

Determining receptor–ligand interaction of human galanin receptor type 3

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ABSTRACT

Galanin is a neuropeptide found throughout the central and peripheral nervous systems of a wide range of species, ranging from human and mouse to frog and tuna. Galanin mediates its physiological roles through three receptors (GalR1–3), all members of the G-protein coupled receptor family. In mapping these roles, receptor subtype selective ligands are crucial tools. To facilitate the ligand design, data on receptor structure and interaction points are of great importance. The current study investigates the mechanism by which galanin interacts with GalR3. Mutated receptors were tested with competitive binding analysis *in vitro*. Our studies identify six mutagenic constructs that lost receptor affinity completely, despite being expressed at the cell surface. Mutations of the Tyr103^{3.33} in transmembrane helix (TM) III, His251^{6.51} in TM VI, Arg273^{7.35} or His277^{7.39} in TM VII, Phe263^{6.63} or Tyr270^{7.32} in the extracellular loop III all result in complete reduction of ligand binding. In addition, docking studies of an *in silico* model of GalR3 propose that four of the identified residues interact with pharmacophores situated within the galanin(2–6) sequence. This study provides novel insights into the interaction between ligands and GalR3 and highlights the requirement for correct design of targeting ligands.

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1. Introduction

In 1983 Viktor Mutt and co-workers identified a novel 29 amino acid peptide in pig intestines named galanin after its N-terminal glycine and C-terminal alanine (Tatemoto et al., 1983). Later galanin has been identified in several species and is shown to be widely distributed in both the central and peripheral nervous systems, as well as in the endocrine system. Galanin is involved in a variety of physiological mechanisms and disease states ranging from appetite and neuroregeneration to seizures and pain, for review see (Lang et al., 2007; Lundström et al., 2005; Runesson et al., 2009b). Three receptors have been identified for the ligand, galanin receptor type 1, 2 and 3 (GalR1–3), all of which are 7-transmembrane G-protein coupled receptors (GPCR). In signaling, GalR1 and GalR3 act mainly via $G_{i/o}$, while GalR2 has $G_{q/11}$ as a major signaling route. The three galanin receptor subtypes have low sequence homology to each other. Sequence identity of human (h) GalR3 versus hGalR1 and hGalR2 is 40% and 64% respectively (for alignment of the 289 residues spanning transmembrane segment (TM) I–TM VII), while the homology of the same receptor in different species is much higher, e.g. between human and rat,

91%, 88% and 88% for receptors 1, 2 and 3 respectively (whole sequences).

Two decades after its discovery, questions still remain about the role of each individual receptor, as sufficient pharmacological tools for adequate mapping of the individual receptors functions are lacking. Consequently, identification of the receptor's binding pocket and important pharmacophores herein are of importance, since this knowledge would greatly facilitate the future design of subtype selective GalR ligands. So far, GalR1 and GalR2 are the most studied of the three receptors, mainly due to availability of transgenic animals and stably transfected cell lines, and a broader distribution pattern in the nervous system. However, since Konkelt et al. presented 1,3-dihydro-1-[3-(2-pyrrolidinylethoxy)phenyl]-3-[[3-(trifluoromethyl)phenyl]imino]-2H-indol-2-one (hereafter referred to as SNAP398299) (Konkelt et al., 2006), a non-peptidergic antagonist towards GalR3, this receptor has gained increased interest, as SNAP398299 has been successfully used as an antidepressant in rodent models of depression (Swanson et al., 2005) and ligands targeting GalR3 are promising drug candidates.

Site-directed mutagenesis studies have been performed on both hGalR1 and hGalR2 to map the binding pocket (Kask et al., 1996; Berthold et al., 1997; Kask et al., 1998; Lundström et al., 2007), and a three-dimensional model of hGalR1 has been presented by Iismaa and co-workers (Church et al., 2002). Altogether these reports indicate that TM helices III, VI and VII together with extracellular loop (ECL) II and III form the binding pocket of

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hGalR1, whereas only TM VI, TM VII and ECL III appear to be involved in galanin binding to hGalR2. Based on the major observations made in these preceding mutagenesis reports on hGalR1 and hGalR2, we here present a study on the receptor–ligand interactions for hGalR3. Here, we have performed site-directed mutagenesis of hGalR3 to elucidate the importance of specific residues corresponding to residues found in GalR1 and GalR2, to be vital for galanin binding. Further, characterization of the ligand binding site was performed by utilizing the subtype selective ligand SNAP398299 (Konkel et al., 2006).

Additionally, *in silico* docking models of the wildtype (wt) hGalR3 and L-Ala-mutated hGalR3 was done in attempt to further explain the binding results. Possible interaction between hGalR3 and the ligand SNAP398299 and a shorter galanin fragment, galanin(2–6), were identified. Hence, the data provide information on receptor–ligand interaction of hGalR3, knowledge that is of importance in future design of ligands with high affinity and specificity.

2. Materials and methods

2.1. The hGalR3 construct and generation of hGalR3 mutants

The human galanin receptor type 3 inserted into pcDNA3.1⁺ plasmid was purchased from Missouri University of Science and Technology cDNA Resource Center (MO, U.S.A.). All mutations, including addition of the FLAG-tag, was performed using the QuikChange[®] II Site Directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions with the addition of 8% DMSO to the reactions. Primers for the mutagenesis were designed using Stratagenes QuikChange[®] Primer Design Program and synthesized by Eurofins MWG Operon (Ebersberg, Germany). To be able to monitor the receptor a FLAG-tag (DYKDDDDK) was introduced at the N-terminal end of the receptor sequence. Plasmids were prepared using QIAGEN Plasmid Maxi/Midi kits (Hilden, Germany) and correct sequence was verified by Eurofins MWG Operon.

