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# Delineation of the peptide binding site of the human galanin receptor

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**Galanin, a neuroendocrine peptide of 29 amino acids, binds to $G_i/G_o$-coupled receptors to trigger cellular responses. To determine which amino acids of the recently cloned seven-transmembrane domain-type human galanin receptor are involved in the high-affinity binding of the endogenous peptide ligand, we performed a mutagenesis study. Mutation of the His264 or His267 of transmembrane domain VI to alanine, or of Phe282 of transmembrane domain VII to glycine, results in an apparent loss of galanin binding. The substitution of Glu271 to serine in the extracellular loop III of the receptor causes a 12-fold loss in affinity for galanin. We combined the mutagenesis results with data on the pharmacophores (Trp2, Tyr9) of galanin and with molecular modelling of the receptor using bacteriorhodopsin as a model. Based on these studies, we propose a binding site model for the endogenous peptide ligand in the galanin receptor where the N-terminus of galanin hydrogen bonds with Glu271 of the receptor, Trp2 of galanin interacts with the $Zn^{2+}$ sensitive pair of His264 and His267 of transmembrane domain VI, and Tyr9 of galanin interacts with Phe282 of transmembrane domain VII, while the C-terminus of galanin is pointing towards the N-terminus of the receptor.**

*Keywords*: galanin/ligand binding/molecular modelling/receptor/site-directed mutagenesis

## Introduction

Galanin, a 29 amino acids long (30 in humans) neuroendocrine peptide, is widely distributed and exerts numerous effects throughout the nervous and endocrine systems of mammals (Bartfai *et al.*, 1993a; Merchenthaler *et al.*, 1993). The involvement of galanin in the regulation of learning and memory (Ögren *et al.*, 1992), pain signalling (Wiesenfeld-Hallin *et al.*, 1992), insulin secretion (McDonald *et al.*, 1985) and growth hormone release (Bauer *et al.*, 1986), feeding (Leibowitz and Kim, 1992) and sexual behaviour (Benelli *et al.*, 1994) implies a great therapeutic potential for drugs acting specifically on the galaninergic systems.

The first galanin receptor cDNA, encoding a member of the G protein-coupled receptor superfamily, was cloned from human Bowes melanoma cell line (Habert-Ortoli *et al.*, 1994). An analysis of the primary sequence shows that this seven-transmembrane (7TM) domain receptor has a high sequence identity (30–31%) to somatostatin (Xu *et al.*, 1993) and δ-opioid receptors (Evans *et al.*, 1992), and binds its endogenous ligand galanin with nanomolar affinity ($K_D \approx 1$ nM). In the Bowes cells, this receptor is coupled to adenylate cyclase via inhibitory G proteins (Heuillet *et al.*, 1994).

In this study we set out to examine which domains and/or single amino acids in the galanin receptor contribute to the high-affinity binding of the large, 29 amino acids long endogenous ligand galanin. Extensive structure–activity relationship studies on the ligand have shown that the N-terminal (1–16) portion is sufficient for high-affinity binding, and that Trp2, Tyr9 and the free N-terminus are vital for the interaction with the galanin receptor (Fisone *et al.*, 1989a; Lagny-Pourmir *et al.*, 1989; Land *et al.*, 1991). Thus the ligand has been described adequately, whereas the receptor was cloned only recently, thereby enabling studies on ligand–receptor interactions focused on the receptor sequence.

Studies on several other neuropeptide receptors (all members of the G protein-coupled receptor superfamily) show that the binding of nonpeptide antagonists to these peptide receptors is dependent upon interactions with only a few amino acids of the receptors, most of which are located in the putative transmembrane regions V, VI and VII of these 7TM receptors (Beinborn *et al.*, 1993; Fong *et al.*, 1993; Gether *et al.*, 1993; Schambye *et al.*, 1995).

In contrast, the mode of the peptide ligand binding to their respective neuropeptide receptors, especially that of endogenous peptides, has not been so well characterized. Data obtained from mutagenesis studies on neurokinin I receptors (Fong *et al.*, 1992) and angiotensin II type I receptors (Schambye *et al.*, 1994), using both non-peptide and peptide ligands to assess the effects of the mutations, suggest the involvement of larger areas of contact between neuropeptide ligands and the receptor surface, including extracellular domains of the receptor and amino acids located close to the top of several transmembrane helices. However, in some cases, like for the SSTR2 somatostatin receptor selective peptide ligand SMS 201–995, it has been shown that only two amino acids of transmembrane regions VI and VII are sufficient to determine the selectivity of the receptor for this peptidomimetic ligand (Kaupmann *et al.*, 1995).

In several studies on peptide receptors the chimeric receptor approach, together with site-directed mutagenesis, has been used to first locate the areas of the receptors involved in ligand binding and then to pinpoint the amino acid residues that are actually making contact with the ligands. Galanin is not structurally related to any other hitherto known neuropeptide. Therefore it is impossible to make chimeras between the galanin receptor and recep-

Porcine $^{+}\underline{NH_3}$-Gly**Trp**ThrLeu**Asn**SerAlaGly**TyrLeuLeu**GlyProHisAla*IleAsp*AsnHisArgSerPhe*His*AspLys*Tyr*GlyLeu*Ala*-$CONH_2$

Human $^{+}\underline{NH_3}$-Gly**Trp**ThrLeu**Asn**SerAlaGly**TyrLeuLeu**GlyProHisAla*ValGlu*AsnHisArgSerPhe*Ser*AspLys*Asn*GlyLeu*ThrSer*-$COO^{-}$

**Fig. 1.** Amino acid sequence of porcine (Tatemoto *et al.*, 1983) and human galanin (Evans and Shine, 1991). The divergent amino acids are in italics. The N-terminal part of galanin essential for binding and agonist activity is underlined. The important pharmacophores of galanin are in bold.

