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THE BINDING SITE OF AMINERGIC G PROTEIN–COUPLED RECEPTORS: The Transmembrane Segments and Second Extracellular Loop

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■ **Abstract** In the current chapter, we review approaches to the identification of the residues forming the binding sites for agonists, antagonists, and allosteric modulators in the family of aminergic G protein–coupled receptors (GPCRs). We then review the structural bases for ligand binding and pharmacological specificity based on the application of these methods to muscarinic cholinergic, adrenergic, dopaminergic, serotonergic, and histaminergic receptors, using the high resolution rhodopsin structure as a template. Furthermore, we propose a critical role of the second extracellular loop in forming the binding site for small molecular weight aminergic ligands, much as this loop dives down into the binding-site crevice and contacts retinal in rhodopsin.

INTRODUCTION

The cloning and sequencing of many unanticipated subtypes of receptors, as well as the identification of altogether unknown receptors, has dramatically altered the landscape of the pharmacology of G protein–coupled receptors (GPCRs) (1). Whereas, classically, receptors were differentiated by the effects of selective drugs, we now find ourselves in a situation in which receptors are defined by their cDNA and inferred amino acid sequence. In many cases, we do not have pharmacological agents that can adequately distinguish between related receptors, and in the case of orphan receptors, the identities of the endogenous ligands are unknown.

Given that a large fraction of drugs target GPCRs, the newly discovered receptors and novel receptor subtypes represent an opportunity for the development of pharmacological agents. A better understanding of the structure of the receptors and of the identity of critical residues and motifs that participate in ligand binding

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would provide a foundation that may ultimately guide and facilitate such drug development efforts. In this review of aminergic GPCRs, we focus upon various approaches to GPCR structure and function and summarize our current understanding of the binding site of aminergic receptors in the context of the high-resolution structure of rhodopsin (2). In a recent review (3), we compared the molecular details of the high-resolution structure of each of the transmembrane segments of bovine rhodopsin with the structural information inferred from experimental probing and sequence analysis and molecular modeling of aminergic GPCRs. In the current chapter, our focus is not upon the detailed structures of the TMs but rather on the role of critical side chains in the binding of antagonist and agonist ligands. Moreover, we propose a role of the second extracellular loop in ligand binding in the family of aminergic receptors.

A GENERAL INDEXING METHOD FOR RESIDUE NUMBERING

To facilitate the comparison of aligned residues in different GPCRs, we use the indexing method introduced by Ballesteros & Weinstein (4), in which the most conserved residue in each transmembrane segment (TM) is given the index number 50. Thus, for example, the Arg in the highly conserved DRY sequence at the cytoplasmic end of TM3 is $Arg^{3.50}$, and the other residues in TM3 are indexed relative to this position, with the preceding $Asp^{3.49}$, and the subsequent $Tyr^{3.51}$. This method, which counts from the most conserved position rather than from inexact inferences of the beginning of the TMs, facilitates comparison among different GPCRs. $Arg^{3.50}$ now takes on significant meaning in any GPCR, and $Arg131^{3.50}$ in the β_2 adrenergic receptor (AR) identifies not only the absolute residue number in the β_2AR sequence but also the position of the aligned residue in other GPCRs. In contrast, unless one's research focuses on these receptors, Arg131 in the human β_2AR or Arg135 in bovine rhodopsin or Arg519 in the human thyrotropin receptor are meaningless residue numbers without reference to a multiple alignment and much counting of residues. The index residues in each of the TMs of bovine rhodopsin are $Asn55^{1.50}$, $Asp83^{2.50}$, $Arg135^{3.50}$, $Trp161^{4.50}$, $Pro215^{5.50}$, $Pro267^{6.50}$, and $Pro303^{7.50}$. All of these are 99%–100% conserved in aminergic receptors and therefore allow unambiguous alignment of the TMs of these receptors.

METHODS TO IDENTIFY BINDING SITE RESIDUES

Affinity Labeling

It is sometimes possible to attach a chemically reactive moiety to an agonist or antagonist ligand while preserving its affinity, thereby producing an affinity label. In many cases, photoactivatable groups are used so that the ligand can bind to the

receptor in a reversible manner, and then bound ligand can be rendered reactive, typically by exposure to UV light. Affinity labeling has the advantage that the residues identified are likely to be in or very near the binding site, and thus the information obtained is more readily interpreted than that from site-directed mutagenesis (see below). A limited number of affinity reagents, however, have been developed for any particular receptor, and it therefore has not been possible to develop a complete picture of the residues involved in binding through affinity labeling. In addition, depending on the particular chemistry employed, not all amino acid residues can be labeled, which can sometimes bias the results toward particular residues and away from others. Nonetheless, despite the technical difficulties of classical affinity labeling, several labeled residues have been identified: these include $Asp^{3.32}$ in TM3 of the muscarinic acetylcholine receptor (5, 6), $Trp^{7.40}$ in TM7 of the β_1AR (7), and fragments containing TM2, TM4, and TM6-TM7 in various aminergic receptors (8–10) (see Table 1A).

Most affinity reagents have employed relatively nonspecific reactive moieties such as azido groups. An interesting and more specific second-generation approach to affinity labeling is to use sulfhydryl-reactive affinity reagents combined with either wild-type receptor or a series of substituted-cysteine mutants (11–13). This method has the advantage that multiple residues near a particular reactive group can be systematically mapped. Moreover, using this approach, which combines features of affinity labeling and the substituted-cysteine accessibility method (see below), one can infer the reactive position, thereby obviating the need for laborious protein sequencing to identify labeled residues.

Site-Directed Mutagenesis

Analyses of the aligned sequences of GPCRs have led to the identification of a number of residues that are highly conserved in subfamilies of receptors but divergent among different subfamilies of receptors. Such residues were assumed, therefore, to play roles in the specific binding of ligands to the receptors within a subfamily. The premier example of such a residue is $Asp^{3.32}$, which is present in all aminergic receptors.

Very often residues are mutated to alanine in an attempt to remove the side chain at a given position, but mutations can also explore the effects of changing side-chain volume, charge, hydrophobicity, and other physicochemical properties. At a number of positions, including $Asp^{3.32}$ in most aminergic receptors, mutations lead to a loss of binding. Such a disruption of binding is often assumed to indicate a disruption of a direct contact between ligand and the mutated residue, but this assumption, although true in the case of $Asp^{3.32}$ (see below), is sometimes unjustified. Mutations can cause indirect effects on the structure of the receptors, sometimes even at the level of interfering with protein folding, posttranslational modification, or surface expression (14). Whereas some have taken the presence in the membrane of a mutant receptor as evidence of normal folding, this need not be the case, as structural rearrangements may not always lead to intracellular

TABLE 1A Residues in the transmembrane domain implicated in ligand binding in aminergic receptors based on affinity labeling, functional complementation of mutations with modifications of ligand, or changes in antagonist affinity[a]

