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Structural Modelling and Mutation Analysis of a Nociceptin Receptor and its Ligand Complexes

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We recently modelled and proposed four ligand-bound conformations for a G-protein-coupled receptor, namely, forms I, II, III and IV, based on the 3D structure and functional evidences for rhodopsin. In this study, the same strategy was applied to a human nociceptin receptor (NR), in order to predict ligand-bound receptor structures. Additionally, site-directed mutagenesis studies were carried out to evaluate these structures. A Thr138Ala mutant demonstrated the same affinity for [Phe^{1}Ψ(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$ as the wild-type receptor; however, the affinity of this mutant for nociceptin was 20-fold lower than that of the wild type. A Ser223Ala mutation showed the same characteristics as those of the wild type. On the other hand, a Gln280Ala mutation reduced the affinity to nociceptin by more than 60-folds. These results suggested that a change in the conformation of NR following agonist binding did not accompany the rigid-body rotation of the sixth transmembrane segment that was reported for an adrenergic receptor and a κ-opioid receptor. NR is potently activated not only by nociceptin but also a synthetic peptide, *i.e.* Ac-RYYRIK-NH$_2$, although the amino acid sequences of both these ligands are completely different. The model explains why both the ligands activate NR and shows that their receptor-bound conformations have similar 3D structures.

Key words: G-protein-coupled receptor, nociceptin receptor, modelling, site-directed mutagenesis.

Abbreviations: F/G-NC, [Phe^1 Ψ(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$; Gα$_{i1}$, α subunit of inhibitory GTP-binding regulatory protein subtype 1; GPCR, G-protein-coupled receptor; G-protein, heterotrimeric GTP-binding regulatory protein; [^{35}S]GTPγS, guanosine 5′-*O*-(3[^{35}S]thiotriphosphate); NR, a human nociceptin receptor; NR-Gα$_i$, a fusion protein of a human nociceptin receptor and a bovine G$_{i1}$ protein α subunit; TM, transmembrane segment; PCR, polymerase chain reaction.

G-protein-coupled receptors (GPCRs) are one of the largest protein superfamilies consisting of ~900 distinct genes in the human genome (*1*, *2*). These receptors receive extracellular signals and activate G-proteins. The binding of an agonist to a receptor induces conformational changes and the formation of a ligand–receptor-G-protein complex. It is estimated that approximately 50% of clinically effective drugs are designed to function as ligands for GPCRs (*3*). Identification of new ligands for GPCRs is, therefore, a key step in developing new drugs. Structural predictions of GPCR–ligand complexes are very attractive concepts, in order to understand the functions of ligands and design new ligands. Based on the crystal structure of rhodopsin (*4*), a typical GPCR, molecular dynamics simulations were often performed to study the formation of the complex between a candidate GPCR and its ligand. With regard to the receptor activation events induced by ligand binding, a two-state model with inactive and active states has been discussed in several studies on structural prediction (*5*).

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On the other hand, experimental evidences of ligand binding for the fluorescence-labelled adrenergic receptor suggested that the structure of a partial agonist-bound receptor is distinct from that of a full agonist-bound receptor (*6*). A recent report on an opioid receptor suggested that full agonist binding involved the rigid-body rotation of transmembrane segment (TM) 6 (*7*), and that a ligand (agonist)-bound receptor would have a different conformation from the rhodopsin crystal structure. These results indicated that GPCRs transformed their structure in more than two forms depending on the bound ligands or their activation states. Rhodopsin has a few distinct states in the photocascade (*8*), and the modelling of photointermediates suggested that each photointermediate has a distinct structure (*6*).