tors for closely related peptides, as, for example, with receptors for neurokinins (Fong *et al.*, 1993; Gether *et al.*, 1993), opioids (Wang *et al.*, 1994), bombesins (Fathi *et al.*, 1993) and endothelins (Takasuka *et al.*, 1994). Although the existence of galanin receptor subtypes has been proposed based on pharmacological data (Rossowski *et al.*, 1990; Bartfai *et al.*, 1993b; Wynick *et al.*, 1993), none of the putative galanin receptor subtype cDNAs has yet been isolated; hence an approach of constructing chimeric receptors based on galanin receptor subtypes is not feasible at present. The existence of radiolabelled non-peptide antagonists to several neuropeptide receptors, which often bind to sites different from peptide ligands, has been exploited widely in mutagenesis studies performed on peptide receptors. These non-peptide ligands are utilized as independent tracers to follow the expression of receptor proteins in cases where the binding of the endogenous peptide ligand under study has been lost after mutagenesis (Fong *et al.*, 1992; Gether *et al.*, 1993; Schambye *et al.*, 1994). Because of a lack of non-peptide ligands to galanin receptors, the expression and affinity of mutant receptors in our study was followed by $^{125}$I-labelled galanin and displacing non-labelled galanin, respectively. Furthermore, the immunological detection of the correct expression of total loss-of-binding mutants was required.

Despite the above-mentioned methodological limitations, we have attempted, via mutagenesis, to identify those residues of the galanin receptor which interact with the endogenous peptide ligand upon binding (Figure 1). We combined the data on the galanin receptor obtained by mutagenesis and molecular modelling with earlier data on pharmacophores of the peptide ligand, obtained by the synthesis of L-Ala-substituted galanin analogues (Land *et al.*, 1991) and [$^1$H]NMR data defining the solution conformation of the ligand (Wennerberg *et al.*, 1990; Morris *et al.*, 1995). These approaches led to the proposal of a model about how the galanin receptor binds its large, 29 amino acids long peptide ligand.

## Results

### *Expression system and biochemical experiments suggesting the importance of histidines for galanin binding*

Human galanin receptor cDNA (GR-cDNA) from the human melanoma Bowes cell line was used as a tool to examine the significance of different regions and amino acids of the receptor for the binding of the endogenous ligand galanin. The GR-cDNA was cloned into a eukaryotic expression vector pRK8, where a potent cytomegalovirus promoter/enhancer drives the transient expression of inserted cDNAs, ensuring a high level of functionally expressed protein in human fibroblast 293 cells ($\geq$500 000 galanin receptors per cell). It appears that the wild-type GR-cDNA from human Bowes melanoma, when transfected into 293 cells, directs the expression of a GR (Figure 2) with the same affinity ($K_D \approx 1$ nM) for [$^{125}$I]galanin as that measured for the GR in the Bowes cells. No specific [$^{125}$I]galanin binding was detected with non-transfected 293 cells.

The treatment of 293 cell membranes expressing the human galanin receptor with 2,4′-dibromoacetophenone (DBAP), a histidine-specific alkylating reagent, prior to the equilibrium binding of [$^{125}$I]galanin caused a total loss of [$^{125}$I]galanin binding (data not shown). The addition of $Zn^{2+}$ ions to the binding buffer inhibited [$^{125}$I]galanin binding to the wild-type human galanin receptor in a concentration-dependent manner, with an inhibition constant of 5 μM (Figure 3). This indicated that several spatially neighbouring histidines could contribute to the high-affinity binding of galanin; these histidines were the first targets in the subsequent mutagenesis study.

### *Point mutations in the putative transmembrane regions III, VI and VII*

Histidines at the top of the transmembrane domain VI (Figure 2), which might be involved in the $Zn^{2+}$ inhibition of galanin binding, were point mutated. The replacement of either His264 or His267 by alanine led to a complete loss of [$^{125}$I]galanin binding (Table I). The mutation of Glu271 of the extracellular loop III to serine resulted in a 12-fold decrease in affinity for galanin. In the putative transmembrane region VII, Phe282 and His289 were point mutated to glycine and serine, respectively (Figure 2). The latter substitution had no effect on the galanin binding (Table I), whereas the Phe282 to glycine mutation rendered the galanin receptor totally unable to bind [$^{125}$I]galanin. The substitution of His112 in transmembrane region III to glutamine did not affect the binding affinity of galanin to the galanin receptor. Table I summarizes the $K_D$ values obtained from binding experiments of [$^{125}$I]galanin to 293 cells transfected with different galanin receptor constructs in relation to the affinity of galanin for the wild-type receptor, and $B_{max}$ values as measures of expression levels.

### *Deletion mutants of the N-terminal extracellular region of the receptor*

To determine the possible role in galanin binding of the net negatively charged N-terminal extracellular domain of the galanin receptor and of the large *N*-glycosylated moieties of the galanin receptor, two galanin receptor mutants were expressed; a unique proline-rich motif containing two glutamic acids (amino acids 17–23 in the galanin receptor) was deleted and replaced with three alanines. In the other mutant, amino acids 7–15 of the galanin receptor, including both putative *N*-glycosylation sites (Asn7 and Asn12), were deleted. In the latter mutant, Glu2 of the galanin receptor was also substituted for alanine. Neither of these N-terminally mutated galanin receptors showed any significant alterations in the binding affinity of [$^{125}$I]galanin (see Table I).