Index	SCAM	RC	Acetylcholine receptor	Adrenergic receptor	Dopamine receptor	Serotonin receptor	Histamine receptor
2.50	X	—	—	—	D80C, D2DR (16) D80A/E, D2DR (17)	D120N, 5H2A (18)	—
2.61	X	—	—	—	V91F, D2DR (19)[spec] F91V, D4DR (19)[spec] F88V, D4DR (20)[spec]	—	—
2.64	X	—	—	F86M, A1AA (21)[spec] H93, B2AR (9)[aff]	—	T134A, 5H2A (22)	—
3.28	X	X	W101A, ACM1 (23, 24)	—	F110L D2DR (19)[spec] L111F, D4DR (19)[spec]	W102F, 5H6 (25)	—
3.29	X	—	L102A, ACM1 (24)	—	M109V, D4DR (20)[spec]	—	—
3.32	X	X	D105A, ACM1 (24) D105N, ACM1 (26) D105, ACM1 (5, 6)[aff] D103E, ACM2 (27)	D113A, B2AR (28) D113N/E, B2AR (29) D113S, B2AR (30)[lig]	—	D155N, 5H2A (18) D155E, 5H2A (14)[lig]	D107A, HH1R (31, 32)
3.33	X	X	Y106A, ACM1 (24)	—	V115C, D2DR (33)	—	—
3.36	X	X	—	—	C114S, D3DR (34) C118K, D2DR (35)	—	—
3.40	X	X	V113A, ACM1 (24)	—	—	—	V122A, HH3R (36)[spec]

4.50	X	—	—	—	W160C, D2DR (37)	—	—
4.56	X	X	—	T164I, B2AR (37)	—	—	W167A/F/M, HH1R (39)
4.59	A	—	P201A, ACM3 (40)	—	P169C, D2DR (37)	—	—
4.61	X	—	—	—	L171C, D2DR (37)	—	—
5.38	X	—	—	—	F189Y, D2DR (19) F189C, D2DR (41)	—	—
5.39	X	—	T187A, ACM2 (27)	V197C, A2AA (12)[aff]	—	—	K200A/M/R, HH1R (39)
5.42	X	X	T192A, ACM1 (13, 42)[aff]	S200C, A2AA (12)[aff] S203A/C/T/V, B2AR (43)[lig]	—	—	*T194A*, *HH1R* (44) D186A/N, HH2R (45)
5.43	X	X	—	C201, A2AA (12)[aff] S204A, B2AR (46)[lig]	S199A, D1DR (47)	—	—
5.46	X	X	—	S204C, A2AA (12)[aff] S207A, B2AR (46)[lig]	S197C, D2DR (41)	S242A, 5H2A (48, 49)[spec] A242S/T, 5H2A (50)[spec] A222S, 5H2C (49)[spec] T196A, 5H6 (51)	T190A/C, HH2R (45) N207A, HH1R (52)[lig]
5.47	X	X	—	—	F198C, D2DR (41)	F243A, 5H2A (53)	—
5.50	X	—	—	—	P201C, D2DR (41)	—	—
6.48	X	X	W400A, ACM2 (27) W503F, ACM3 (40)	—	W386C, D2DR (54)	W336A, 5H2A (55) W327A, 5H1B (56)	—

(*Continued*)

TABLE 1A (*Continued*)

Index	SCAM	RC	Acetylcholine receptor	Adrenergic receptor	Dopamine receptor	Serotonin receptor	Histamine receptor
6.51	X	X	Y381A/F, ACM1 (57) Y403A, ACM2 (27)	—	F389A, D2DR (58) F389C, D2DR (54)	F339A/L/Y, 5H2A (22, 59, 60)	—
6.52	X	X	N382A, ACM1 (42, 57) N404A/Q, ACM2 (27) N507A/D/S, ACM3 (61)	—	—	F340A/L/Y, 5H2A (22, 59, 60) F331A, 5H1B (62)	F433A/M, HH1R (39)
6.55	X	X	—	N293L, B2AR (63)^{lig}	H394L, D2DR (64) H393C, D2DR (54) H349L, D3DR (65)	—	F436A/M, HH1R (39)
7.35	A	—	—	F359Y, B1AR (66)	—	—	—
7.39	X	X	Y404A/F, ACM1 (23) Y529F, ACM3 (67)	F412N, A2AA (68)^{spec} N312Q/T, B2AR (69)^{spec}	—	N386V, 5H1A (70, 71) T355N, 5H1B (72–74)^{spec}	—
7.40	X	—	—	W330, B1AR (7)^{aff}	—	W367A, 5H2A (55)	—
7.42	ND	X	*C407S, ACM1* (75)	—	—	—	—
7.43	X	X	Y533F, ACM3 (67)	—	Y417F, D2DR (76) Y416C, D2DR (77)	Y370A, 5H2A (55)	—
7.45	X	—	—	—	N418C, D2DR (77)	—	—
7.46	—	—	—	—	S419C, D2DR (77) S391A, D2DR (78) S420L/N/V, D2DR (79)	—	—

TABLE 1B Representative residues implicated in ligand binding in aminergic receptors based on changes in agonist affinity and/or potency[a]

RI	Acetylcholine receptor	Adrenergic receptor	Dopamine receptor	Serotonin receptor	Histamine receptor
2.50	D69N, ACM2 (80)	D79A, B2AR (28)	D80V, D2DR (17)	D82N, 5H1A (81) D120N, 5H2A (18)	—
3.28	W101A/F, ACM1 (23, 24) W99A, ACM2 (27)	—	—	—	—
3.29	L102A, ACM1 (24)	—	—	—	—
3.32	D105A, ACM1 (24) D105N, ACM1 (26) D103E, ACM2 (27)	D125A, A1AB (82) D113N/E, B2AR (29) D113S, B2AR (30)	—	D116N, 5H1A (81) D155E, 5H2A (14) D155N, 5H2A (18) D106N, 5H6 (25)	D107A, HH1R (31, 32)
3.33	Y106A, ACM1 (24) Y148F, ACM3 (67)	—	—	—	—
3.36	S109A, ACM1 (24)	—	C114S, D3DR (34)	S159A/C, 5H2A (83)	—
3.40	*V113A, ACM1* (24)	—	—	—	—
4.50	W192F, ACM3 (40)	—	—	W200A, 5H2A (55)	—
4.56	—	T164I, B2AR (38)	—	—	W167A/F/M, HH1R (39)
4.59	P201A, ACM3 (40)	—	—	—	—
4.61	—	—	—	F185A, 5H1B (84)	—
5.38	I188A/C, ACM1 (13)	Y203A, A1AB (82)	—	—	—
5.39	T189C/S, ACM1 (13) T187A, ACM2 (27) T231A, ACM3 (67)	V185A, A1AA (85)^{spec} A204V, A1AB (85, 86)^{spec}	—	—	K200A/M, HH1R (87)

(*Continued*)

TABLE 1B (*Continued*)