Application of the photointermediate structural models to the functionally distinct states of biogenic amine receptors showed that four functionally different ligands—inverse agonists, antagonists, partial agonists and full agonists—bind the corresponding receptor structures. Therefore, we recently proposed four ligand-bound structures for the cationic biogenic amine receptors; these structures include form I (inverse agonist bound), form II (antagonist bound), form III (partial agonist bound) and form IV (full agonist bound). These forms

correspond to the structural models of the photointermediate, *i.e*. metarhodopsins I, Ib (opsin), I_{380} (a rhodopsin mutant) and II, respectively (*9*). Here, we extend this modelling strategy to peptide receptors whose ligands are larger than biogenic amines, such as adrenaline and dopamine.

The nociceptin receptor (NR) belongs to the GPCR family and shares significant sequence homology with opioid receptors. It influences various biological effects including spinal analgesia, supraspinal hyperalgesia and inhibition of locomotor activity and learning (*10*, *11*). Since NR ligands are expected to be worthy of clinical investigation, the 3D structure of NR is desired for a rational drug design (*12*). Nociceptin (*13*, *14*), a 17-amino acid peptide (FGGFTGARKSARKLANQ), is an endogenous ligand for NR. Although it shares significant homology with the opioid peptide dynorphin A (YGGFLRRIRPKLKWDNQ), nociceptin does not bind to opioid receptors. All opioid peptides require tyrosine as the N-terminal residue; thus, its absence in nociceptin is unique. We assume that the molecular interactions between NR and nociceptin significantly differ from those in the opioid receptor-ligand complexes. Furthermore, a synthetic peptide, *i.e*. Ac-RYYRIK-NH_2, shows potent agonist activity for NR despite the absence of a sequence homology with nociceptin. It will be intriguing to elucidate the role of the N-terminal phenylalanine and to investigate how nociceptin and Ac-RYYRIK-NH_2 that have completely different sequences activate NR.

To understand the molecular recognition of NR and the change in its conformation that is induced by ligand binding, we constructed molecular models of the nociceptin–receptor complex for forms III and IV. One of the most important aims of our modelling is to compare the receptor-bound conformation of nociceptin with that of the peptide Ac-RYYRIK-NH_2, in order to clarify why these ligands with different sequences are able to activate NR potently. Moreover, to examine our proposed structures, we focused on three residues, namely, Thr138, Ser223 and Gln280, and mutated these residues to alanine. The activity of the mutants was measured using a nociceptin receptor-$G\alpha_{i1}$ fusion protein (NR-$G\alpha_i$) (*15*, *16*). A series of these computational modelling (dry prediction) and mutational experiments (wet examination) for several GPCRs will facilitate the understanding of the general schemes of GPCR–ligand interactions.

MATERIALS AND METHODS

Three-dimensional Modelling of the Nociceptin-Receptor Complex—Previously reported 3D structural models of metarhodopsins I_{380} and II (*9*) were used for the construction of human nociceptin receptor models. Calculation methods for NR modelling were also described previously (*9*). The ligand-binding space was explored using the binding site module of Insight II (Accelrys Inc., San Diego, USA), and amino acid and peptide fragments were docked into the binding site.