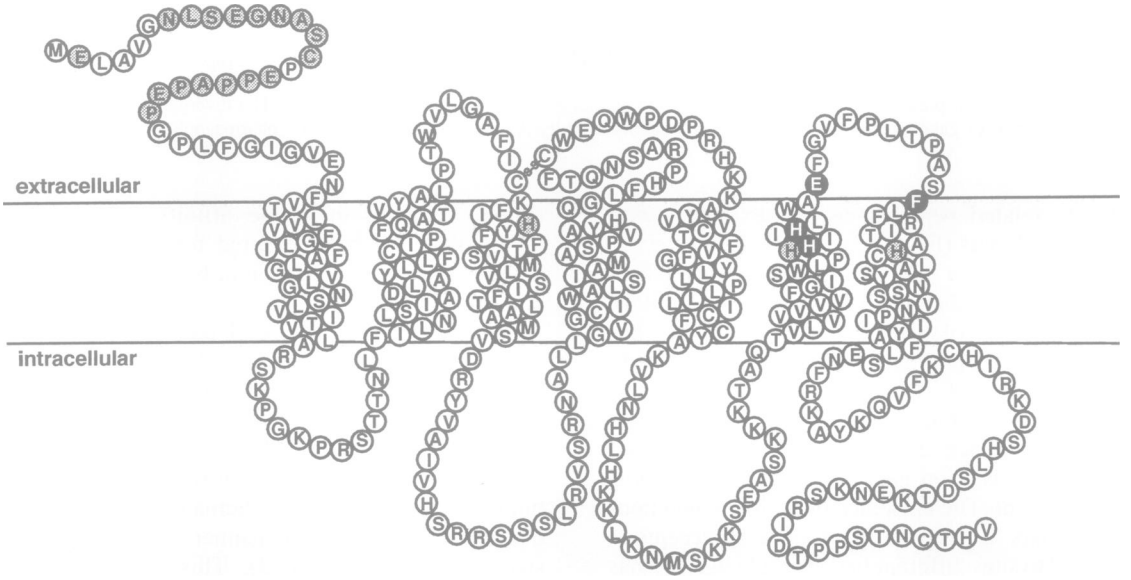



**Fig. 2.** Schematic diagram of human galanin receptor based on the cDNA sequence, showing putative transmembrane helices and the locations of amino acids mutated in this study. Mutated amino acids affecting galanin binding are in black; amino acids mutated or deleted, but having no impact on the binding affinity of galanin, are shaded.

### *Immunodetection of the loss-of-binding receptor constructs on the cell surface*

To assess whether the mutant receptors leading to a total loss of [$^{125}$I]galanin binding were indeed expressed in transfected 293 cells and correctly inserted into the plasma membrane, we used the immunofluorescence technique to evaluate the expression of mutant receptor proteins. Because specific antibodies against the human galanin receptor were not available, the FLAG™ epitope was introduced into the N-terminal portion (replacing amino acids 2–15) of the galanin receptor. The receptor construct carrying the FLAG™ tag in its N-terminus bound [$^{125}$I]-galanin upon transfection into 293 cells with the same affinity as the wild-type receptor (see Table I). It was therefore chosen for the immunological detection of all loss-of-binding GR mutants, such as the His264 to alanine, His267 to alanine and Phe282 to glycine mutants of GR. As shown in Figure 4, these GR mutants were expressed at the cell surface and the expression levels per cell varied within a 10-fold range compared with that of the wild-type FLAG™-tagged GR.

### *Molecular modelling of the interaction of galanin with the human galanin receptor*

A homology-based model of the human galanin receptor was constructed using the 3-D structure of bacteriorhodopsin as a template. The recently published low-resolution cryoelectron microscopy structure of bovine rhodopsin crystals (Schertler *et al.*, 1993; Unger and Schertler, 1995) does not provide precise coordinates to enable the positioning of individual amino acids. Instead, in our study we took into account the results of the multiple sequence alignment of members of the G protein-coupled receptor superfamily and data obtained from site-directed mutagenesis studies (Baldwin, 1993; Schwartz, 1994). This model was used in conjunction with a model of the galanin peptide conformation in solution to identify possible interactions.

CD and [$^{1}$H]NMR studies have shown that galanin

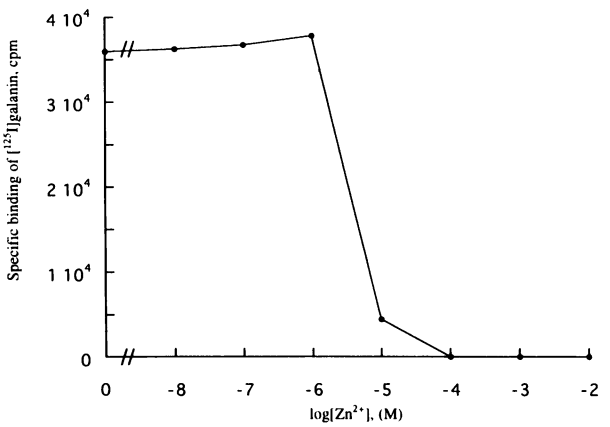



**Fig. 3.** The inhibition by $Zn^{2+}$ of the specific binding of [$^{125}$I]galanin to human galanin receptors transiently expressed in 293 cells. A representative of two independent experiments is shown. Each point is the mean of duplicates.

may adopt an α-helical conformation in trifluoroethanol (Wennerberg *et al.*, 1990), and a recent NMR study indicated that galanin-(3–11) may also adopt an α-helical conformation in aqueous solution (Morris *et al.*, 1995). Therefore, we chose to use an α-helical conformation of galanin in the modelling study in which the α-helix was slightly kinked because of *Pro13*, separating the N-terminal 1–12 fragment important for binding from the C-terminal 14–29 portion of the peptide which contributes little to interactions with the receptor, as shown by studies on galanin fragments.

We used the putative ionic or hydrogen bond interaction between the free N-terminus of *Gly1* in galanin and Glu271 of the receptor as an anchoring point, which enabled us to position the N-terminal portion of galanin relative to the receptor. In our model, galanin is located at the top of the transmembrane regions of the receptor, with the N-terminal part located close to transmembrane region VI. To achieve maximal overlap between the

**Table I.** Binding characteristics of galanin to human galanin receptor clones

| Clone No. | Region of hGR | Mutation | $K_D$ (nM) | $K_{D(mut)}/K_{D(wt)}$ | $B_{max}$ (pmol/mg protein) |
|---|---|---|---|---|---|
| 1 | wild-type hGR | – | 2.1 ± 1.7 (4) | – | 0.7 |
| 2 | N-terminal | NLSEGNASC(7–15) | – | 0.8 (2) | 0.6 |
| 3 | portion | E2A; EPPAPEP(17–23)AAA | – | 0.5 (2) | 0.5 |
| 4 | | ELAVGNLSEGNASC(2–15) DYKDDDK (FLAG™ tag) | – | 0.3 (2) | 0.7 |
| 5 | TM domain III | H112Q | – | 0.6 (2) | 0.1 |
| 6 | TM domain VI | H264A | – | >100 (3) | ND |
| 7 | | H267A | – | >100 (3) | ND |
| 8 | EC III | E271S | – | 12.5 (3) | 1.3 |
| 9 | TM domain VII | F282G | – | >100 (2) | ND |
| 10 | | H289S | – | 0.9 (2) | 0.8 |