RI	Acetylcholine receptor	Adrenergic receptor	Dopamine receptor	Serotonin receptor	Histamine receptor
5.42	T192A, ACM1 (13)[aff] T192A, ACM1 (42) T190A, ACM2 (27) T234A, ACM3 (67)	S188A, A1AA (86) S207A, A1AB (82) S203A/C/T/V, B2AR (43)	S198A, D1DR (47) S193A, D2DR (78, 88)	S199A, 5H1A (81) S212A, 5H1B (62)	T194A, HH1R (44) D186A/N, HH2R (45)
5.43	—	S204A, B2AR (46)	S199A, D1DR (47) S194A, D2DR (78, 88)	T200A, 5H1A (81) S239A, 5H2A (53, 89)	—
5.46	A196C/G, ACM1 (13)	S192A, A1AA (86) S207A, B2AR (46)	S202A, D1DR (47) S197A, D2DR (88, 90)	S242A, 5H2A (48, 49)[spec] A242S, 5H2A (50)[spec] A222S, 5H2C (49)[spec] T196A, 5H6 (51)	N198A, HH1R (32, 44) T190A/C, HH2R (45) N207A, HH1R (52)
5.47	—	—	F198A, D2DR (58)	F243A, 5H2A (53)	—
5.50	P242A, ACM3 (40)	—	—	—	—
6.48	W400A, ACM2 (27) W503F, ACM3 (40)	—	—	W336A, 5H2A (55) W327A, 5H1B (56)	—
6.49	—	—	L286Y, D1DR (91) L387A, D2DR (58)	—	—
6.51	Y381A/F, ACM1 (57) Y403F, ACM2 (92) Y506F, ACM3 (67)	F289A, B2AR (93)	F389A, D2DR (58)	F339Y, 5H2A (60)	—
6.52	N382A, ACM1 (42, 57) N404Q, ACM2 (27) N507A/S/D, ACM3 (61)	F290M, B2AR (93)	—	F340L, 5H2A (22, 55, 59) *F331A, 5H1B* (84)	F433A/M, HH1R (39)

6.55	—	N293L, B2AR (63, 94) M292L, A1AA (85)[spec]	H349L, D3DR (65)	*S334A, 5H1B* (95)	F436A/M, HH1R (39)
7.35	W400A/F, ACM1 (23)	Y308A/F, B2AR (66, 96)[spec]	—	—	—
7.36	—	I309A, B2AR (96)[spec]	—	D352A, 5H1B (56)	—
7.39	Y404A/F, ACM1 (23) Y529F, ACM3 (67)	F412N, A2AA (68) N312Q/T, B2AR (69)	*T369V, D3DR* (65)	*N386V, 5H1A* (71) T355N, 5H1B (72, 74)	—
7.40	—	—	—	W367A, 5H2A (55)	—
7.42	C407S, ACM1 (75)	Y338A, A1AB (82)	—	—	—
7.43	Y533F, ACM3 (67)	—	—	Y370A, 5H2A (55)	—
7.45	—	N318A, B2AR (28)	N351D, DBDR (97)	—	—
7.46	—	—	—	S393A, 5H1A (98)	—

[a]Listings include the wild-type residue in single letter code, the residue number, and the residue to which the residue was mutated, followed by the receptor name in Swiss-Prot format (http://www.expasy.ch/cgi-bin/lists?7tmrlist.txt). Most of the mutations can be found in tGRAP Mutant Database (http://tinygrap.uit.no/) (99). The criteria we used for inclusion in this table of representative mutations were: (*a*) binding or function was detected or the presence of receptor protein in the membrane was confirmed by immunoblotting, (*b*) mutation at a given index position must have produced a change in affinity or potency of greater than fivefold in two or more different receptors, and (*c*) the mutation must be a single residue change (or a double mutation in which the second mutation was without effect when studied alone). (This criterion was added because a number of mutants with significant effects involved mutation of several positions, and it was not possible to determine which of the residues was responsible for the effect). Mutations involving the (D/E)RY motif, which are thought to affect activation state and not involve ligand binding were excluded.

Abbreviations in tables:

SCAM, substituted-cysteine accessibility method: X = accessible and protected by ligand in D2 receptor; A = accessible but not protected by ligand; ND = no binding detected in Cys mutant at this position.

RC, retinal contact residues: X = solvent accessible surface area calculation in the absence of retinal significantly greater than in the presence of retinal.

[aff], residues that were affinity labeled by either antagonist or agonist derivatives.

[spec], residues that were mutated to those of other subtypes (or the same subtype in a different species) with substantial conversion of pharmacological specificity.

[lig], residues implicated in direct contact by receptor mutagenesis with complementary structural modification of ligand.

italic, mutations that increased affinity (excluding residues designated [spec]).

retention. Thus, it can be extremely difficult to differentiate direct and indirect effects of mutations on function. The structure of the λ phage receptor maltoporin, for example, showed that approximately half of the mutated residues that had been implicated in λ phage recognition are located in the protein interior (15). Thus, mutation of these buried residues alters λ phage binding indirectly.

EFFECTS OF MUTATIONS ON RECEPTOR ISOMERIZATION Studies with constitutively active mutants (CAMs) and with overexpression of wild-type receptors have led to the realization that GPCRs spontaneously isomerize to varying extents from an inactive state(s) to an active state(s) (100–102). Agonists, because of their greater affinity for the active than the inactive state(s), promote this isomerization, which leads to a greater fraction of receptors in the active state(s) that binds and activates G proteins. The measured IC_{50} (or the calculated apparent K_i) by itself cannot tell us about the actual dissociation constants of the agonist for the inactive and the active receptors because it is a hybrid value that depends on these values as well as the propensity of the receptor to isomerize to the active state (103).

Interpretation of the effects of mutation on the binding of agonists, therefore, is fraught with complexity. Effects on affinity can be produced by effects on receptor isomerization as well as by altering direct interaction of the mutated residue with ligand. Such effects on isomerization can occur in either direction, resulting either in a gain in affinity in the case of increased isomerization to the active state or in a loss of affinity in the case of decreased isomerization to the active state. Thus, constitutively active receptors have a higher affinity for agonists, even in the absence of G protein, because more of the receptor is in the high-affinity active state, which optimizes existing interactions and/or adds new interactions to bound agonist (101). It would be incorrect to infer that this gain in affinity results from better contact of the mutated residue with ligand. This is relatively simple to accept in the case of a residue at the cytoplasmic end of TM6, a classical spot for the production of CAMs, but this logic is identical in the case of mutations in any portion of the receptor, including residues in the TMs that are being considered as possible binding-site residues. Examples of increases in ligand affinity produced by mutations in aminergic GPCRs are indicated in Table 1 in italics. Like all mutations producing effects on agonist affinity, these must be interpreted with caution.

The temptation to infer direct effects is perhaps even greater in the case of decreased affinity. This can be illustrated by the example of $Asp^{2.50}$ in TM2, which is conserved not only in aminergic receptors but in nearly all GPCRs. Early studies in the β_2AR found that, unlike the case for $Asp^{3.32}$, the mutation of which abolished the binding of both agonists and antagonists, mutation of $Asp^{2.50}$ greatly reduced the affinity of agonists without a significant effect on antagonist binding (28). Initially, this was interpreted as evidence for a direct contact of agonists but not antagonists with $Asp^{2.50}$. This inference was based on the assumption that if antagonist binding was normal, then a change in agonist binding resulting from this mutation must be caused by an alteration of a direct interaction of the mutated residue. But if such a mutation prevents or greatly decreases

the propensity of a receptor to assume the active state, this will manifest as a decrease in the binding affinity of the compound as well as a decrease in the potency of activation (if activation can still be measured). Our current interpretation is that $Asp^{2.50}$ is relatively far from the binding site (16), and that this residue plays a key role in the process of receptor activation and in the modulatory effects of sodium on activation (17, 104, 105). In addition, $Asp^{2.50}$ has been proposed to form part of a sodium-binding site (79). Nonetheless, although our current models make it seem unlikely, without a crystal structure of the receptor bound to the particular agonist we cannot absolutely rule out direct contact with $Asp^{2.50}$.