Based on site-directed mutagenesis studies, it was suggested that Asp130 electrostatically interacts with the nociceptin N-terminus (*17*) and is very important for ligand binding of NR (*18*). Thus, we assumed that the cationic amine at Phe^1 in nociceptin forms a salt bridge with Asp130, similar to the corresponding Asp130 in the adrenergic receptor that interacts with the cationic amine group. Since the receptor-bound conformation of nociceptin is yet to be elucidated, we attempted to position several fragments such as Phe^1, Gly^2-Gly^3, Phe^4, Thr^5-Gly^6-Arg^7 and the remaining C-terminal fragment (Arg^8-Gln^{17}) in a putative ligand-binding cleft. In this step, two conformations of the N-terminal Phe^1 residue, namely, mode 1 and mode 2, were found to be possible; these modes were generated by manually docking the residue into the ligand-binding cleft, with an ionic interaction between the N-terminal cationic amino group of nociceptin and the carboxylate of the Asp130 side chain in TM3 of NR. Subsequently, two Gly^2-Gly^3 residues were attached to the Phe^1 residue, followed by attachment of the Phe^4 residue. The remaining peptide fragment Thr^5-Gly^6-Arg^7 was located at a binding space near the extracellular surface, and the C-terminal fragment was positioned at the extracellular surface area of the receptor. The ligand-binding space was estimated by the Binding-Site module installed using the Insight II software (Accelrys Inc., San Diego, USA). Protonated phenylalanal (H_3N^+-Phe^1-H) was then docked into the ligand-binding cleft situated at a distance of <3.0 Å between the N-terminal cationic amine and the carboxylate oxygen of the essential residue Asp130 (*18*) at the extracellular site of TM3 using the form III conformation model. After energy minimization of the complex structure, 12 different conformations of the phenyl propanal portion of the phenylalanal residue were generated by rotating the N-Cα bond by 30°; these conformations were converged to three conformations by optimizing them by using the molecular dynamics/minimization procedure. Two of the three conformations, *i.e*. mode 1 and mode 2, were selected for the further construction of nociceptin, since the remaining conformation did not sterically allow the further extension of the peptide structure. Then, a glycylglycinal (Gly^2-Gly^3) portion was connected to phenylalanal to form FGG-al guided by the estimated ligand-binding space. After optimization of the conformation of FGG-al, initial receptor-bound structures of the N-terminal-pentapeptide portion were completed by the connection of the Phe^4-Thr^5-H portion to FGG-al guided by the estimated ligand-binding space. The dipeptide fragment Gly^6-Arg^7 was located at a binding space near the extracellular surface, and the remaining C-terminal fragment was placed at the extracellular surface area of the receptor.

Two complex structural models of nociceptin in form IV conformation were constructed in a similar manner. These initial complex models were energy-minimized and then optimized using the molecular dynamics/minimization procedure with Discover 3 (Acelrys Inc., San Diego, USA) without any constraints between the ligands and the receptors, while the all Cα-carbons of the receptor were tethered at the original positions. The lowest-energy structure was selected as an energy-optimized complex model.

Three-dimensional Models of the F/G-NC-Receptor Complex—In the complex structure, the carbonyl group

of the peptide bond of Phe^1-Gly^2 in nociceptin was converted to a methylene group by using the BUILDER module of Insight II (Accelrys Inc.). Subsequently, the initial structure was energy minimized, followed by energy optimization by using Discover 3.

Three-dimensional Models of the Ac-RYYRIK-NH_2-Receptor Complex—The Phe^1-Gly^2-Gly^3 fragment of nociceptin was replaced with N-acetylarginine at the ligand-binding site with an ionic interaction between Asp130 and the cationic guanidium moiety. The Phe^4-Thr^5 fragment of nociceptin was then replaced with the Tyr^2-Tyr^3 moiety, followed by replacement of the remaining residues. The initial steric hindrances were removed by energy minimization without any constraints between the ligands and the receptors. The minimized structure was then optimized using the molecular dynamics/minimization procedure with Discover 3. During the minimization/optimization procedure, all the Cα-carbons of the receptor were tethered at their original positions. The lowest energy structure was selected as the energy-optimized complex model.

Materials and Reagents—We obtained [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]nociceptin (90 Ci/mmol) from PerkinElmer (Boston, USA). Synthetic nociceptin and Ac-RYYRIK-NH_2 were purchased from Peptide Institute (Osaka, Japan). F/G-NC was obtained from Tocris (Bristol, UK). Restriction enzymes were purchased from Toyobo (Tokyo, Japan).