The binding affinities of [$^{125}$I]galanin to mutant human galanin receptor clones are presented in relation to the affinity for the wild-type galanin receptor. Expression levels are calculated from the specific binding of [$^{125}$I]galanin, as determined from displacement experiments. The number of independent experiments for each clone is given in parentheses. Mutants are described by the amino acid(s) deleted or mutated, followed by the position number(s) in the wild-type receptor and by the residue or sequence inserted. Amino acids are given in the one-letter code. ND, not determined.

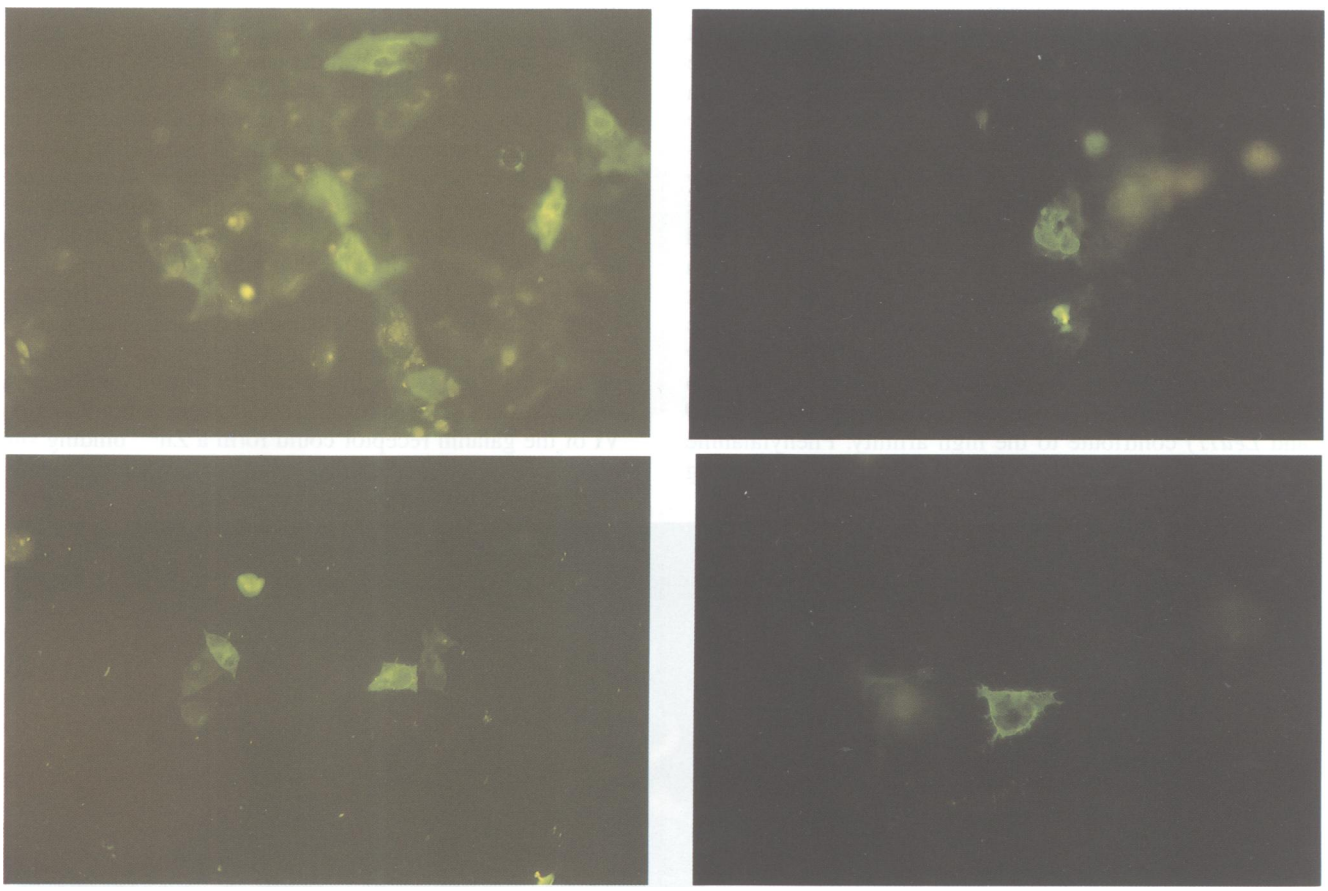


**Fig. 4.** Immunodetection of FLAG™-tagged human galanin receptor and mutants thereof, leading to an apparent loss of galanin binding on the cell surface. From top left clockwise: wild-type GR, galanin receptor mutant His264Ala, galanin receptor mutant Phe282Gly and galanin receptor mutant His267Ala.

receptor and galanin, the C-terminus of galanin was directed towards transmembrane region I and the N-terminal extracellular domain of the receptor. The proline (*Pro13*)-induced kink in the backbone of galanin directed the C-terminal part of galanin away from the transmembrane helices towards the N-terminus of the receptor (Figure 5).