THE IDENTIFICATION OF DIRECT LIGAND CONTACTS There are a limited number of elegant examples of functional complementation of binding or activation in which alterations in putative binding site residues were combined with reciprocal changes in the putative functional group in the ligand thought to contact this site. For example, mutations and modifications of agonists have been used to study the role of conserved serines in TM5 of the β_2AR: an examination of the affinities and activating potencies and efficacies of a series of agonists with or without key hydroxyl moieties (OH) on the phenyl ring led to the proposal of a direct contact between $Ser204^{5.43}$ and the meta-OH, and $Ser207^{5.46}$ and the para-OH, respectively (46). Related analysis of β_2AR mutants has also led to the proposal that both $Ser203^{5.42}$ and $Ser204^{5.43}$ participate in an interaction with the meta-OH (43). Similarly, the direct interaction of the β-OH of catecholamine agonists with $Asn293^{6.55}$ in TM6 of the β_2AR was inferred from the effects of mutagenesis (94) and further supported by simultaneous modification of the ligand that showed gain of function (63).

In many aminergic receptors, mutation of the completely conserved $Asp^{3.32}$ abrogates binding altogether. Remarkably, in the β_2AR, catechol esters and ketones, which substitute a hydrogen bonding interaction for the normal electrostatic interaction between the protonated amine and $Asp^{3.32}$, were able to activate the mutant in which $Asp113^{3.32}$ was mutated to serine ($D113^{3.32}S$) (30). Likewise the 5-hydroxytryptamine (5-HT) analog gramine, which is one carbon shorter than the agonist *N*,*N*′-dimethyltryptamine, had greater efficacy at the $D155^{3.32}E$ 5-HT_{2A} mutant receptor (in which the length of the carboxylate side chain was increased by one carbon) than at the wild-type receptor, whereas *N*,*N*′-dimethyltryptamine was more efficacious at the wild-type receptor than at $D155^{3.32}E$ (14).

Thus a role of the highly conserved $Asp^{3.32}$ in binding muscarinic cholinergic, adrenergic, dopaminergic, serotonergic, and histaminergic agonists and antagonists has been supported by work through multiple approaches, which are listed below in rough order of increasingly firm support that the mutated residue contacts ligand: the residue is conserved in aminergic receptors and is predicted to be in the extracellular half of the transmembrane domain; the mutation of this residue abrogates ligand binding (33, 90); the mutation of this residue abrogates ligand binding and receptor expression is confirmed by immunoblotting (28, 82); agonist

affinity and/or potency is reduced but detectable (25, 29, 81); antagonist affinity is reduced but detectable (18, 24, 26, 31, 32); a direct interaction of the side chain with agonists has been supported through complementary effects of mutations and modifications of ligands (14, 30); the residue has been directly affinity labeled (5, 6).

This hierarchy is the basis for our division of the literature into Tables 1A and 1B. In Table 1A we list representative examples of affinity-labeled residues, of residues that likely contact ligands based on complementation experiments in which the ligand was modified, and of mutations that either altered antagonist binding or abrogated binding but with confirmation of receptor expression through immunoblotting or preservation of agonist activation. Table 1B lists representative examples of mutations that altered agonist binding and/or potency. Whereas many of these positions overlap with examples from Table 1A and are likely to be contact residues, others, such as $Asp^{2.50}$ (see above) are more likely examples of indirect effects.

Second-Site Revertant Mutations

$Asp^{2.50}$ is conserved in nearly all GPCRs, but when the gonadotropin releasing hormone (GnRH) receptors were cloned, it was noted that these receptors often contained an Asn at 2.50 (106). Sequence analysis of correlated mutations also demonstrated that instead of the highly conserved $Asn^{7.49}$, an Asp is present at 7.49 in the GnRH receptor. This led to the proposal that the residues at these two positions interact in GPCRs, and that the side chain identities are exchanged in the GnRH receptor. Indeed, whereas mutation of one of these residues disrupts receptor function, the exchange of these two residues reconstitutes function in the GnRH receptor, the $5\text{-}HT_{2A}$ receptor, and other GPCRs as well (106–109). This second-site revertant effect has been taken as evidence that these two residues interact directly or via a network of hydrogen bonding interactions, and such an interaction has been incorporated into molecular models of GPCRs that predict the presence of significant kinks in TM7 (77, 110). A number of other second-site revertant mutations in various GPCRs (111–115) have also been used to infer the packing and orientation of the TMs, which are the framework for the binding site.

Although the identification of second-site revertant mutants can be a powerful method for inferring residue interactions, a cautionary shot across the bow comes from the solution of the crystal structures of several dihydrofolate reductase mutants. The primary D27S mutation, located in the substrate-binding pocket, greatly reduced catalytic activity as compared to the wild-type enzyme (116). The combination of F137S and D27S partially restored catalytic activity. Phe137, however, was found in the crystal structure to be on the surface of the molecule, outside of the catalytic center and approximately 15 Å from residue 27! Thus, the double mutant underwent an extended structural perturbation, which was propagated between the two widely separated sites via a perturbation in both solvent and polypeptide

backbone structure. These authors concluded with a prescription that would certainly reduce the size of the literature on GPCRs: "Our findings reemphasize the unpredictability of structural effects due to mutation and hence, the importance of determining crystal structures of mutant proteins under study" (116).

Mapping the Surface of the Binding-Site Crevice with the Substituted-Cysteine Accessibility Method

The rhodopsin-like receptor family, which contains the aminergic receptors among others, binds agonists present in the extracellular medium and couples this binding to the activation of intracellular G proteins. The binding sites of these receptors are formed among their seven TMs and are accessible to charged, water-soluble agonists, such as dopamine. Thus, each of these binding sites is contained within a water-accessible crevice, the binding-site crevice, which extends from the extracellular surface of the receptor into the transmembrane domain. The surface of this crevice is formed by residues that contact specific agonists and/or antagonists and by other residues that play a structural role and may affect binding indirectly.

To identify the residues that form the surface of the binding-site crevice in the human dopamine D2 receptor, we have used the substituted-cysteine accessibility method (SCAM), a method initially developed to map channel-lining residues in ligand-gated ion channels (117). Consecutive residues in the membrane-spanning segments are mutated to cysteine, one at a time, and the mutant receptors are expressed in heterologous cells. The surface-accessibility of a cysteine is inferred from the irreversible effects of sulfhydryl-specific reagents on binding. If ligand binding to a cysteine-substitution mutant is near normal, we assume that the structure of the mutant receptor is similar to that of wild type and that the substituted cysteine lies in a similar orientation to that of the wild-type residue. Sulfhydryls facing into the binding-site crevice should react much faster with charged sulfhydryl-specific methanethiosulfonate (MTS) reagents (118) than should cysteines facing into the lipid bilayer or the protein interior. Experimental support for the validity of such an approach comes from a study in the aspartate chemotaxis receptor of the accessibility of engineered cysteines to reaction with another hydrophilic, sulfhydryl-specific alkylating agent (119). In the $\alpha 2$ helix of the periplasmic domain, a striking correlation was observed between the measured chemical reactivity of each engineered cysteine and the calculated solvent accessibility of the β-carbon at the corresponding position in the crystal structure.