2.2. Cell culture, transfection of hGalR3 constructs and membrane preparation

HEK293T cells were cultured in Dulbecco's modified essential media with Glutamax I supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin and kept in a 37 °C, 5% CO₂, humidified environment. Cell culture reagents were all from Invitrogen (Stockholm, Sweden) and cell plastics from Greiner Bio-One GmbH (Frickenhausen, Germany). The cells were seeded in 150 mm Petri dishes 24 h prior to transfection with linear polyethyleneimine, MW 25,000 (Polysciences Inc, PA, U.S.A.) according to the protocol of CELLnTEC Advanced Cell Systems (Bern, Switzerland). After an additional 48 h membranes were prepared as follows; the cells were trypsinated and transferred to a centrifuge tube. The cells were centrifuged for 1000 × g at 4 °C for 10 min, washed with cold phosphate buffered saline (PBS) and centrifuged again. The pellet was then suspended in cold HM-EDTA buffer (25 mM HEPES, 5 mM MgCl₂, 10 mM EDTA, pH 7.4) and was allowed to lyse for 45 min on ice. The cell lysates were centrifuged at 45,000 × g for 30 min at 4 °C. The supernatant was discarded and replaced by cold assay buffer (25 mM HEPES, 5 mM MgCl₂, pH 7.4) and centrifuged again at 45,000 × g for 30 min at 4 °C. The pellet was resuspended in cold assay buffer with the addition of 1% protease inhibitor cocktail (Sigma Aldrich, St. Louis, U.S.A.), the cell lysate was separated with a 27-gauge needle and frozen in aliquots at –80 °C until use.

2.3. Ligands: rat galanin, galanin(2–11) and SNAP398299

Rat galanin (amino acid sequence: GWTLNSAGYLLGPHAIDNHRFSFDKHLGT amide) and galanin(2–11) (amino acid sequence: WTLNSAGYLL amide) were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis as described previously (Runesson et al., 2009a). SNAP398299 was a kind gift from Lundbeck Research U.S.A., Inc. Paramus, NJ, U.S.A. (Konkel et al., 2006).

2.4. Confocal microscopy

To monitor the expression of FLAG-labeled hGalR3 receptor mutants, HEK293T cells were seeded on coverslips in 12-well plates and transfected as described above. 48 h after transfection, the cells were washed three times with PBS, fixed with 4% *p*-formaldehyde in PBS for 10 min on ice and washed two times with PBS. Permeabilization was performed in 0.5% Triton X-100 in PBS for 10 min at RT followed by washing with PBS. Non-specific binding was blocked in 5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. The cells were washed in PBS once before incubated with primary antibody; anti-FLAG (Sigma–Aldrich, St. Louis, U.S.A.) diluted 1:1000 in 5% BSA in PBS for 1 h. Thereafter, the cells were washed 2 × 10 min in 0.1% Tween-20 in PBS. Rhodamine red-labeled goat anti-mouse secondary antibody (Molecular Probes, Stockholm, Sweden) was added at a dilution of 1:250 in 5% BSA in PBS for 1 h followed by two washes with 0.1% Tween-20 in PBS

for 10 min each and one wash with PBS. Finally, the cells were washed in H₂O and mounted on slides. Fluorescently stained cells were examined using a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a Leica 40× objective illuminated by an argon laser. Images were recorded and stored using Leica LCS software.

2.5. Competitive binding studies

Ligand binding studies at the galanin receptor mutants were assessed by equilibrium binding on cell membranes harvested 48 h after transient plasmid transfection. Equilibrium binding experiments were performed with 0.15 nmol/L [¹²⁵I]-porcine galanin (2200 Ci/mmol; PerkinElmer Life Science, Boston, MA, U.S.A.) and 35 µg membrane preparation in a total volume of 200 µL assay buffer, containing various concentrations of peptide (10^{–5} to 10^{–11} mol/L) in the presence of 0.3% (w/v) BSA. The binding reaction was incubated for 30 min at 37 °C while shaking and terminated by rapid filtration over glass fiber filters (Millipore MultiScreen Assay System; Millipore, Stockholm, Sweden) pre-soaked in 0.3% polyethyleneimine solution. Filters were washed with assay buffer and the remaining radioactivity was counted in a β-counter (Packard Instrument Company, Meriden, CT, U.S.A.) using SuperMix cocktail scintillation liquid (PerkinElmer Life Sciences, Boston, MA, U.S.A.). EC₅₀ values were calculated using Prism 5 (GraphPad Software Inc., San Diego, CA, U.S.A.) and converted into K_i values using the equation of Cheng–Prusoff with a K_D value for the radioligand of 2.23 nM (Borowsky et al., 1998).

2.6. Protein templates for homology modeling

The crystal structures of turkey beta-1 Adrenergic Receptor (PDB id 2VT4) (Warne et al., 2008), human beta-2 Adrenergic Receptor (PDB id 2RH1) (Cherezov et al., 2007), and squid Rhodopsin from *Todarodes pacificus* (PDB id 2Z73) (Murakami and Kouyama, 2008) were chosen as templates for the homology model. The proteins in all three crystal structures belong to the class A rhodopsin like family within the GPCR superfamily. GPCRs are characterized by seven α-helical transmembrane helices (Probst et al., 1992). The sequence identity for the alignment of hGalR3 to 2VT4, 2RH1 or 2Z73 was 24%, 26%, and 23%, respectively. The structure files of the templates were obtained from the PDB website (Berman et al., 2000). Homology prediction of TM regions was performed using the Alignment Explorer program (Van Durme et al., 2006). Below, amino acid residues are designated both by their positions in the hGalR3 sequence and the two-number identifier of Ballesteros and Weinstein (1995) appearing as a superscript.

2.7. Sequence alignment and topology prediction

A multiple sequence alignment between hGalR3 and the three templates were created using Kalign 2.0 (Lassmann and Sonnhammer, 2005), a method employing the Wu–Manber string-matching algorithm. TMHMM 2.0 (Krogh et al., 2001) was used to obtain a transmembrane topology prediction for hGalR3. In the alignment, the predicted transmembrane segments and the loop regions of hGalR3 overlapped with the seven α-helices found in the crystal structures of the templates. In a second step the boundaries of the transmembrane segments predicted by TMHMM 2.0 were used to adjust the alignments by manually aligning the start of predicted transmembrane segments and reducing gaps in the middle of these segments. Further, additional factors suggested to be involved in important structural and functional features among GPCR sequences, such as conserved cysteine residues and the DRY motif, were incorporated into the manual alignment.