Based on this orientation of galanin and on the mutagenesis data showing the importance of His264, His267 and Phe282 for galanin binding, we could identify other possible sites of interaction between the peptide and the receptor. In our model, *Trp2*, *Asn5* and *Tyr9*, which are crucial pharmacophores for galanin binding, are facing the receptor cavity. Thus, in this model *Trp2* interacts with His264 and/or His267 in transmembrane region VI. In addition, *Asn5* may interact with His112, and the mutation of His112 to glutamine did not change the affinity of the mutant receptor for [$^{125}$I]galanin. *Tyr9* of galanin could interact with Phe282 of transmembrane region VII by aromatic–aromatic interactions. His289 is

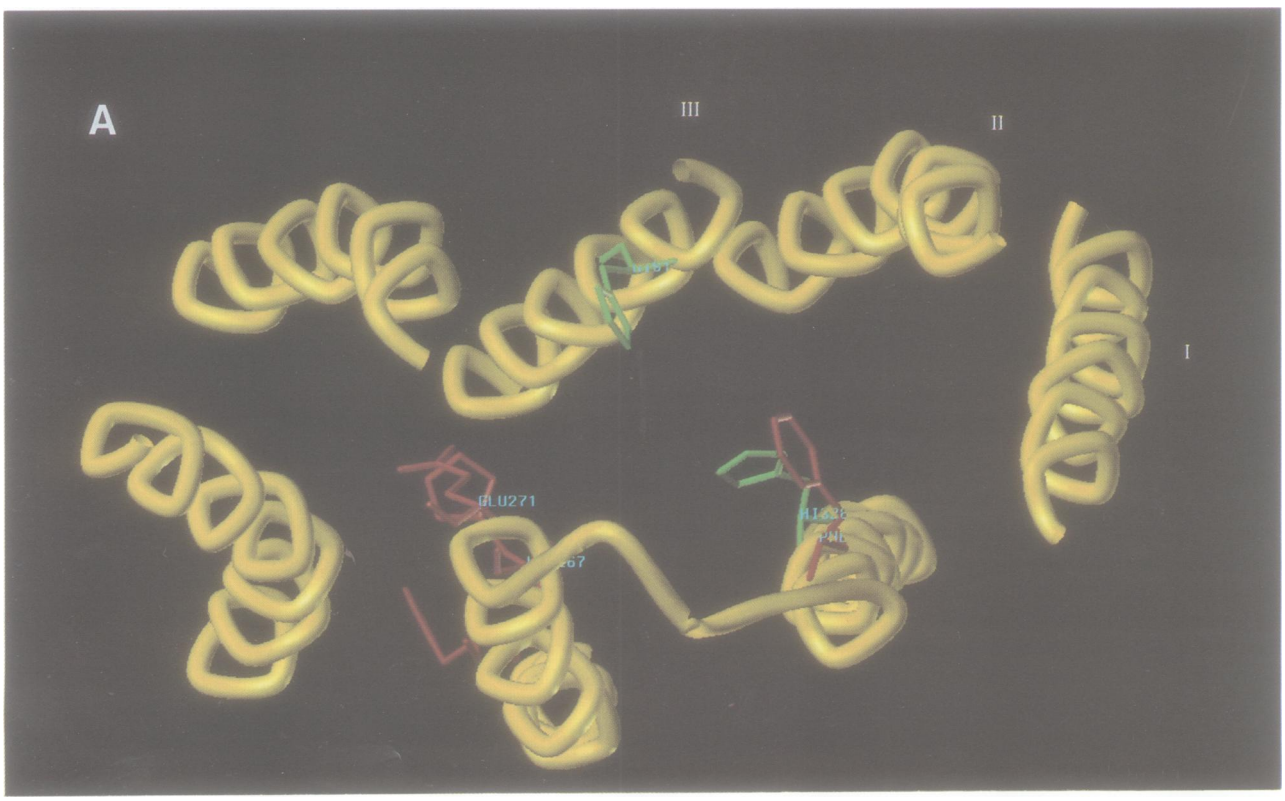
located deeper in the membrane, thus being unable to interact with the peptide, as also shown by the lack of effect on the binding affinity of [$^{125}$I]galanin when mutating His289 to serine (Figure 5).

## Discussion

Galanin exerts a variety of physiologically important effects, making the galaninergic system a potential target for therapeutic applications. Understanding the molecular basis of interactions between the galanin receptor and its endogenous ligand could greatly facilitate the screening for such compounds. Therefore, we have undertaken this study in which we attempt, using site-directed mutagenesis in combination with molecular modelling, to identify key amino acids of the galanin receptor interacting with the major pharmacophores of galanin.

In most species galanin is a 29 amino acids long peptide with an amidated C-terminus. The N-terminal first 15 amino acids are highly conserved throughout all studied species (Figure 1). There is no species difference in the affinity of human, porcine or rat galanin to human galanin receptors: all bind with a $K_D$ ~1 nM. Structure–activity relationship studies have demonstrated that the well-conserved N-terminal part of galanin is both necessary and sufficient for high-affinity binding to galanin receptors and for agonist activity (Fisone *et al.*, 1989a; Land *et al.*, 1991), whereas the C-terminal portion of galanin contributes marginally to the binding affinity but is thought to increase the proteolytic stability of the peptide (Bedecs *et al.*, 1995). The amino acids with central importance for high-affinity galanin binding are *Trp2* and *Tyr9*, but also *Asn5* and hydrophobic amino acids around *Tyr9* (*Leu10* and *Leu11*) contribute to the high affinity. Phenylalanine or tyrosine substitutions of *Trp2* diminish the binding affinity of galanin to its receptors in the rat brain, but not to the same extent as substitutions for alanine or isoleucine (Lagny-Pourmir *et al.*, 1989), indicating that interactions between aromatic and polar amino acids in the galanin–galanin receptor complex make a major contribution to the overall free energy of binding. Acetylation of the N-terminal $NH_2$ group of galanin leads to a 20- to 60-fold decrease in the binding affinity, pointing to the requirement of a free N-terminus for the high-affinity interaction with the receptor (Land *et al.*, 1991). [$^1$H]NMR studies on human galanin in aqueous solutions suggest that galanin-(3–11), i.e. a large portion of the N-terminal galanin, may adopt an α-helical structure in aqueous solution (Morris *et al.*, 1995). Therefore, when modelling the galanin binding site and docking the ligand, we assumed that galanin interacts as an α-helix with its receptor.