Gene Constructions—Complimentary DNA (cDNA) encoding NR-$G\alpha_i$ was constructed by two-step polymerase chain reaction (PCR) using KOD-plus polymerase (Toyobo) as follows. First, to amplify the human NR gene from the human genome (Clonetech, Calfornia) and the bovine $G\alpha_{i1}$ gene from the cDNA clone that was kindly provided by Dr Nukada (Tokyo Institute of Psychiatry) and Prof. Tatsuya Haga (Gakusyuin University), a first PCR was performed using primers 5′-AAAGCGGCC GCATGGAGCCCCTCTTCCCC-3′ (forward) and 5′-CGC TCAGCGTACAGCCCAT TGCGGGCCGCGGTACCG-3′ (reverse) for the NR gene, and 5′-ATGGGCTGTACGCT GAGCG-3′ (forward) and 5′-TTTTCTAGATCAGAAGAGA CCACAGTCTTTTAGG-3′ (reverse) for the $G\alpha_{i1}$ gene. The temperature program was 20 cycles of 94°C for 30 s, 60°C for 15 s, and 68°C for 1 min. Next, to fuse both genes, a second PCR was performed using the forward primer for the NR gene and the reverse primer for the $G\alpha_{i1}$ gene by using both the first PCR products as templates. The temperature program was 20 cycles of 94°C for 30 s, 60°C for 15 s, and 68°C for 3 min. This fused gene was inserted into pFastBac1 (Life Technologies) using the *Not*I and *Xba*I restriction sites.

The NR-$G\alpha_i$ mutant genes were prepared by two-step PCR using KOD-plus polymerase and the following primers. NociR-F, 5′-AAAGCGGCCGCATGGAGCCCC TCTTCCCC-3′; for the Thr138Ala mutation: 138F, 5′-ATGTTCACCAGCGCTTTCACCCTAACT-3′ and 138R, 5′-AGTTAGGGTGAAAGCGCTGGTGAACAT-3′; for the Ser223Ala mutation: 223F, 5′-ATCTTCCTCTTCGCCTT CATCGTCCCC-3′ and 223R, 5′-GGGGACGATGAAGG CGAAGAGGAAGAT-3′; for the Gln280Ala mutation: 280F, 5′-TGGACGCCTGTCGCCGTCTTCGTGCTG-3′ and 280R, 5′-CAGCACGAAGACGGCGACAGGCGTCCA-3′; and 314Gi1: 5′-GCTAGCACAAAGAGTTGG-3′.

The first PCR was performed using the primer pairs NociR-F and 138R, 138F and 314Gi1, NociR-F and 223R, 223F and 314Gi1, NociR-F and 280R and 280F and 314Gi1; the PCR conditions were 30 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 2 min. The second PCR was performed using the primer pairs NociR-F and 314Gi1 and the pair of the first PCR products as templates; the PCR conditions were 30 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 3 min. The second PCR products were inserted into wild-type NR-$G\alpha_i$/pFastBac1 by using the *Not*I and *Sma*I restriction sites. All DNA sequences were confirmed by a DNA sequencer (model 310; Applied Biosystems).

Expression of Fusion Proteins in Sf9 Cells—The wild-type and three mutated NR-$G\alpha_i$ genes in pFastBac1 were used to produce a recombinant baculovirus using a Bac-to-Bac system (Life Technologies, CA, USA). Sf9 cells were grown at 27°C and infected with the recombinant virus at a density of 3×10^6 cells/ml. After 48 h of infection, the cells were harvested and homogenized in a homogenizing buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-KOH (pH 7.0), 1 mM EDTA and 10 mM $MgCl_2$) with a protease inhibitor cocktail (Sigma, Boston, USA). Plasma membrane was precipitated by centrifugation at 50,000 rpm for 2 h (SCP70H ultracentrifugator and SW28 rotor; Hitachi), and the precipitate was resuspended in the homogenizing buffer described earlier. The membrane suspension was stored at –80°C. Protein concentration was determined using the protein assay kits (Bio-Rad).

[^{35}S]GTPγS-Binding Assay—Ten micrograms of membrane fractions expressing NR-$G\alpha_i$ fusion proteins were incubated in 100 μl of 20 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 160 mM NaCl, 1 mM dithiothreitol, 100 pM [^{35}S]GTPγS, 10 μM GDP, and 10 mM $MgCl_2$ at 30°C for 30 min on 96-well microplates. [^{35}S]GTPγS bound to the membrane was trapped on a GF/B glass fiber filter (Whatman, London, UK), washed three times with 300 μl of cold 20 mM potassium phosphate buffer (pH 7.2), and counted with a liquid scintillation counter (LS6500, Beckman Coulter).