If binding is irreversibly altered by an MTS reagent, we infer that the MTS reagent has reacted covalently with the sulfhydryl of the engineered cysteine. Much like site-directed mutagenesis studies, the inhibitory effects of the addition of SCH_2CH_2X to the cysteine could be due to steric block, electrostatic repulsion, indirect conformational changes, or a combination of these. Although we do not know the detailed mechanism of the alterations in binding, the alteration itself allows us to infer that reaction has occurred. Furthermore, we infer that if the MTS reagents

react with an engineered cysteine, then the cysteine is on the water-accessible surface of the protein and that the corresponding wild-type residue is also accessible on the water-accessible surface of the protein.

Our studies of the dopamine D2 receptor (16, 19, 33, 37, 41, 54, 77, 120, 121) have allowed us to identify the surface of the binding-site crevice (Figure 1, for a 3D version, follow the Supplemental Material link on the Annual Reviews homepage at http://www.annualreviews.org/) and to infer the secondary structure of the membrane-spanning segments forming this surface as well as the electrostatic potential within the crevice. We have obtained evidence for the presence of kinks in a number of the TMs and have identified two clusters of aromatic residues critical to receptor structure and ligand recognition, one between TM2-TM3-TM7 and another between TM5 and TM6.

AMINERGIC GPCR STRUCTURE AND MOLECULAR MODELING

Despite enormous efforts, prior to the new millennium, no high-resolution structure of any GPCR had been solved. Low-resolution structures that include a 9 Å projection structure of bovine rhodopsin (122) and a 7.5 Å structure of frog rhodopsin (123) have been used to guide the development of a molecular model of the TMs of rhodopsin (124). Molecular models for many other GPCRs were based on this model and on inferences from sequence alignments, analyzed in terms of conservation and physico-chemical properties. These models provided a structural context, putatively shared by all rhodopsin-like GPCRs (3) for incorporating constraints derived from biophysical and mutagenesis experiments into mechanistic hypotheses (4, 125). These models have been useful in rationalizing experimental results and formulating hypothesis for further testing.

The landmark high-resolution structure of bovine rhodopsin to 2.8 Å (2) has revolutionized the field by providing the first high-resolution GPCR structure. This structure and its compatibility with the rhodopsin literature have been reviewed elsewhere (126). In comparing the SCAM data from the dopamine D2 receptor and mutagenesis data from a number of aminergic GPCRs, we have inferred that the structure of these receptors may be remarkably similar to that of rhodopsin, even to the extent of local perturbations in regular helical structure (3). New homology models based on the high-resolution rhodopsin structure, as well as on future GPCR structures, will likely promote a significant advance in the accuracy and predictive power of the resulting models.

To review and illustrate, from a three-dimensional perspective, the role of TM residues in forming the binding site, we have constructed models of the dopamine D2 receptor and the α_{1A}AR. These models were built by homology modeling using Modeller (127) with the bovine rhodopsin structure as a template. The models were further refined by molecular dynamics using CHARMM (128). In Figure 1

we have shown each TM of the dopamine D2 receptor, except for TM1 for which no ligand contacts have been proposed, in the context of the adjacent helices seen from the inside of the binding-site crevice from the perspective of ligand. On this structure, we have shown in purple the side chains and backbone of the residues at positions implicated in ligand binding in aminergic receptors by site-directed mutagenesis and/or by affinity labeling and that were accessible in SCAM studies (See Table 1A). Of note, in the D2 receptor, cysteines substituted for all of the residues shown in purple were protected from reaction of sulfhydryl reagents by the presence of ligand within the binding site, with the exception of 4.59 and 7.35. $Gly^{7.42}$ in the D2 receptor did not tolerate mutation to cysteine but is shown in purple based on the data in Table 1A in other receptors. In yellow, we show additional residues that are accessible and protected in the D2 receptor but that are either not implicated in binding in other studies or are not studied. Note that these are by and large contiguous with the purple ligand-contact residues but located further intra- or extracellularly within the binding-site crevice. This is consistent with the fact that contact residues are a subset of the surface of the binding-site crevice.

The Binding Site

It is likely that different ligands can dock in different exact orientations and positions (53), and it is difficult to make generalizations that will be true of every receptor in its interaction with every ligand. Nonetheless, as summarized in Table 1, a number of the interactions of aminergic GPCRs with their ligands appear to involve similar positions in the binding-site crevice. Thus, the completely conserved $Asp^{3.32}$ likely makes a direct contact with the protonated amine of all aminergic ligands (see Table 1). In certain receptors and with certain ligands, this interaction with the protonated amine is shared with the residue at 3.36, one turn below 3.32 (83). In cholinergic and histaminergic receptors, the residue at 3.40, yet another turn more toward the cytoplasm, is also involved in ligand binding and specificity (24, 36).

Many aminergic ligands also form critical interactions with residues in TM5. The critical TM5 residues are not as conserved as is $Asp^{3.32}$, but the positions and interactions nonetheless appear to be conserved. Thus, in catecholamine receptors critical interactions exist between $Ser^{5.42}$ and $Ser^{5.46}$ and the meta-OH and para-OH moieties of catecholamine agonists (43, 46, 47, 78, 82, 86, 88, 90), and antagonists appear to interact with $Ser^{5.42}$ as well (43). In some catecholamine receptors an additional interaction of the meta-OH with $Ser^{5.43}$ also appears to take place (46, 47, 78, 88). Histamine, via its imidazole nitrogens, forms hydrogen bonding and/or ionic interactions with the H_1 and H_2 receptors: in H_2 receptor, $Asp^{5.42}$ interacts with the N^{τ} nitrogen of histamine (45), whereas in H_1 receptor, $Asn^{5.46}$ interacts with the N^{τ} nitrogen (32, 44, 52). In the H_2 receptor, $Thr^{5.46}$ is thought to interact with the N^{π} nitrogen of histamine through a hydrogen bond (45). In the H_1 receptor, $Lys^{5.39}$ may interact with the N^{π} nitrogen of histamine through a

hydrogen bond (87), but Thr$^{5.42}$ may also interact with this N$^{\pi}$ nitrogen (44). In various serotonin receptors, Ser and Thr at 5.42 and 5.43 also appear to make important hydrogen bonding interactions with serotonergic ligands (53, 62, 81, 89). As discussed below, the difference in affinity for mesulergine between the human and rat 5-HT$_{2A}$ receptors results from a Ser$^{5.46}$ to Ala substitution (48), and the differences in affinity between the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors for this drug result from a similar substitution at 5.46 (49). This Ser is thought to form a hydrogen bond with the N-1 nitrogen of unsubstituted tryptamines and ergolines (129). In cholinergic receptors, mutation of Thr$^{5.39}$ and Thr$^{5.42}$ impaired agonist binding and/or activation (13, 27, 42, 67), and T$^{5.42}$C was affinity labeled by an agonist derivative (13). It has been suggested that the acetyl methyl group of acetylcholine binds in the vicinity of these side chains (130). In addition, in the M1 receptor, Ala$^{5.46}$ has also been implicated in agonist binding (13).