Because of methodological limitations, we could not use chimeric receptors or 'segment exchange' approaches to draw a rough map confining amino acids important for galanin binding to certain areas of the receptor. Instead, to select a starting point for mutagenesis studies, preliminary experiments using DBAP, a histidine-specific alkylating agent, were performed to assess the importance of several histidines in transmembrane regions VI and VII for galanin binding, because in various studies on other neuropeptide receptors histidines in these regions and in transmembrane region V have been demonstrated to be involved in both peptide and non-peptide ligand binding (Fong *et al.*, 1993; Gether *et al.*, 1993; Schambye *et al.*, 1995). Because incubation with DBAP abolished all galanin binding (data not shown), we assumed that histidines in the human galanin receptor were important for ligand binding. A unique stretch of three histidines in transmembrane region VI of the galanin receptor could form a $Zn^{2+}$ binding site because zinc ions inhibit galanin binding to transiently



expressed human galanin receptors with an inhibition constant of ~5 μM (Figure 3). Thus, one or several spatially adjacent histidines of the receptor are involved in galanin binding, similar to the findings on mutated neurokinin I receptors (Elling *et al.*, 1995). The assessment of the importance of these histidines for galanin binding was the starting point for our mutagenesis studies. The substitution of His264 or His267 for alanines resulted in a total loss of galanin binding to the mutant receptors (Table I). Because all mutant receptor constructs leading to a complete loss of galanin binding were expressed at about the same level as the wild-type receptor in 293 cells, and also inserted into the plasma membrane as shown by immunofluorescence techniques (Figure 4), we conclude that these mutations indeed affect galanin binding. The importance of these single amino acid mutations suggests that His264 and/or His267, both of which point towards the interior of the receptor according to our molecular modelling study (Figure 5), might interact with *Trp2*, the most important pharmacophore in galanin (Land *et al.*, 1991). The involvement of tryptophan in the receptor–ligand interactions is not unusual; two tryptophans were pinpointed as the crucial amino acids involved in the ligand–receptor interaction in a recent study on the human growth factor/growth factor receptor, combining mutagenesis and X-ray crystallography (Clackson and Wells, 1995). It is conceivable that strong interactions between the *Trp2* of galanin and the histidines in positions 264 and/or 267 of the GR also correspond to the majority of the free energy of binding for this ligand–receptor pair. Assuming that the *Trp2* of galanin interacts with histidines in transmembrane region VI of the receptor, a negatively charged amino acid or a residue able to participate in hydrogen bonding in the receptor, thereby docking the free N-terminus of galanin, should reside in close proximity to His264 and His267. A mutation of the only possible candidate (Glu271 of the extracellular loop III) to serine causes a 12-fold drop in the affinity of galanin to the receptor. This affinity could correspond to a loss of free energy of binding compatible with the loss of one hydrogen bond between Glu271 of the receptor and the N-terminus of galanin (Table I). Assuming that the docking site for