2.8. Homology modeling

Three-dimensional models for hGalR3 sequence were constructed using MODELLER9v1 (Sali and Blundell, 1993). Initially, 5 models were constructed based on the multiple sequence alignments generated by Kalign 2.0. From these a preliminary model was chosen based on model quality scorings: the lowest DOPE (Discrete Optimized Protein Energy) statistical potential (Shen and Sali, 2006) and the highest GA341 score, which combines statistical potential function, target–template sequence identity and a measure of structural compactness (Eswar et al., 2003; Melo et al., 2002; Melo and Sali, 2007). The model was then visually inspected with a focus on the location and orientation of residues corresponding to residues identified as important in ligand binding in hGalR2 (Lundström et al., 2007). This model then underwent loop refinement of ECL I, II, III and the N-termini. Loop conformations were selected by clustering and visual inspection. Energy profiles were created for the loops and loops with the lowest energies were chosen for the final model. For this study, hGalR3 models with and without disulphide bonds between TM II and ECL III has been tested, the latter one to keep the binding cavity open and maximize the ligand binding property.

2.9. Docking of ligands

All calculations in this section were done with the Schrödinger suite of programs (Schrödinger Inc., Mannheim, Germany). The ligands were constructed and optimized using the Maestro program. Due to computational complexity flexible ligand docking is limited to 32 rotatable bonds. When selecting all σ–σ and π–σ bonds as rotatable, this limit is reached by just five amino acids in galanin. Given this limitation

galanin(2–6) was used for the docking studies, as this is the five residue long galanin fragment that retain most of the interactions identified in earlier studies. The first glycine moiety of the galanin sequence is of minor importance in binding, at least to hGalR2 (Wang et al., 1997) and an α -alanine-scan of galanin(2–11) display that two of the pharmacophores for binding of galanin(2–11) to GalR2 is situated inside galanin(2–6) (Lundström et al., 2005). Therefore a five amino acid long galanin fragment (WTLSN), starting from the second position, was chosen for the docking studies. The SNAP398299 synthetic compound was also used (Konkel et al., 2006).

Molecular docking of the ligands into the receptor structure was done using Glide. The Protein Preparation Wizard was used to add missing hydrogen atoms and to optimize hydrogen bond network of the protein. Protein structure was optimized with Macro Model (OPLS2005 force field). Intermolecular interactions were pre-calculated on the grid representing extracellular half of receptor and centered on selected residues in the binding site. Receptor flexibility was derived by in place temporary alanine mutations and van der Waals (vdW) radii scaling. Finally, fully flexible ligands were subsequently docked onto the grid and the potential energy of complexes was minimized.

Analysis of the docking results was based on three factors: estimation of ligand binding energy, contact analysis, and clustering of docked poses. First, RMSD clustering was employed to select representatives of each cluster and narrow down the number of possible solutions. Secondly, Glide Score and E-model scoring functions were applied to rank all poses. Glide Score is an empirically derived scoring function which combines lipophilicity, hydrogen bonding, ionic interactions, vdW interactions and solvation terms, each term is a weighted sum of function based on nominal optimal distances typical for each interaction type (Friesner et al., 2004). E-model is the sum of the Glide Score, ligand–receptor molecular mechanics interaction energy and the ligand strain energy (Friesner et al., 2004). Presence of ligand contacts with conserved residues found as important in GalR1 and GalR2 studies (Berthold et al., 1997; Kask et al., 1998; Church et al., 2002; Lundström et al., 2007) was additionally accounted. Low energy clusters with members showing preference in binding to these residues were preferred. In the contact analysis, two residues were assumed to be in contact if the atomic distances were below 3.0 Å cutoff.

3. Results

3.1. Expression and displacement studies of the FLAG-labeled hGalR3

After transfection, expression of the receptor on the plasma membrane of HEK293T cells was confirmed using immunostaining

and detection of the FLAG epitope. In Fig. 1 (upper middle panel) we show that there is a clear expression of receptor in cells transfected with the FLAG-labeled wild type receptor. Little or no receptor was detectable in untransfected cells (Fig. 1; upper left panel). We pursued by showing that the introduction of a C-terminal FLAG epitope does not alter the affinity of the receptor towards either rat galanin, galanin(2–11) or SNAP398299. The affinity of galanin towards hGalR3-wt and FLAG-hGalR3 was 4.1 and 5.1 nM, respectively (Table 1, Fig. 2). The affinity of the two ligands galanin(2–11) and SNAP398299 were somewhat higher, with a K_i ranging from 200 to 300 nM (Table 1), which is consistent to what is previously published for galanin(2–11) (Lu et al., 2005; Runesson et al., 2009a).

3.2. Displacement of ligands at selected hGalR3 mutants

The mutated amino acids were selected based on comparison between the galanin receptor subtypes. Sequence alignment was used and also results from previous mutagenesis studies of hGalR1 and hGalR2. Several conserved residues throughout the three receptors have been shown to be important in hGalR1 and hGalR2; these include residues Phe^{3.33}, His^{6.51}, Phe^{6.63}, and Tyr^{7.32}. Two residues of GalR1 reported not to interact with galanin, one residue in ECL III (Arg^{7.35}) and one residue in TM VII (His^{7.39}), was also analyzed to identify potential differences between receptor subtypes. To further investigate the influence of the upper part of TM VII a Leu278^{7.41}Ala hGalR3 construct was also produced. In total, seven mutations were done in GalR3; namely Tyr103^{3.33}Ala, His251^{6.51}Ala, Phe263^{6.63}Ala, Tyr270^{7.32}Ala, Arg273^{7.35}Ala, His277^{7.39}Ala and Leu278^{7.41}Ala (Fig. 3).

