₃ mAChR together with the hitherto unknown M₄ and M₅ subtypes. Consequently, whereas pharmacological approaches defined three mAChR subtypes, molecular biological techniques revealed the existence of five [1].

The proliferation of known receptor subtypes identified by homology cloning was not unique to mAChRs. For example, there are five dopamine receptors and fourteen 5-hydroxytryptamine (5-HT) receptors! Consequently, cloning neurotransmitter receptors has presented new therapeutic targets to the pharmaceutical industry and raises the prospect of improved drug select-
ivity. In addition, the use of specific, or degenerate, primers to amplify sequences by PCR has resulted in a proliferation of GPCR cDNAs. An alternative strategy was developed for cloning the tachykinin NK$_2$ receptor (NK2R), which binds the neuropeptide substance K, and the technique has been quite widely used subsequently for cloning other neurotransmitter receptors. A cDNA library was transcribed in vitro and the mRNA microinjected into *Xenopus* oocytes (see below). Substance K-evoked electrophysiological responses were obtained if full-length NK2R mRNA was present in the mixture of mRNAs injected. Following stepwise fractionation of the library, a single functional cDNA was isolated by this ‘expression screening’ [2].

The result of all these various cloning strategies is that sequence databases currently contain the sequences of over 800 GPCRs from a variety of eukaryotes. Although GPCRs exhibit key structural features (see later), sequence identity does not necessarily mean that the cDNA obtained encodes a physiologically functional receptor. The recent explosion in genomic information and bioinformatic analysis of databases has generated over 100 putative GPCR clones for which there are no known agonists or physiological roles (‘orphan receptors’). Occasionally, these orphan receptors are later identified, e.g. RDC4 encodes a 5-HT$_{1D}$ receptor [3].

Although sequence information has expanded our knowledge of the plethora of receptors, it is not possible to classify subtypes on the basis of sequence alone, because the same receptor subtype expressed by a different species may have a different primary sequence. For example, the NK$_1$Rs from rat and human have 22 divergent residues out of 407, and the NK$_2$R expressed by the rat is eight residues shorter than the human homologue. Usually species-specific differences in sequence occur in domains that are unimportant for normal receptor function, as there is no evolutionary pressure to preserve structural motifs. However, the proliferation of synthetic analogues of naturally occurring neurotransmitters has resulted in some species differences in binding or signalling being manifested; e.g. bovine, rat and human V$_{1a}$-vasopressin receptors have different affinities for the extensively used antagonist [d(CH$_5$)$_3$Tyr(Me)$_2$]AVP (where AVP is [Arg$^8$]vasopressin) [4].

The first GPCRs cloned ($\beta_2$-adrenergic receptor and mAChRs) lacked introns, but subsequently it has been demonstrated that many GPCR genes do possess introns. This has resulted in splice variants adding a further layer of receptor heterogeneity. For example, the D$_2$-dopamine receptor exists in short (D$_{2S}$) and long (D$_{2L}$) forms. D$_{2L}$ has an alternative spliced exon that generates a 29-amino-acid insert in the third cytoplasmic loop between residues 241 and 242 of D$_{2S}$. D$_{2L}$ predominates, but there are only slight pharmacological differences between the two forms and these are restricted to the benzamide derivative drugs. However, it seems that inhibition of stimulated adenylate cyclase by D$_{2L}$, but not by D$_{2S}$, has an absolute requirement for a specific G-protein, G$_i$α2 [5].

LGICs, such as ionotropic glutamate receptors (GluRs) and GABA$_A$ receptors (where GABA is $\gamma$-aminobutyric acid), are multi-subunit complexes
for which many different subunits have been cloned to date. For example, there are six α, four β, four γ, one δ, one ε and three ρ subunits of the GABA<sub>A</sub> receptor, and that does not include splice variants. It has been established that different subunit composition confers different properties on the resulting receptor. As many of these receptors have a pentameric quaternary structure, this gives rise to a vast array of functional heterogeneity.