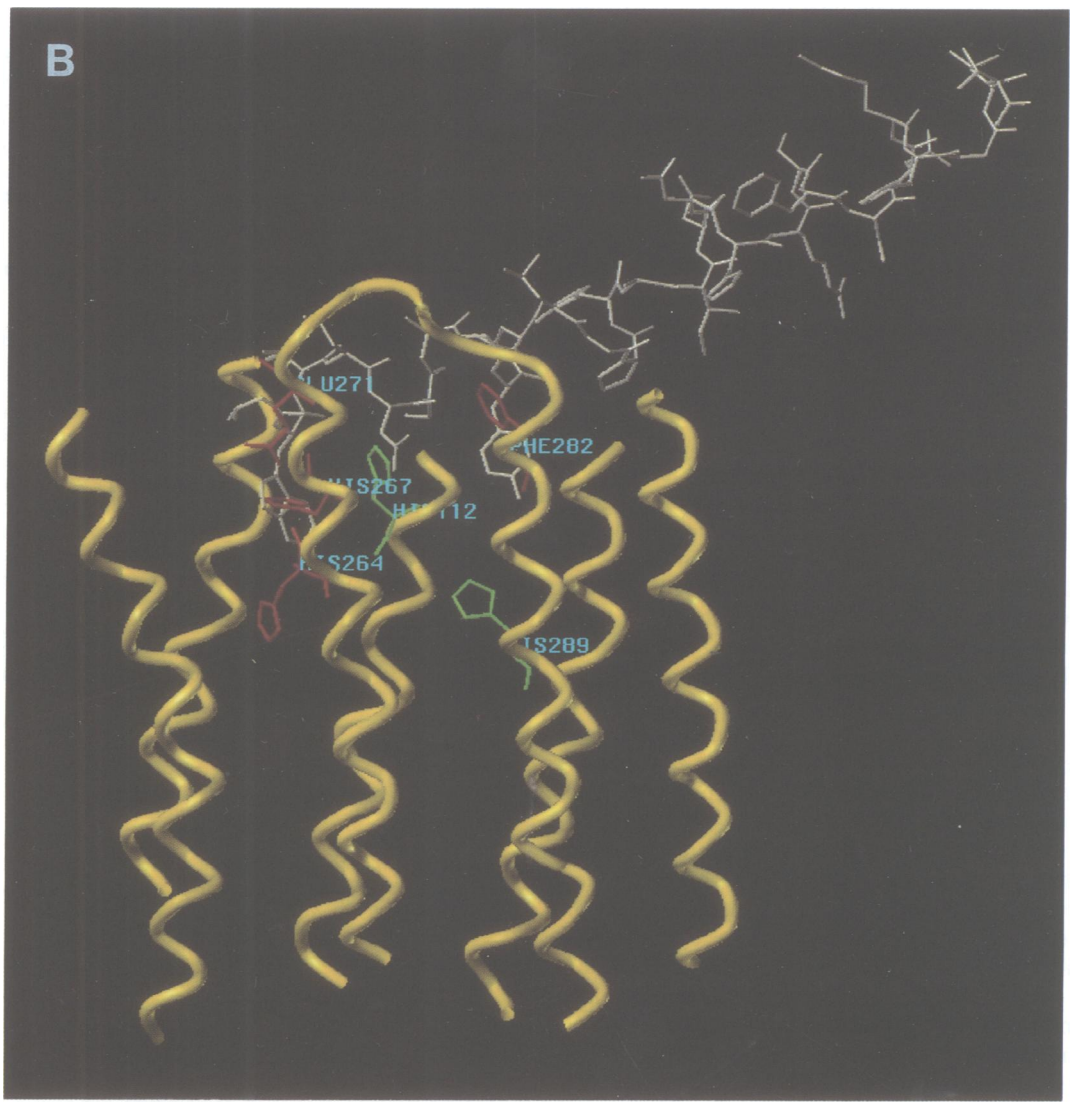



**Fig. 5.** Model of the human galanin receptor (top view, **A**) and of the galanin–galanin receptor complex (lateral view, **B**). Amino acid residues found to be important for galanin binding are in red; amino acids oriented towards the interior of the receptor surface, but not essential for galanin binding as shown by mutagenesis, are in green. Note the interactions between His264, His267 and *Trp2* of galanin, Phe282 and *Tyr9* of galanin, and Glu271 and the N-terminus of galanin, respectively.

the first two amino acids of the ligand in the receptor consists of Glu271, His264 and/or His267, the orientation of galanin relative to the receptor can be predicted. The free energy contribution to high-affinity galanin binding of *Tyr9* and the two hydrophobic amino acids (*Leu10* and *Leu11*) next to it, suggests that galanin has to bind the receptor surface in a manner enabling interactions between *Tyr9*, *Leu10* and *Leu11* and amino acids with aromatic or polar side chains in a hydrophobic milieu. The orientation of galanin in all other directions but towards transmembrane regions VII and I and the N-terminal extracellular part of the galanin receptor would place *Tyr9* of galanin outside the receptor surface. Molecular modelling shows that the most likely region in the receptor providing these interactions is transmembrane VII, where *Tyr9* could interact with His289 or Phe282. However, the mutation of His289 to serine did not alter the ability of the receptor to bind galanin, whereas the substitution of Phe282 for glycine yielded a mutant receptor which was expressed at the same level as the wild-type construct (Figure 4) but had lost the ability to bind galanin. Hence, it is very likely that the second most important pharmacophore in galanin, *Tyr9*, interacts with the Phe282 of the human galanin receptor. Mutation of the bulky phenylalanine to a small glycine could affect the receptor conformation required for ligand binding, but because the molecular modelling indicates that Phe282 resides at the very top of transmembrane region VII, we believe that the loss of binding in this case is caused by a disruption of the aromatic–aromatic interactions rather than by changes in the overall conformation around amino acid Phe282. It should be noted that all amino acids mutated in this study reside on the top of transmembrane domains or close to the membrane surface where the peptide ligand is expected to bind; thus the 7TM conformation of the galanin receptor is not likely to be altered to an extent where the interaction with G proteins, and thereby signal transduction, would be affected. Although His112 of transmembrane region III has, because of its position in the receptor, a great potential to interact with *Asn5* of galanin, the mutagenesis (H112Q) did not affect the affinity of galanin to the receptor. The deletion of different stretches of the N-terminal extracellular domain of the receptor, containing the two putative *N*-glycosylation sites (Asn7 and Asn12), did not affect the binding affinity of galanin, although the native receptor is glycosylated, carrying 13–14 kDa glycosylation as calculated by the difference between the SDS gel-determined molecular mass (Lorinet *et al.*, 1994) and the primary structure of the receptor known from its cDNA sequence (Habert-Ortoli *et al.*, 1994).

In summary, combining mutagenesis and modelling data on the receptor with data on conformation and pharmacophores of the ligand, we propose a galanin binding site model of the human galanin receptor (Figure 5). The N-terminal part of galanin, which was shown earlier to be necessary and sufficient for high-affinity galanin binding, enters the area of the receptor between the transmembrane domains VI and VII, where *Trp2* interacts with His264 and/or His267 of transmembrane domain VI, *Tyr9* makes contact with the Phe282 of transmembrane domain VII and the positively charged free N-terminus of galanin is attracted to Glu271. The C-terminal part of galanin may contribute marginally to the binding affinity via interactions with the N-terminal extracellular region of the receptor, but this contribution is indeed very small; two relatively large deletions made in the N-terminal part of the galanin receptor, which had no effect on the binding affinity of galanin, show that this region does not contribute to any detectable extent to ligand recognition in the human galanin receptor in Bowes cells (Table I).

The binding site of the galanin receptor for its endogenous ligand of 29 amino acids accommodates the N-terminal half up to *Tyr9* and possibly *Leu10* and *Leu11*. This peptide binding site is considerably larger than those described in 7TM receptors binding monoaminergic ligands. The binding site provides at least three ligand–receptor interactions, Trp–His, Tyr–Phe and $NH_2$ group–Glu, which taken together may account for the nanomolar dissociation constant of the peptide–receptor interaction.

## Materials and methods

### Materials

All enzymes were purchased from New England Biolabs, with the exception of Pfu polymerase which was obtained from Stratagene. [$^{125}$I]Galanin (porcine and human) and [$^{35}$S]dATP were purchased from NEN. Oligonucleotide synthesis was carried out on a Gene Assembler (Pharmacia), using reagents purchased from Pharmacia and Sigma. All other reagents were bought from Sigma. Porcine and human galanin was synthesized and kindly provided by Dr Ülo Langel (Stockholm University, Stockholm, Sweden) (Figure 1). The mammalian expression vector, pRK8, was kindly provided by Prof. P.H.Seeburg (Heidelberg, Germany).

### Cloning of human galanin receptor cDNA and mutagenesis of the receptor cDNA

The human galanin receptor cDNA from human Bowes melanoma cell line was PCR-cloned and inserted into the *Eco*RI and *Not*I cleaved mammalian expression vector pRK8 (a derivative of pRK5; Schall *et al.*, 1990).