Ligand-Binding Assay—Twenty micrograms of the membrane preparation were incubated in 200 μl of a solution containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.14 mg/ml bacitracin, 0.2 mg/ml bovine serum albumin, 20 nM of [^{3}H]nociceptin and various concentrations of cold ligands at 30°C for 90 min. After incubation, the membranes were collected on a GF/B glass fiber filter (Whatman, London, UK) and washed with 20 mM potassium phosphate buffer (pH 7.2). The radioactivity was measured using a liquid scintillation counter (LS6500, Beckman Coulter).

RESULTS

Modelling of the Receptor–ligand Complexes—Since nociceptin and its related ligand peptide analogues exhibit agonist activity, we expected that these ligands bind partial agonist-bound (form III) or full agonist-bound receptor model (form IV), as described in our

previously proposed conformations of the biogenic amine receptors (*9*). Thus, using the homology modelling method, we constructed receptor–agonist complexes based on the main chain conformations of forms III and IV that were derived from the structural models of metarhodopsins I_{380} and II, respectively, which were photointermediates in the rhodopsin photocascade (*8*). The NR structure of form IV contained the rotated conformation of TM6 and thus was distinct from that of form III. Hence, the ligand-binding region in TM6 was very different in these two forms. In particular, Gln280 in TM6 was located outside the ligand-binding site in form IV, whereas the residue was oriented toward the binding site in form III.

First, we explored the plausible interactions between nociceptin and NR in form III (partial agonist-bound form). Two binding modes for the N-terminal Phe^1 residue of nociceptin were possible for the initial manual model building (Fig. 1). In the first mode (mode 1), the aromatic moiety of Phe^1 was oriented toward the intracellular side, when the positive N-terminal amino group of nociceptin bound Asp130 in TM3 (Fig. 1A). This ionic interaction was analogous to the binding of cationic amines of the adrenergic receptor ligands with the carboxylate of Asp113, which is at an analogous position of Asp130 in the NR sequence and is highly conserved within the biogenic amine receptors. In the second mode (mode 2), the N-terminal Phe^1 residue was oriented toward the extracellular side (Fig. 1B). The Gly^2-Gly^3 portion in mode 2 was located at a site occupied by an aromatic group of Phe^1 in mode 1. These differences in binding modes 1 and 2 caused a divergence in the interaction between the Gln280 side chain in TM6 and the main chain carbonyl groups of nociceptin. In mode 2, the two main chain carbonyl groups of Gly^2 and Gly^3 were proximal to the side chain amide protons of Gln280, whereas no appreciable interactions occurred between Gln280 and the main chain carbonyl groups in mode 1. The carbonyl oxygen of the Phe^1-Gly^2 peptide bond in mode 2 was located proximal to the sulphur atom of Met134 in TM3, thereby suggesting a favourable sulphur-oxygen interaction (Fig. 2, blue dotted lines). The following Phe^4 residue bound at a similar site in both the modes. The next three residues (Thr^5-Gly^6-Arg^7) also positioned themselves in a similar fashion in modes 1 and 2. Finally, the remaining C-terminal residues were located at the second extracellular loop and a negatively charged extracellular surface. This is consistent with a previous result that the displacement of Leu^{14} and Ala^{15} of nociceptin with Arg and Lys, respectively, led to an increase in the affinity for the nociceptin receptor (*19*).