In addition to splice variants, receptor differences can be generated by mRNA editing [6]. AMPA (α-amino-3-hydroxy-5-methylisoxazolepropionat) and kainate receptors are LGICs and are subtypes of GluRs. The electrophysiological properties of these channels can be dramatically altered by editing of channel subunit mRNAs at several sites. Receptor mRNA editing capacity is important for normal brain function, since transgenic mice that were incompetent at editing the GluR-B subunit mRNA developed epilepsy and died soon after birth. Editing has now been reported for a GPCR; the 5-HT<sub>2C</sub> receptor can have different residues at three positions in the second intracellular loop and this affects the efficiency of stimulation of its effector phosphoinositidase C, suggesting that mRNA editing regulates 5-HT signalling [7]. Research over the next few years will establish if this mechanism of regulating neurotransmitter receptor function is widespread.

Studies on human populations have revealed that some neurotransmitter receptors exhibit polymorphic variation. For example, there are at least six different polymorphisms of the β<sub>2</sub>-adrenergic receptor, some of which affect receptor function. The N-terminus variants Arg<sup>16</sup> → Gly and Gln<sup>27</sup> → Glu differ in their down-regulation following exposure to agonist, whereas Thr<sup>164</sup> → Ile variants exhibit different ligand binding and effector coupling [8]. Interestingly, the nocturnal form of asthma has been associated with the enhanced down-regulation shown by the Gly<sup>16</sup> β<sub>2</sub>-adrenergic receptor variant.

**Approaches to determining the structures and physiological functions of native neurotransmitter receptors**

**Insights from the distribution and/or disruption of receptor expression**

Pharmacological characterization of the individual receptor subtypes and detailed analysis of receptor structure/function at the molecular level were difficult in the past. This was because the specificity of the tools available was not usually sufficient to overcome the extreme complexity of the tissue. For example, we now know that many of the antagonists utilized to localize receptor distribution by autoradiography, or to characterize receptors by blocking their activation, were actually binding to several different receptor subtypes. Consequently, instead of using pharmacological probes such as radioligands to localize receptors, recent investigations have employed DNA probes. Detailed analysis of isoreceptor sequences enables cDNA probes to be constructed which are subtype-specific. Cellular distribution can then be
addressed by hybridization of the probes to the receptor mRNA using Northern blot analysis and/or in situ hybridization. This strategy can be sufficiently stringent to establish whether mRNAs for different isoreceptors are present in the same cell. By selecting the probe sequence carefully, detection can be restricted to an individual subtype or can include all members of a neurotransmitter receptor family.

Receptor blockade by an antagonist or covalent modification of G-proteins by bacterial toxins have been widely used to establish physiological roles for individual neurotransmitters. An alternative strategy is to reduce, or ablate, expression of individual receptors or G-proteins using molecular biological approaches. Injection or transfection of antisense oligonucleotides or full-length antisense cDNA has been employed to knock out components of the neurotransmitter signalling apparatus [9]. The rate of degradation of the target protein is crucial (proteins with a long half-life may take days to be affected) and the technique can be compromised by RNA secondary structure interfering with hybridization or by the antisense probe cross-hybridizing with non-target mRNA. These various factors often result in antisense probes generating a knock-down (a reduction) rather than a knock-out (an elimination). Therefore the extent of the protein knock-out must be quantified directly in each case.

Homologous recombination between chromosomal DNA and introduced cDNA can be used to transfer a modification, or disruption, of a cloned gene into the genome of living mice. In this way ‘knock-out’ (KO) mice have been genetically engineered in order to study the physiological function of neurotransmitter receptors. Despite the extraordinary costs involved, an ever-increasing number of KO mice have been developed to elucidate the physiological roles of neurotransmitter receptors. For example, this strategy has established that mice lacking the mGluR4 subtype of metabotropic GluRs have impaired synaptic plasticity. In addition, KO mice establish unambiguously if responsiveness to a neurotransmitter in a tissue/region is conferred by a single gene. Thus pharmacological differences had led to the suggestion that neuronal and smooth muscle B2-bradykinin receptors were different subtypes, but ablation of the B2 receptor eliminated bradykinin responses in both the superior cervical ganglia and the uterus [10]. As the absence of a receptor subtype simplifies interpretation of data, KO mice lacking one isoreceptor can be very useful for studying the roles of related subtypes still expressed by the mice. A potential problem with KO mice is that ablation of a neurotransmitter receptor may lead to flawed development. Consequently, it is likely that KO mice in the future will have targeted gene disruption that can be regulated with respect to time and/or tissue.