The cluster of aromatic residues in TM6 is highly conserved among aminergic GPCRs and includes Trp$^{6.48}$, Phe/Tyr$^{6.51}$, and Phe$^{6.52}$. These residues have been implicated in ligand binding and/or in receptor activation in many aminergic receptors (22, 27, 39, 40, 54–60, 67, 84, 92, 93). Curiously, the cholinergic receptors, for which the endogenous ligand does not have an aromatic ring, contain an Asn at 6.52, and mutation of this residue impaired antagonist and agonist binding (27, 42, 57, 61), consistent with an interaction of the 6.52 position in this receptor as well. Trp$^{7.40}$ is completely conserved in all aminergic receptors, and in β_1AR this residue was affinity labeled by an antagonist derivative (7). Mutagenesis studies in the 5-HT$_{2A}$ receptor support an interaction of Trp$^{7.40}$ with serotonergic ligands as well (55).

The highly conserved Trp$^{4.50}$ was inferred to be accessible based on SCAM studies (37) and inferred to contact ligands based on mutagenesis studies in 5-HT$_{2A}$ receptor (55) and M3 cholinergic receptor (ACM3) (40). Curiously, this residue faces out toward lipid, TM2, and TM3 in the rhodopsin structure (2). Several additional TM4 residues that were inferred to be accessible in the D2 receptor (37) also face outward in the rhodopsin structure (2). Although this inconsistency might represent a significant difference in the structures of rhodopsin and other "rhodopsin-like" receptors, there are other possible explanations, such as a potential role of this surface in receptor dimerization (3).

Structural Bases of Pharmacological Specificity

Early mutagenesis studies focusing on residues that were conserved within families of receptors led to the identification of conserved residues that could not be responsible for differences in specificity within these subfamilies. These include many of the residues discussed above, including Asp$^{3.32}$, Trp$^{6.48}$, Phe/Tyr$^{6.51}$, and Trp$^{7.40}$. Such highly conserved residues, although implicated in ligand binding, cannot account for the pharmacological differences among these receptors, and these must arise instead from differences in their sequences and folded structures.

In a number of receptors, single residues have been identified that differ among related subtypes and that when mutated, partially or fully interchange pharmacological specificity (summarized in Table 1). In the α_{1A}AR, Phe86$^{2.64}$ was found to be responsible for antagonist selectivity between the 1A and 1D subtypes (21). Differences in antagonist selectivity between the rat and human histamine H_3 receptors result from a substitution of Val$^{3.40}$ with Ala (36). The difference in affinity for mesulergine between the human and rat 5-HT_{2A} receptors results from a Ser$^{5.46}$ to Ala substitution (48), and the differences in affinity between the human 5-HT_{2A} and 5-HT_{2C} receptors for this drug result from a similar substitution at 5.46 (49). Tyr308$^{7.35}$ was shown to play a role in agonist selectivity for β_2AR relative to β_1AR (66, 96). In addition, the residue at position 7.39 contributes to the specificity of multiple receptors, including the α_2AR and β_2AR (68, 69) and the 5-HT_{1A} and 5-HT_{1B} receptors (70, 72, 74).

A number of studies with chimeric receptors demonstrated the contribution of multiple residues/domains to creating high-affinity binding sites (131–133). There are fewer cases in which individual residues not adjacent in primary sequence have combined effects on specificity. Simultaneous substitution of Ala$^{5.39}$ and Leu$^{6.55}$ in the α_{1B}AR with the aligned Val$^{5.39}$ and Met$^{6.55}$ from the α_{1A}AR converted the pharmacological specificity for several specific agonists (86). An example from another rhodopsin-like receptor is the conversion of the pharmacological profile of a mammalian angiotensin receptor to that of an amphibian receptor with the substitution of 13 residues scattered throughout TM2–TM7 (134).

In exploring the bases for pharmacological specificity in the dopamine D2 and D4 receptors, we have used the accessibility pattern determined by SCAM to narrow our initial screen and to identify structural determinants of pharmacological specificity (19). We reasoned that the residues that form the surface of the binding-site crevice but that are not conserved in the D2 and D4 receptors are the best candidates for determinants of the pharmacological differences between these receptors. Of the more than 90 nonidentical residues within the TMs of the D2 and D4 receptors, only 20 residues in the TM2 through TM7 segments were determined to be accessible by SCAM in the D2 receptor. Six of these 20 residues represent conservative aliphatic substitutions between the two receptors, whereas the other 14 residues are not conserved.

In a D2 receptor background, we mutated these 14 nonconserved accessible residues to the aligned D4 residue one at a time and/or in combination. We also made the reciprocal substitutions in a D4 receptor background. The combined substitution of four to six of these residues was sufficient to switch the affinity of the receptors for several chemically distinct D4-selective antagonists by three-orders of magnitude in both directions (D2 to D4-like and D4 to D2-like) (19). The mutated residues, at positions 2.60, 2.61, 2.64, 3.28, 3.29, and 7.35, form a cluster in the binding-site crevice. We could rationalize these data in terms of a set of chemical moieties in the ligands interacting with the divergent aromatic microdomain in TM2–TM3–TM7 of the D2 and D4 receptors. These findings emphasize the importance of considering the role of three-dimensional structural microdomains

in receptor function (135) as opposed to simply considering the structural and functional roles of single residues or stretches of residues in the primary structure.

We have noted that the residues that are critical for pharmacological specificity often are one turn above, and sometimes but less frequently one turn below, the critical "conserved" contact residues. As discussed, these residues include 3.28 and 3.29, one turn above 3.32, and 7.35 and 7.39, which are above and adjacent to 7.40. Moreover, $Asn^{6.55}$, which is one turn above 6.51 and 6.52, interacts with the β-OH of epinephrine (63). In contrast, $His^{6.55}$ is conserved in D2-like receptors and is thought to contact particular ligands (54, 64, 65). $Phe^{6.55}$ in H_1 receptor also appears to play a role in ligand binding (39). Thus, although the physicochemical properties of the side chains at 6.55 vary considerably, this position, one turn above the aromatic cluster, often forms important ligand interactions. In addition, the residues at 2.61 and 2.64 are highly variable and at approximately the same depth into the transmembrane domain as 3.28, 3.29, 6.55, 7.35, 7.36, and 7.39.

As indicated in Table 1A there is a remarkable extent of overlap between the positions of the proposed ligand-contact residues and the positions of the retinal contact residues in rhodopsin (3). Thus, to achieve specificity for different agonists, these receptors have evolved different residues in similar positions on the surface of the binding-site crevice, thereby presumably maintaining a conserved mechanism for coupling agonist binding to receptor activation. Although different positions and residues may play more and may play less critical roles in different receptors and with different ligands, the residues on the surface of this crevice are potential contacts for novel drugs, even if the residues are not contacts for existing compounds.

Receptor Activation

Using indirect methods, some progress has been made toward an understanding of the conformational changes that are associated with GPCR activation (136–139). Ultimately, however, it may require structures of multiple receptors bound to multiple ligands, including inverse agonists, agonists, and antagonists, for us to flesh out the details of the conformational changes associated with receptor activation. In addition, different agonists may channel individual receptors to different G proteins and thereby to different second messenger systems, a phenomenon that would require the existence of multiple active conformations (140–143), further demonstrating the need for additional high-resolution structures.