All mutated receptor constructs were expressed in the plasma membrane (Fig. 1). Six of the mutated receptors completely lost its ligand affinity and showed no detectable binding with either galanin

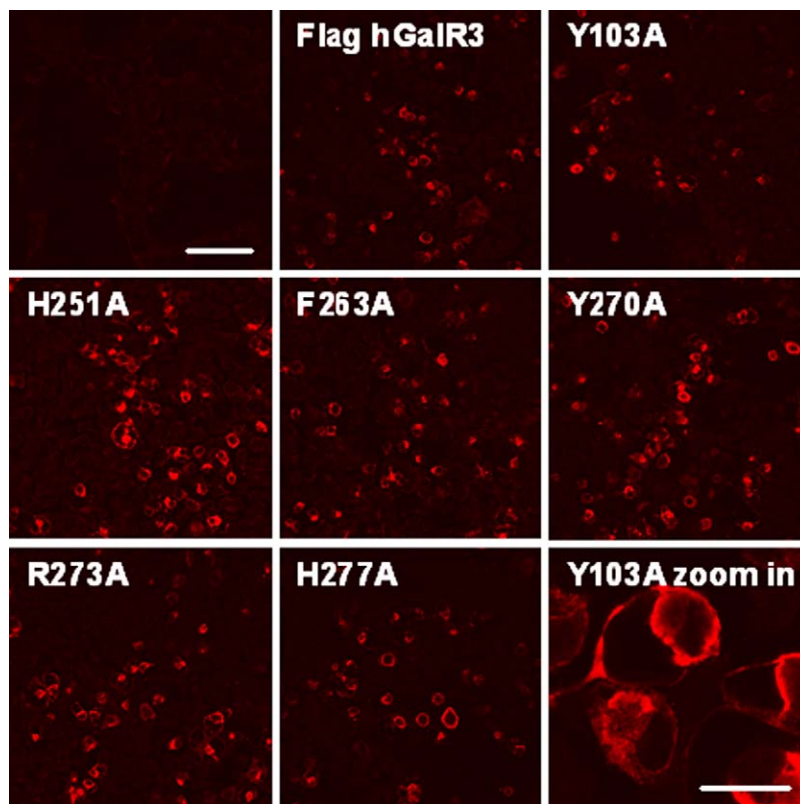


Fig. 1. Confocal microscopy images displaying membrane expression of untransfected HEK293T cells, wt-hGalR3, FLAG-labeled hGalR3 and FLAG-labeled hGalR3 mutants on HEK293T cells. A secondary antibody with rhodamine labeling towards the FLAG-labeling is used. In the zoom-in frame you can see that the receptor is expressed on both the nuclear and cellular membranes. Bar = 50 μ m (Y103^{3.33}A zoom in = 10 μ m).

Table 1

K_i values for rat galanin, galanin(2–11) and SNAP398299 binding at wt-hGalR3, FLAG-hGalR3 and FLAG-hGalR3 mutants. The results are presented as means \pm SEM from 3 experiments performed in duplicate. NB—no binding, NT—not tested.

Receptor	$K_i \pm$ SEM (nM) galanin	$K_i \pm$ SEM (nM) SNAP398299	$K_i \pm$ SEM (nM) galanin(2–11)
wt-hGalR3	4.1 ± 2.0	329 ± 14	197 ± 8
FLAG-hGalR3	5.1 ± 2.3	285 ± 29	179 ± 12
Tyr103 ^{3,33} Ala-hGalR3	NB	NB	NT
His251 ^{6,51} Ala-hGalR3	NB	NB	NT
Phe263 ^{6,63} Ala-hGalR3	NB	NB	NT
Tyr270 ^{7,32} Ala-hGalR3	NB	NB	NT
Arg273 ^{7,35} Ala-hGalR3	NB	NB	NT
His277 ^{7,39} Ala-hGalR3	NB	NB	NT
Leu278 ^{7,41} Ala-hGalR3	15.1 ± 1.3	49.4 ± 2.1	NT

or SNAP398299 in ligand concentrations below 10^{-5} M (Table 1). Analysis of the Leu278^{7,41}Ala mutant showed an approximately threefold reduction in affinity for galanin, with a K_i value of 15.1 nM. In contrast, the SNAP398299 compound displayed a fivefold increase in affinity for Leu278^{7,41}Ala mutant (Table 1). When analyzed in the *in silico* docking, the residue Leu279^{7,41} was identified to be situated inside the binding pocket but no interaction could be detected with either of the two ligands analyzed.

3.3. Molecular docking to hGalR3

Both galanin(2–6) and SNAP398299 compound are bound primarily between TM II, III and VII and ECL I and II. We found the binding sites for the two galanin poses to be similar and formed by TM II, III, and VII (Fig. 4, as supplementary data we present the coordinates of GalR3 and top galanin and SNAP398299 poses saved in PDB format). However, the binding site for the two SNAP398299

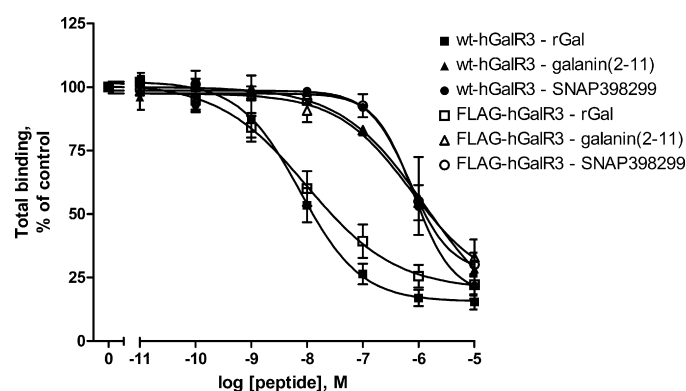


Fig. 2. Receptor displacement studies of hGalR3-wt and FLAG-hGalR3 by rat galanin, galanin(2–11) and SNAP398299. Data from three experiments performed in duplicate, K_i values are presented in Table 1.

poses differs slightly. SNAP398299 pose 1 is bound between TM II, III and III and ECL I and II while pose 2 is bound by TM II and VII and ECL II and III. Both ligands are anchored deep inside the receptor and are stabilized by interaction of polar residues with either an oxygen (SNAP398299) or an amino group (galanin(2–6)) as well as by hydrophobic burial of the indole ring (galanin(2–6)) or pyrrolidine ring (SNAP398299). While the Trp2 indole ring in galanin(2–6) and the pyrrolidine ring of SNAP398299 occupy the same place in the binding pocket, differences in binding mode are observed on the other ends of the ligands which form different contacts with the extracellular loops (Fig. 4). It should also be noted that the hGalR3 model created with disulphide bond between TM II and ECL III show no binding to either ligand studied.