**Insights from the expression of cloned recombinant receptors**

The pharmacological properties of a receptor can be influenced by many factors, including variability in receptor reserve and differences in the cellular context, such as type/level of G-proteins, effectors and membrane lipid.
These complexities can be circumvented by expressing different receptor subtypes in an identical cellular environment using recombinant expression in cultured cells. Furthermore, a homogeneous receptor population is obtained if the cultured cell does not express the receptor endogenously. Obviously, the appropriate post-translational processing, G-proteins, effector systems, kinases etc. must be present to enable the receptors to be functional and suitably regulated. It is for this reason that mammalian cells are the most commonly used for both stable and transient expression, but important results have also been obtained using Xenopus oocytes, yeasts and insect cells transfected with baculovirus.

Expression of recombinant receptors in these systems has revealed that a single GPCR can couple to multiple effectors and that the signalling pathway to which a GPCR couples is dependent on the level of expression and cell type [11]. Consequently, more is not always better when considering receptor expression, particularly if data obtained in recombinant systems are being extrapolated to effects in the whole animal. It has long been recognized that the efficacy of agonists is influenced by the abundance of receptors. It was not surprising, therefore, that overexpression of GPCRs in recombinant systems often resulted in elevated basal activity. This can be explained by the receptors existing in two interconvertible forms, inactive (R) and active (R*), which are in equilibrium. Agonists stabilize the active form, altering the position of the equilibrium in favour of R*, whereas antagonists do not differentiate between R and R* and, therefore, lack intrinsic activity. Increasing the receptor density can inadvertently increase the abundance of R*, due to the pre-existing R ↔ R* equilibrium, and generate an elevated basal activity. However, studies on recombinant expression systems have revolutionized receptor theory. It was demonstrated that, although some antagonists have null intrinsic activity, many antagonists reduce basal activity and, therefore, possess negative efficacy (Figure 2). It was proposed that antagonists with negative efficacy stabilize R and shift the equilibrium in favour of the inactive conformation (Figure 2) [12]. These ligands are termed inverse agonists, a term originally used with respect to GABA\textsubscript{A} receptors to describe a class of benzodiazepines which induced a reduced affinity for GABA. As for classical agonists, they can exhibit a range of intrinsic activities.

Observations of inverse agonism with GPCRs have generally been restricted to recombinant overexpression systems, raising doubts as to its relevance. However, the theory does translate to whole animals, as transgenic mice with cardiac-specific overexpression of β\textsubscript{2}-adrenergic receptors exhibited a maximal baseline atrial rate and contractility in the absence of agonist, and the antagonist ICI-118,551 functioned as an inverse agonist [13]. Consequently, inverse agonists could have a unique therapeutic role in treating disease states resulting from overexpression of receptors or constitutively active receptors where mutation has shifted the equilibrium from R towards R*. Known examples of the latter are few, but include the luteinizing hormone receptor and the thyrotropic
hormone receptor, the constitutive activities of which underlie familial male precocious puberty and hyperfunctioning thyroid adenomas, respectively.