THE SECOND EXTRACELLULAR LOOP

A remarkable feature of the bovine rhodopsin structure is that the second extracellular loop (E2) dives down into the transmembrane domain and forms two β-strands, one of which contacts retinal (2). It is unknown whether the general structure of E2 and its involvement in ligand binding are shared features among

rhodopsin-like GPCRs such as the aminergic receptors. Whereas the extracellular loops have been found to be important in ligand binding of GPCRs with large molecular weight ligands, such as peptide receptors (144), their role in aminergic GPCRs has received much less attention.

Removal of the disulfide bond between $Cys^{3.25}$ and the conserved Cys in E2 (Cys_e2) by mutagenesis severely disrupted the function of M1 muscarinic acetylcholine receptor (ACM1) (75). Ligands protected the β_2AR from reduction by dithiothreitol (145), which suggests that the extracellular conserved disulfide bridge is intimately related to the binding site and may be physically nearby. This disulfide bond between position $Cys^{3.25}$ and Cys_e2 is also responsible for stabilization of the high-affinity state of the β_2AR (146).

Several reports implicate E2 in ligand specificity in aminergic and other small molecule-ligand GPCRs. Perez et al. found that substitution of three consecutive residues in E2 changed the ligand specificity for particular antagonists from that of α_{1B}AR to that of α_{1A}AR, and vice versa (147). Similarly, substitution of E2 and TM5 altered the subtype-specificity of the 5-HT_{1D} receptor to that of the 5-HT_{1B} receptor, and vice versa (148). Substitution of a single residue in E2 was also sufficient to interconvert the pharmacological specificity of canine 5-HT_{1D} and human 5-HT_{1D} receptor (149). In adenosine receptor, in which the binding site is also formed in the transmembrane domain (144), several glutamate residues in E2 are critical for ligand recognition (150, 151).

Thus, although it has been argued that the presence of E2 within the transmembrane domain may be a feature unique to rhodopsin (126, 152), we propose that E2 may enter into the binding-site crevice of aminergic and certain other small molecule-ligand GPCRs. Although it is currently difficult to envision the entrance route of ligands into the binding-site crevice and the potential associated conformational rearrangements of E2, the data reviewed above are highly suggestive of a direct role of residues in E2 in ligand binding. In order to explore the feasibility of such a hypothesis, we have extended our modeling analysis to E2 of the α_{1A}AR (Figure 2A, B, follow the Supplemental Material link on the Annual Reviews homepage at http://www.annualreviews.org/). We have assumed that the dimensions of the transmembrane domains of aminergic receptors are similar to those of bovine rhodopsin, that the aligned extracellular boundaries of the TMs are similar, and that there is a conserved disulfide bond from $Cys^{3.25}$ to Cys_e2. Given that E1 (5–7 residues) and E3 (mostly 6–8 residues) are short in all aminergic receptors, it is less likely that they penetrate sufficiently into the binding-site crevice to play a direct role in ligand binding (Figure 2A). In contrast, E2 is significantly longer (13–31 residues) and may reach into the binding-site crevice and form a lid over bound ligand, as it does in rhodopsin. The conserved proline(s) at the extracellular end of TM4 may play an important role in the structure and/or dynamics of E2.

The distance between $Cys^{3.25}$ and the extracellular end of TM5 is 16–18 Å in rhodopsin; thus, in aminergic receptors, the stretch of only 4–7 residues between Cys_e2 and the extracellular end of TM5 is likely to be in an extended state, or partly

in a β-strand as in rhodopsin, in order to reach Cys$^{3.25}$. Furthermore, it is likely that the residues upstream of Cys_e2 are more extracellularly located relative to the stretch downstream of Cys_e2, in order to leave sufficient room in the binding-site crevice for ligand (Figure 2, follow the Supplemental Material link on the Annual Reviews homepage at http://www.annualreviews.org/). Given these geometrical constraints, two or three residues immediately following the conserved Cys_e2 may face the binding pocket and be located near other key binding residues within the transmembrane domain.

In rhodopsin several residues around Cys_e2, including Glu181, Ser186, Cys187 (Cys_e2), Gly188, Ile189, and Tyr191, contact retinal (2). Although it is difficult to use our standard indexing in this region because of the variable sequence length between TM5 and Cys_e2, aligning the E2 sequences on Cys_e2 reveals the surprising result that two of the three E2 residues identified by Perez and colleagues as able to convert specificity of the α_{1A}AR to that of α_{1B}AR (147) are aligned with the E2 retinal-contact residues. Thus, α_{1A}AR Gln177 and Ile178 align with Gly188 and Ile189 in rhodopsin. In contrast, Asn179 is aligned with Asp190, which in rhodopsin faces up toward the extracellular milieu. In the α_{1A}AR model shown in Figure 2, the side chains of Gln177 and Ile178 are in similar orientations as the aligned residues in rhodopsin and are positioned to form part of the binding site. In Figure 2B, we have illustrated the antagonist WB4101 docked within the binding-site crevice with interactions between Asp$^{3.32}$ and the protonated amine, and between Ser$^{5.42}$ and Ser$^{5.46}$ and a methoxy oxygen. This also positions the aromatic ring in a position in which it can interact with Phe$^{6.52}$. The side chains of Gln177 and Ile178 from E2 point down and interact with ligand, with the Gln interacting with the protonated amine and the Ile with the aromatic ring. Remarkably, Gln189 in 5-HT$_{1D}$ receptor, the residue shown to be partially responsible for the specificity of ketanserin (149), is also aligned with Gly188 in rhodopsin and Gln177 in α_{1A}AR, which suggests a wider role of this position in ligand binding.

E2 has also been implicated in ligand-ligand allosteric interactions in muscarinic acetylcholine receptors. By switching the acidic sequence EDGE immediately before Cys_e2 to the neutral residues LAGQ (the aligned sequence in ACM1), the affinity of ACM2 for the allosteric modulator gallamine was reduced, whereas the converse substitution of acidic sequence into ACM1 significantly increased the affinity for gallamine (153, 154). In addition, mutations at positions 3.28 and 7.35 decreased the affinity of gallamine in ACM1 (23). The authors inferred that these residues contact gallamine, and since gallamine slows the dissociation of the antagonist N-methylscopolamine, they speculated that gallamine may bind just extracellular to the antagonist binding site and function as a cap. On the basis of our homology model, it is difficult to envision access to 3.28 from the extracellular milieu if E2 is within the binding site. E2, however, may contact 3.28 and 7.35, and mutation of these residues may affect gallamine binding indirectly by modulating the interaction of E2 with the binding site. Therefore, it is tempting to speculate that gallamine indeed functions as a cap, by binding above the E2 “lid” and stabilizing its closure, and thereby slowing the rate of dissociation of the “trapped”

ligand. This hypothesis raises the possibility that novel allosteric modulators might be targeted to this region in other aminergic receptors.

Amiloride analogues accelerate the off-rate of antagonists in the α_{2A}AR and α_{1A}AR (155–157) and in dopamine receptors as well (158, 159). The structural features of the receptors responsible for these effects are unknown, but curiously, mutation of 3.29 in the dopamine D4 receptor increased the affinity of methylisobutylamiloride 30-fold (160). Whether E2 plays a role in the effects of amiloride and its modification by mutation of 3.29 requires further investigation.