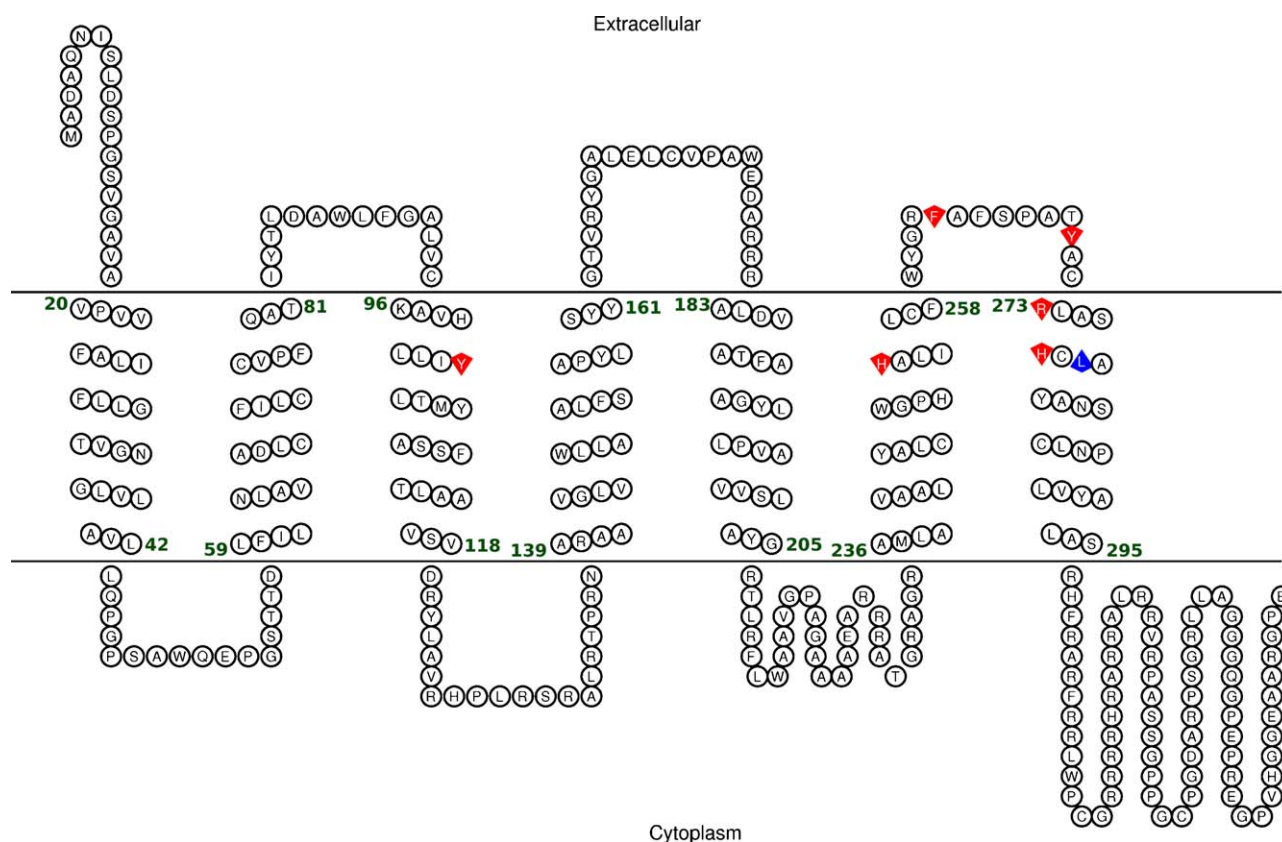


Fig. 3. TM topology of GalR3 created with transmembrane region prediction. Mutated amino acids affecting ligand binding *in vitro* (Tyr103^{3,33}, His251^{6,51}, Phe263^{6,63}, Tyr270^{7,32}, Arg273^{7,35} and His277^{7,39}) are shown in red and residues having no impact on the binding (Leu279^{7,41}) are depicted in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

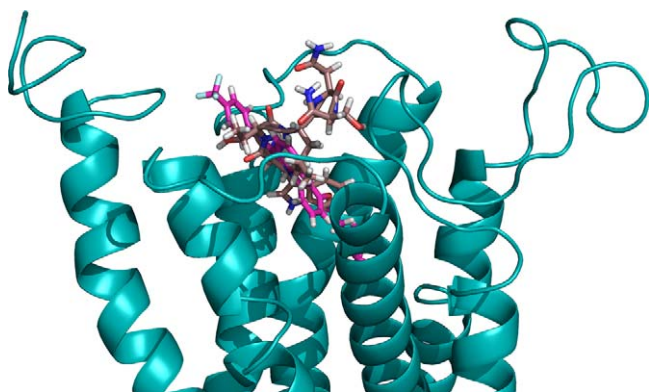


Fig. 4. Computational modeling of hGalR3 and SNAP398299. Top ranked poses of SNAP398299 (pink) and galanin(2–6) (brown) are shown docked to the GalR3 model showing deep ligand binding in the inter TM helices cavity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Galanin pose 1 has an E-model score of -79.35 and has a tighter fit with a higher (403) number of contacts within 5 \AA compared to pose 2 (374) (Supplementary Table 1). Both poses are stabilized by vdW interactions of Trp2 with residues Arg273^{7.35}, His277^{7.39}, Tyr103^{3.33}, Tyr161^{4.61} and Tyr166^{4.66}, with the addition of residue His251^{6.51} in pose 2. Pose 1 is further stabilized by the vdW interaction between Thr3 and Leu85^{2.67} while in pose 2 the aliphatic carbon of Thr3 contacts with Ile82^{2.64}. In pose 1 three hydrogen bonds are observed between Ser6 and Ala92^{3.22}, Trp2 and Gln79^{2.61} and between Thr3 and Ile82^{2.64} (Fig. 5A), in comparison to the 2 hydrogen bonds formed between Trp2 and Gln79^{2.61}, and Thr3 and Phe90^{3.20} in pose 2 (Fig. 5B).