Many neurotransmitter receptors signal by coupling to $G_s$ or $G_q$ to raise, respectively, cAMP or intracellular Ca$^{2+}$. Stable recombinant cell lines expressing reporter genes have been engineered in order to characterize receptor signalling and to facilitate high-throughput screening of putative ligands by pharmaceutical companies. For instance, a cAMP reporter cell line has been generated by stable expression in Chinese hamster ovary (CHO) cells of the firefly luciferase gene under the control of cAMP response elements. These cells generated a dose-dependent increase in luciferase expression in response to elevated cAMP [14]. In another example, receptor-mediated rises in intracellular Ca$^{2+}$ were studied using cells stably co-transfected with the calcium-sensitive photoprotein, apoaequorin, and $G_{16}\alpha$. The $G_{16}$ subunit was transfected following
observations that it allowed a wide range of GPCRs to couple to phospholipase Cβ [15].

**Receptor architecture and function**

A primary objective in our understanding of how neurotransmitter receptors selectively bind ligands and generate intracellular signals is the identification of key functional domains/residues within the receptor protein. Two basic strategies have been employed: (i) replacement of individual residues by site-directed mutagenesis; and (ii) use of chimaeric receptors in which whole domains have been substituted by the corresponding domain from a different, but related, receptor. Engineered constructs are then characterized in recombinant expression systems. A potential problem with these techniques is that changes in function following structural modification may not necessarily indicate a direct role for a given domain or residue, but instead reflect changes in overall receptor conformation, membrane insertion, trafficking, protein half-life etc.

**Ligand binding**

The synergistic approaches of molecular biology and protein chemistry have resulted in an explosion of information aimed at defining the ligand binding site and receptor–G-protein contact sites for GPCRs. As space is limited, only the main themes arising out of the work from many laboratories are presented, and the reader is directed to reviews [1,16] as a source of primary references. The binding site for small ligands, such as the biogenic amines acetylcholine, dopamine, noradrenaline etc., is buried approx. 11–15 Å (1.1–1.5 nm) within the hydrophobic core of the GPCR. An Asp residue, positioned approximately one-third of the way down TM3, is conserved throughout this subfamily of GPCRs and provides a counter-ion to the charged amine head-group of both agonists and antagonists. A role for this Asp in ligand binding has been confirmed by site-directed mutagenesis [1,16] and by affinity labelling/radio-sequencing of the mAChR with [3H]propylbenzilylcholine mustard [17]. Other binding epitopes are provided by residues from TM5 and TM6. For example, the hydroxy groups of catecholamines and 5-HT hydrogen-bond with serine residue(s) located one turn apart in TM5. Residues at analogous positions to these serines have been implicated in agonist binding to mAChRs and histamine receptors. In fact, a series of conserved Ser, Thr and Tyr residues located on helices TM3–TM7 are in the same general plane as the conserved Asp in TM3. Mutation of any one of these decreases agonist, but not antagonist, binding, indicating subtle binding-site differences between the two classes of ligand. The occurrence of key residues, particularly the Asp in TM3, is diagnostic that a GPCR has a biogenic amine as the natural ligand, and this has aided subsequent identification of orphan receptors.

Size considerations alone dictate that the binding site for peptide neurotransmitters is not confined within the TM helical bundle, but must also
involve extracellular domains (Figure 3). Site-directed mutagenesis, deletion and chimaeric receptor constructs have indeed established that both the extracellular surface and the TM domains contribute to peptide ligand binding energy and ligand selectivity. For example, three residues in the N-terminus and three residues in the first extracellular loop are critical for high-affinity binding of neurokinin peptides to the NK1 receptor. This contrasts with biogenic amine GPCRs, where extensive deletion of the extracellular domains was not detrimental to binding [16]. Mutation of individual residues has also indicated that the antagonist and agonist binding sites are not identical. This is particularly apparent for non-peptide antagonists, developed by the pharmaceutical industry for their superior stability relative to peptides. These are usually small molecules which bind to a pocket formed by the top of TM3–TM7, with no significant contribution from extracellular domains. Consequently, very few residues contribute to both the agonist and the non-peptide antagonist binding sites, despite the competitive binding observed. This implies that allostery or mutual exclusion effects, rather than overlapping intermolecular interactions, underlie competitive ligand binding between such compounds.