In summary, the detailed relationship of E2 to ligand entry and binding and to a possible role in allosteric modulation may differ among GPCRs. With additional experimentation and new high-resolution structural information, such details will ultimately be revealed. It is possible that E1 (23) and/or E3 (161) in particular receptors may also participate in ligand entry, binding and/or modulation, but because of the length of E2 and its putative position within the binding-site crevice, it is much more likely than the other parts of the extracellular domain to participate, along with residues in the transmembrane domain, in the binding of small molecular weight ligands and in the determination of their specificity of interaction.

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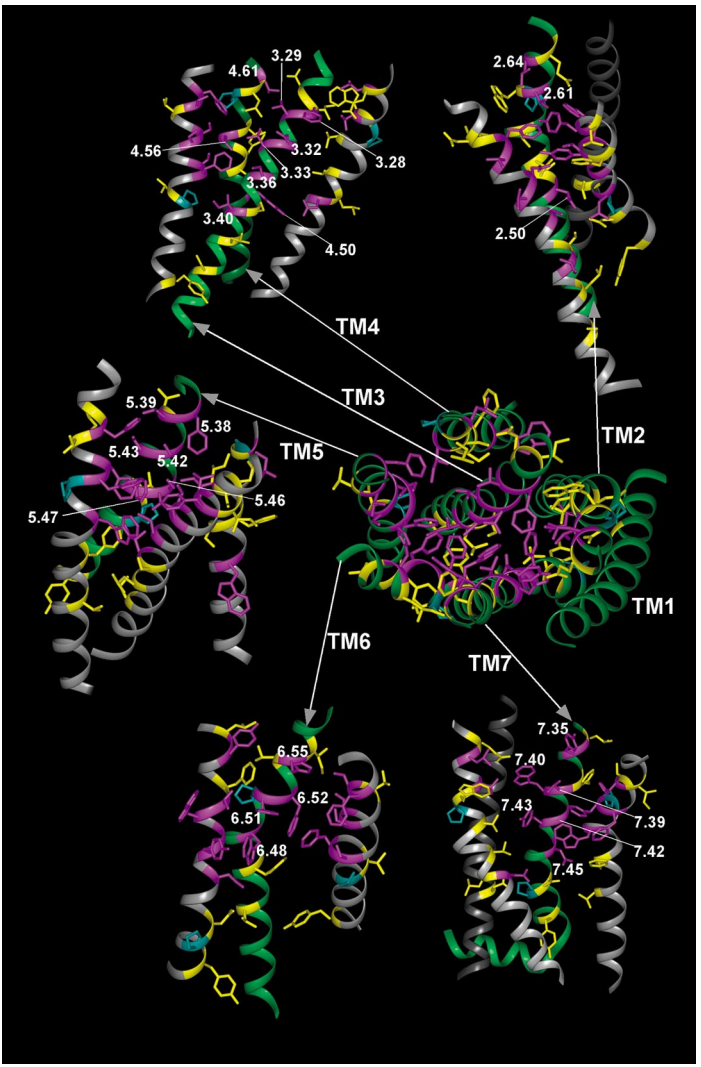

Figure 1 A 3-D molecular model of the dopamine D2 receptor based on the bovine rhodopsin high-resolution structure. The view on the middle right is an extracellular view; side views of TM2–TM7 are shown surrounding this view, from the perspective of ligand within the binding site. The side chain and backbone of residues at positions implicated in ligand binding in aminergic receptors and accessible in the binding-site crevice based on SCAM studies in the D2 receptor (see Table 1A and text) are shown in purple. The side chain of other residues found to be accessible and protected in SCAM studies of the D2 receptor (see text) are shown in yellow. Index numbers are shown for the purple residues. The featured TMs within each view are shown with a green ribbon, and the context is provided by the neighboring helices shown in gray. Prolines within TMs are shown in cyan.

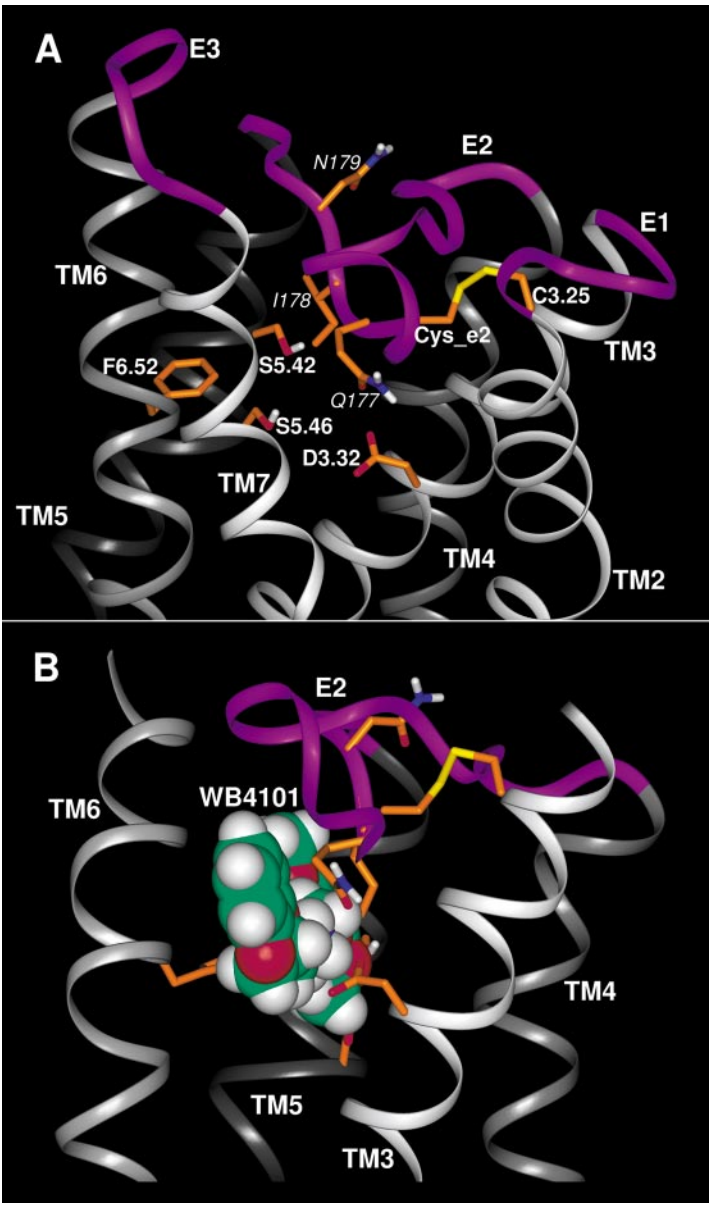

Figure 2 A 3-D molecular model of the α_{1A}AR based on the bovine rhodopsin high-resolution structure. Panel A shows a side view illustrating the positions of the three extracellular loops, as well as disulfide bond between Cys_e2 and C3.25 residues in the TMs and in E2 that are implicated in ligand binding (see text). Panel B shows a slightly tilted view from a more intracellular perspective with WB4101 docked within the binding site, making contacts with TM residues and with Gln177 and Ile178 from E2 (see text).