SNAP398299 pose 1 represents a bent SNAP398299 conformation, which is the result of rotations in the O-ethyl-[2-N-pyrrolidine] moiety ($\sim 100^\circ$) and is docked closer to the surface of the binding pocket compared to the energetically preferred linear conformation of pose 2. In pose 1, one hydrogen bond was formed between Gln79^{2.61} and the ligand (Fig. 5C) and in pose 2 the ligand was stabilized by the formation of two hydrogen bonds between Arg273^{7.35} and Tyr161^{4.61} respectively (Fig. 5D). The residues in non-polar contact with the two poses were Ala268^{7.30}, Arg273^{7.35}, His251^{6.51}, His277^{7.39}, Ile82^{2.64}, Leu274^{7.36} and Tyr161^{4.61} for pose 1 and His251^{6.51}, His277^{7.39}, Ile102^{3.32}, Ile82^{2.64}, Leu274^{7.36}, Leu85^{2.67} and Tyr103^{3.33} for pose 2 (Supplementary Table 2). Specificity of the hGalR3–SNAP398299 binding arises mainly from a high number of polar interactions. The smaller dimensions of the SNAP398299 than galanin allow a better fit into the tight binding pocket and are consequently preferred over any pose stabilized mainly by amino acids located on the extracellular loops of the receptor.

3.4. Docking to mutated hGalR3

Residues in hGalR3 that were shown to be essential for galanin binding *in vitro* and interact with galanin(2–6) *in silico* were exchanged for alanine and thereafter underwent the same docking procedure as described in Section 3.3, to confirm the results from the receptor displacement studies and to verify the docking model. It was studied if the receptor ligand complex may be created and also, how similar the resulting binding modes are compared to wt hGalR3. The galanin(2–6) fragment were docked outside the binding pocket, between helices 5 and 6 and the extracellular loops, and were not able to fit in between transmembrane helices. These conformations show no reproducibility in docking attempts indicating no significance in that type of binding. This indicates

that the mutated residues, namely Tyr103^{3.33}, His251^{6.51}, Arg273^{7.35} and His277^{7.39}, plays an important attracting role in the otherwise unfavorable conditions of the GalR3 binding pocket, which is too tight to comfortably host the bulky galanin ligand. The only exception is the Arg273^{7.35}Ala mutant, in which galanin(2–6) is docked inside the binding pocket reaching depths comparable to the smaller SNAP398299 ligand. These contacts were not present in the wt-hGalR3 dockings and this pose has a lower E-model score of -106.13 . This can be explained by the significantly smaller alanine side-chain. Removing the Arg273^{7.35} side-chain, which is pointed directly in to the binding pocket, creates additional space to host the ligand.

4. Discussion

Compelling evidence has implicated a role for galanin and GalR1–3 in the regulation of several physiological disorders (reviewed in Runesson et al., 2009b). Early studies of the galaninergic system, including mutagenesis studies, mainly focused on the GalR1 subtype, wherefore GalR1 is the most well characterized entity of the three receptors. However, since the introduction of several subtype preferential ligands (Liu et al., 2001; Sollenberg et al., 2006; Konkelt et al., 2006; Runesson et al., 2009a; Robertson et al., 2010) have the other receptor subtypes gained increased interest. To further delineate the galaninergic system, highly selective tools are needed. The present study aimed to identify important pharmacophores in hGalR3. Identification of receptor residues that are involved in ligand binding is a fundamental step in elucidating and understanding the principles of ligand–receptor interactions. Such studies have been performed on GalR1 and GalR2, delineating both conserved and subtype different interaction with galanin receptor ligands (Kask et al., 1996; Berthold et al., 1997; Kask et al., 1998; Church et al., 2002; Lundström et al., 2007).

In the present study, we utilized the FLAG-label to be able to confirm the expression of the mutated receptors on the plasma membrane by immunofluorescence techniques. The FLAG-labeled wt-sequence displayed wt-receptor properties (Table 1) for all tested ligands, galanin, SNAP398299 and galanin(2–11).

Previous studies of hGalR1 have shown that a Phe115^{3.32}Ala exchange affect the interaction between the receptor and galanin and Phe115^{3.32} was suggested to interact with Gly1 in galanin (Berthold et al., 1997). In contrast, the Phe106^{3.33}Ala mutation in hGalR2 did not affect galanin binding (Lundström et al., 2007). To identify differences between the galanin receptor subtypes, residue Tyr103^{3.33} in GalR3 corresponding to Phe106^{3.33} in hGalR2, was mutated. Interestingly, the Tyr103^{3.33}Ala mutant displayed no ligand binding, emphasizing that Tyr103^{3.33} in hGalR3 is a necessity for ligand binding. The *in silico* docking identified vdW interactions between the N-terminus in galanin(2–6) and the Tyr103^{3.33} in GalR3, in line with the proposed function in hGalR1; as the residue responsible for docking the N-terminal part of galanin receptor ligands (Berthold et al., 1997). The interaction between the N-terminus of galanin and either residue Phe115^{3.32} in hGalR1 or Tyr103^{3.33} in hGalR3, but not Phe106^{3.33} in hGalR2, may explain the importance of retaining Gly1 in galanin analogues to retain high affinity towards GalR1 and GalR3 (Sollenberg et al., 2006; J. Runesson et al., unpublished data). However; this interaction remains to be clarified by further mutations in this region of the binding pocket in the different receptor subtypes.