All single amino acid mutations were introduced by the PCR overlap technique using two sets of primers on plasmid carrying the human galanin receptor, yielding overlapping intermediate products and in most cases also embodying a diagnostic cleavage site to facilitate the screening of recombinants. After the final round of PCR with external primers, an extended PCR product carrying the mutation was double digested with either *Bst*XI–*Not*I or *Ppu*MI–*Not*I. The cleavage product corresponding to the fragment of the expected size was excised and eluted from the agarose gel using a Jetsorb kit (Genomed). After ligation into the vector and transformation into *Escherichia coli* strain DH5α, recombinants were selected by restriction analysis. Plasmids corresponding to the correct mutants were purified by an alkali lysis method using a Qiagen kit (Qiagen).

Two N-terminal deletion mutants were made by cleaving out the fragments with *Eco*RI–*Bsp*EI and *Bsp*EI–*Xma*I, respectively. To ensure the integrity of the open reading frame, religation was performed with the help of short oligonucleotide adapters also containing diagnostic cleavage sites.

All mutations were verified by sequencing around the expected positions of mutations using cyclic sequencing (Cyclist kit; Stratagene).

As the N-terminal extracellular part was found not to be important for binding, the FLAG™ epitope (AspTyrLysAspAspAspAspLys; IBI) was introduced into the N-terminus of the receptor, replacing amino acids 2–15, to confirm by immunodetection the correct expression and presence in the plasma membrane of mutant receptor constructs leading to a complete loss of binding.

### Transient expression of human galanin receptor cDNA constructs

For the transient expression of wild-type and mutant human galanin receptor constructs, human kidney fibroblast 293 cells (ATCC) were grown to 50% confluency in minimal essential medium (Gibco) [with Earle's salts, 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin] in 5% (v/v) $CO_2$-enriched air at

37°C. For binding studies, 2–5 μg plasmid DNA per dish were transfected by calcium phosphate precipitation. For immunodetection, 0.1 μg plasmid DNA per chamber were transfected to cells grown on chamber slides (Nunc). In both cases, cells were used in binding studies 40–48 h after transfection.

### *Binding experiments*

The transfected 293 fibroblast cells were harvested in ice-cold 5 mM HEPES binding (HBB) buffer containing 2.5 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.3), and centrifuged at 400 *g* for 10 min at 4°C. The pellet was resuspended in HBB supplemented with 0.05% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) bacitracin. To assess the effect of the histidine-specific modifying reagent DBAP on the galanin binding (Fisone *et al.*, 1989b), the membranes were pretreated prior to binding with 1 mM DBAP for 30 min at 37°C, and subsequently washed before the binding experiment using $^{125}$I-labelled porcine galanin. There was no detectable difference in the binding affinities of either porcine or human galanin to the recombinant human galanin receptor. Therefore porcine galanin was used as both the tracer and displacer throughout the binding studies. The equilibrium binding experiments were performed in a final volume of 400 μl HBB containing 0.1 nM [$^{125}$I]galanin (NEN), transfected 293 cell membranes and increasing concentrations of non-labelled galanin ($10^{-11}$–$10^{-6}$ M). Samples were incubated for 30 min at 37°C in a shaking water bath. The incubation was terminated by the addition of 2×10 ml ice-cold HBB, followed by rapid filtration over Whatman GF/C glass fibre filters presoaked for 2–3 h in 0.3% (v/v) polyethylenimine solution. Radioactivity retained on the filters was determined in a Packard gamma counter. The specific binding amounted to at least 90% of the total binding. To determine the effect of $Zn^{2+}$ on galanin binding, varying concentrations of $ZnCl_2$ ($10^{-8}$–$10^{-2}$ M) were added to Tris-buffered equilibrium binding mixtures (no BSA added). Protein was assayed according to Peterson (1977). All tubes and pipette tips were coated with Sigmacote (Sigma). $IC_{50}$ and $K_D$ values were calculated using a non-linear regression analysis program of the Kaleidagraph software package on a Macintosh computer.

### *Immunodetection of constructs bearing the FLAG™ epitope*

The transfected cells grown on chamber slides were washed repeatedly with PBS at room temperature, fixed with 2% formaldehyde, washed with PBS, incubated for 1 h at room temperature with anti-FLAG™ M2 antibody (IBI; 5 μg/ml) and washed several times again with PBS. Incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse monoclonal IgG1 antibodies (Caltag) was carried out for 30 min at room temperature. After the final washing step, cells were treated with anti-fading reagent. Slides were mounted and photographed under ×500 magnification using a fluorescent microscope (Zeiss).

### *Molecular modelling of the galanin receptor*

The galanin receptor model was constructed using Sybyl 6.1 (Tripos Associates Inc., St Louis, MO). The amino acid sequence of the human galanin receptor (Habert-Ortoli *et al.*, 1994) and experimental coordinates of bacteriorhodopsin (Henderson *et al.*, 1990) were used to construct a model of the human galanin receptor similar to a strategy described previously for the muscarinic m1 receptor (Nordvall and Hacksell, 1993). In brief, the models are based on a presumed homology in 3-D structure between bacteriorhodopsin and the G protein-coupled receptors. α-Helices were constructed from the primary structure of the galanin receptor. Transmembrane regions were built upon hydropathy plots and multiple sequence alignments of a number of G protein-coupled receptors. The relative rotations of the helices were estimated by considering conserved amino acids and hydrophobic moment plots. The α-helices ($\phi$ = –55.02 and $\psi$ = –50.43) were constructed from the amino acid sequences of the transmembrane regions, and proline kinks were considered. Side-chain conformations from rotamer libraries were used. Fitting of the backbone of these helices onto the backbone of bacteriorhodopsin produced the transmembrane bundle of the receptor. Only the extracellular loop between transmembrane regions VI and VII was included in the modelling. This loop was constructed using the Loop Search feature in Sybyl. The side chains were adjusted manually to avoid any overlap produced by the fitting procedure. The search for possible interaction points between galanin and its receptor was carried out manually in Sybyl.

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