**G-protein coupling and receptor activation**

Molecular biological and biochemical strategies have established that multiple intracellular domains contribute to receptor–G-protein coupling. In particular, residues in the N- and C-terminal portions of the third intracellular loop (i3)
located near to the membrane have been implicated in the recognition and selection of G-proteins. Despite conservation of function, sequence identity is not displayed by these regions, even between related isoreceptors. However, homology is thought to exist at the level of secondary structure, as these domains are highly charged and are predicted by modelling programs to form amphipathic α-helices juxtaposed to the membrane. Synthetic peptides corresponding to GPCR intracellular loops can activate G-proteins in vitro, suggesting that the unoccupied receptor constrains the G-protein coupling domains in an inactive conformation (R). Agonist binding induces allosteric changes which relax this constraint, thereby facilitating receptor–G-protein interaction. Currently, these conformational changes associated with receptor activation are poorly understood. Mutation of the C-terminus of i3 has generated constitutively active receptors, indicating that an active receptor conformation (R*) can be induced without agonists and suggesting a constraining function for this region of the i3 loop [18]. The conserved Asp-Arg-Tyr (DRY) motif at the base of TM3 is important for receptor–G-protein coupling. Molecular modelling and engineered constitutively active receptors have suggested that the Arg in this DRY motif acts as a switch during $R \leftrightarrow R^*$ conversion. Activation of receptor, by either agonist or mutation, causes the Arg side chain to move out of a polar pocket formed by TM helices 1, 3, 6 and 7, concomitantly favouring coupling of R to the G-protein [19].

**GPCR dimerization**

It has been suggested that TM1–TM5 and TM6–TM7 of GPCRs are independently folded. When these two regions were cloned in different vectors and co-expressed, a functional receptor was reconstituted, notwithstanding the absence of a covalent bond between TM5 and TM6. Co-expression of $\alpha_5/M_3$ and $M_3/\alpha_5$ chimaeric GPCRs reconstituted some functional mAChR activity indicative of intermolecular interaction, i.e. GPCR dimer formation [20]. Furthermore, an inactivating mutation in a GPCR could be ‘rescued’ by co-expression with the appropriate part of the wild-type receptor. This has culminated in the hypothesis that GPCRs undergo a rearrangement upon activation which involves ‘domain swapping’ [21]. What is not clear currently is whether dimerization is obligatory to function or merely coincidental. For example, dimerization of nicotinic AChRs occurs, but is not a prerequisite for acetylcholine-induced channel opening.

**Future perspectives**

The model of GPCR structure will continue to be refined. In particular, structural information on the loops is required so that their role in ligand recognition and G-protein coupling can be defined. Improved computer models will aid rational selective drug design by indicating differences between isoreceptors in ligand contact points. A major gap in our understanding is a precise
molecular description of GPCR activation, including conformational changes and the effect of dimerization (if any). A high-resolution structure would result from crystallographic studies. Currently, it is very difficult to obtain high-quality crystals of membrane proteins in general, but enormous efforts are being made in this direction. Crystals of receptor when unoccupied and when binding agonist or inverse agonist will provide insight into receptor activation processes.

Summary

- Neurotransmitter receptor proteins can be divided into GPCRs and ligand-gated ion channels. Members within each family utilize a similar signalling mechanism and are structurally related.
- Multiple receptor subtypes are expressed for individual neurotransmitters, generating orders of complexity that have been exploited for therapeutic intervention.
- GPCRs have been cloned and extensively studied by using a range of molecular biological strategies, particularly recombinant expression systems. These have located functional domains for ligand binding and G-protein coupling.
- A combination of molecular biology and computer modelling has increased our understanding of receptor activation and will aid drug design.

Due to limitations of space, I must apologize to the many contributors to this field that I have not been able to cite in this article. I am grateful to Dr. David R. Poyner (University of Aston, Birmingham, U.K.) and Dr. Mary Keen (University of Birmingham) for their constructive comments. I thank the Wellcome Trust, the BBSRC and the MRC for supporting my research in the area of GPCRs.

References