Two conserved histidine residues, His^{6.51} and His^{6.52} are present in TM VI, are, both proposed to be potential interaction partners with Trp2 in galanin (Lundström et al., 2007). Further sequence homology comparison indicates a conserved histidine residue in TM VII, the His^{7.39} residue. Mutation of the His^{6.51} residue displayed different results in studies of the hGalR1 and the

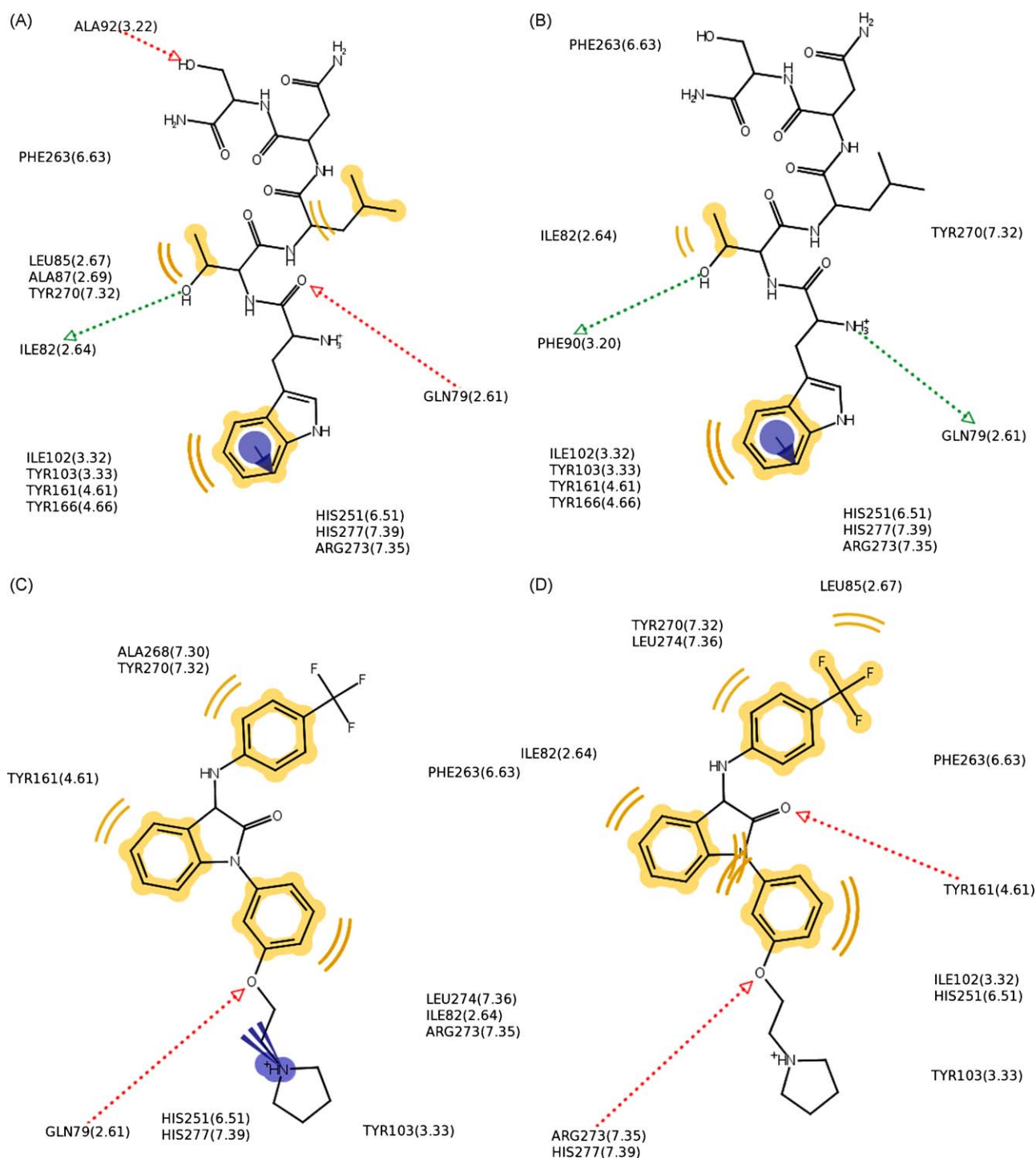


Fig. 5. The 2-D pharmacophore model generated by LigandScout. This model displays the structural formula of the ligand showing the active site of the hGalR3 model with the ligand. Two alternative poses for the tested ligands, galanin and SNAP398299, respectively, is presented. The hydrogen bond features are depicted in green (hydrogen bond donor) and red (hydrogen bond acceptor) vectors. The hydrophobic and aromatic interactions are depicted by the yellow spheres and the blue spheres respectively. Residues contributing to these interactions are also listed in [Supplementary Tables 1 and 2](#). The residues shown outside of the boxes are the selected “important residues” added manually to the figures. (A) galanin(2–6) pose 1. (B) Galanin(2–6) pose 2. (C) SNAP398299 pose 1. (D) SNAP398299 pose 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

hGalR2. The His263^{6.51}Ala mutation in hGalR1 did not affect the affinity for galanin, and was also proposed to point away from the binding pocket in the presented hGalR1 model (Berthold et al., 1997). However, His252^{6.51} in hGalR2 is essential for ligand binding (Lundström et al., 2007). His^{6.52} has been shown to be essential in both hGalR1 and hGalR2 (Kask et al., 1998, Lundström et al., 2007). The importance of His^{7.39} residue has only been evaluated in hGalR1, were no effect on ligand binding could be

detected. This prompted us to evaluate His252^{6.52} and His277^{7.39} in hGalR3 to detect differences between the receptor subtypes. Strikingly, mutation of either residue resulted in complete abolishment of detectable ligand binding. Surprisingly, the His252^{6.52}–ligand interaction was not identified in our *in silico* docking of hGalR3. Instead interactions were found between Trp2 and two other histidine residues, His251^{6.51} and His277^{7.39}. This discrepancy is not clear, although it may highlight the requirement

to further optimize the *in silico* model when additional GPCR structures are available.

In close proximity to His277^{7,39}, essential in hGalR3, is Leu278^{7,41} situated. Leu278^{7,41} resides in the binding pocket of the *in silico* model, although no interaction was found with the ligands in the docking experiments. The Leu278^{7,41}Ala receptor mutant had a slightly lower binding for galanin (threefold) and a slightly higher binding for the SNAP398299 compound (fivefold). Replacement of the leucine residue with the smaller alanine residue could allow the SNAP398299 compound to slightly shift its occupancy in the binding pocket and therefore its interactions with the receptor.

Previous studies have hypothesized the importance of both ECL II and ECL III. Several mutations were introduced in ECL II in hGalR1 that did not affect the ligand binding, suggesting that ECL II is not strongly involved in ligand interactions (Berthold et al., 1997; Kask et al., 1998). Later studies on hGalR2 thus focused on ECL III, where two essential aromatic residues were identified, Phe264^{6,63} and Tyr271^{7,32}. These, and homologous residue in hGalR1, have been suggested to have aromatic-aromatic interaction with Tyr9 in galanin (Berthold et al., 1997; Kask et al., 1998; Church et al., 2002; Lundström et al., 2007). In this study, we show that these two residues conserved between hGalR2 and hGalR3, Phe263^{6,63} and Tyr270^{7,32}, are also essential for detecting ligand binding on hGalR3. Unfortunately, the *in silico* model could not identify all interaction partners for galanin, due to the technical limitations of ligand size used within the *in silico* docking experiments. Compared to other mutated residues, Phe263^{6,63} and Tyr270^{7,32} are situated more distant to the bound ligand. However, these residues create a cluster of hydrophobic and aromatic residues together with Pro267^{7,29}, Ala268^{7,30} and Phe265^{6,65} in ECL III and Pro12 in the N-terminus in the *in silico* model. Therefore, we propose that this cluster forms a crucial interaction point with the hydrophobic fragment AGYLL of galanin.

The Arg285^{7,35}Ala mutation in hGalR1 had no detectable effect on ligand binding (Berthold et al., 1997), while the Arg273^{7,35}Ala hGalR3 mutant showed no detectable binding. Docking results from the *in silico* model confirmed potential interaction between Arg273^{7,35} and Trp2 in the galanin fragment and also with the SNAP398299 compound, see Supplementary Tables 1–2 and Figs. 4 and 5.

In the absence of a crystal structure of hGalR3, in complex with a ligand, little is known about the exact orientation of galanin ligands in the binding pocket. This is an obstacle shared with most members in the GPCR family. Homology models of GPCRs in combination with site-directed mutagenesis have been successfully used in the past to provide a structural framework for both ligand binding and functional studies (see Böhme and Beck-Sicking, 2009). Our model of hGalR3 displays a relatively narrow and deep binding pocket compared to what has been suggested for hGalR1 and hGalR2 (Kask et al., 1998; Lundström et al., 2007). The narrow binding pocket could contribute to the sensitivity of this receptor and hence absolute correct fit could be crucial for sufficient binding of a ligand to hGalR3, explaining the striking loss in binding after a single amino acid substitution in six of the receptor constructs.

Even if the galanin fragment, galanin(2–6), gives no detectable signal in competitive binding studies with full length galanin (data not shown), *in silico* docking of galanin(2–6) can further emphasize important contributions to the interactions seen by full length galanin *in vitro*. The residues identified *in vitro* were also identified *in silico* as possibly important for ligand binding based on proximity to docked ligand, galanin(2–6) or SNAP398299. Along with performing docking on the wt hGalR3, we performed *in silico* L-Ala-mutation of the interesting residues; i.e. the putative interactions with the galanin(2–6) fragment; in order to additionally clarify the presented *in vitro* results. Docking experiments after

side chain replacements resulted in complete loss of binding with the galanin(2–6) peptide for three of the tested receptors mutants. Surprisingly, in docking experiments with the Arg273^{7,35}Ala mutation, galanin(2–6) where bound deeper along the receptor Z-axis than any other galanin(2–6)-receptor complex, reaching a ligand position comparable to the SNAP398299 complexes. Current docking techniques do not allow for receptor flexibility and thus may cause occurrence of steric clashes, or in a less critical case, non-optimal side chain rotamers not resolved after simple energy minimization procedure. This results in relatively underestimated binding energies in otherwise correct complexes. In this situation deletion of a residue important for binding has lower energetic costs (smaller contribution to dG of attracting interactions of Arg273^{7,35} than steric repulsion) and results in an overall drop of binding energy. To further verify this explanation, calculation of free energy might be applied to estimate its contribution.

In this context, we wanted to elucidate our *in silico* model further by testing its interaction with the 60 amino acid residue long galanin-like peptide (GALP). The amino acid sequence of GALP(9–21) is identical to that of galanin(1–13) (Ohtaki et al., 1999). Previous studies have identified GALP to interact with GalR1–3 *in vitro* and receptor affinity data for GALP suggest that the presence of the N-terminal fragment, GALP(1–8), is well tolerated by all galanin receptor subtypes (Ohtaki et al., 1999; Lang et al., 2005). In our model, a manually added extension with a helical structure of the amino acids present in GALP(1–9) on the N-terminus of galanin(2–6), is indeed tolerated in pose 1 (see Fig. 5A). In contrast, an equivalent extension on the N-terminus of galanin(2–6) in pose 2 (see Fig. 5B) requires a slight conformational change of the ECL III (RMSD 2.79 Å) and a 180 degree rotation of the N-terminal amino group in galanin(2–6). This dissimilarity suggests that GALP interacts with hGalR3 in a manner similar to pose 1. However, since the present docking program does not allow receptor flexibility (see above) which could potentially remove the induced steric clashes in pose 2, characterization of this interaction remains inconclusive and can be only speculative at this time.

In conclusion, the present study identifies six conserved amino acid residues in the transmembrane helices and the extracellular domains of hGalR3 that are crucial for galanin binding using site-directed mutagenesis studies (Tyr103^{3,33}, His251^{6,51}, Phe263^{6,63}, Tyr270^{7,32}, Arg273^{7,35} and His277^{7,39}). The complete loss of affinity as a result of a single amino acid substitution further strengthens the view of hGalR3 to be extra sensitive, why mutation of one important amino acid is able to affect binding dramatically. The identification of essential amino acid residues in GalR3 for binding of galanin receptor ligands should provide a rationale basis for the design of drugs targeting GalR3, facilitating future understanding of the galaninergic signaling events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2010.08.018.

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