In form IV, there is an ionic interaction between the N-terminal Phe^1 of nociceptin and the conserved Asp130

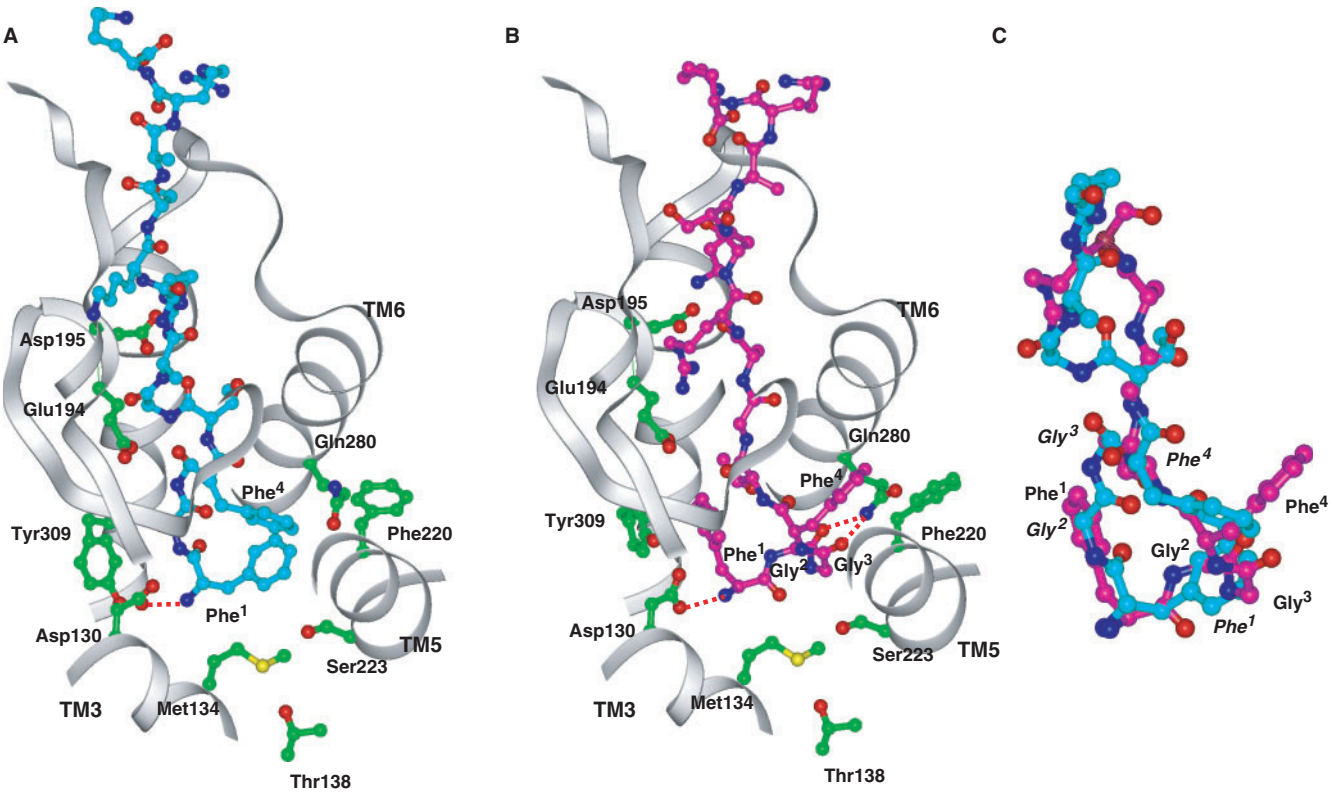

Fig. 1. **Molecular models of the nociceptin-receptor complexes in form III.** (A) binding mode 1; (B) binding mode 2; (C) two conformations of the N-terminal portion (Phe^1-Arg^8) of nociceptin in modes 1 and 2 are superimposed. Only the selected amino acid residues in the ligand-binding cleft are shown, and the hydrogen atoms are excluded for clarity. The carbon atoms of the nociceptin receptor are coloured green; the carbon atoms of nociceptin in mode 1 and those in mode 2 are coloured cyan and magenta, respectively. The nitrogen and oxygen atoms are coloured blue and red, respectively. The red broken lines indicate electrostatic interaction.

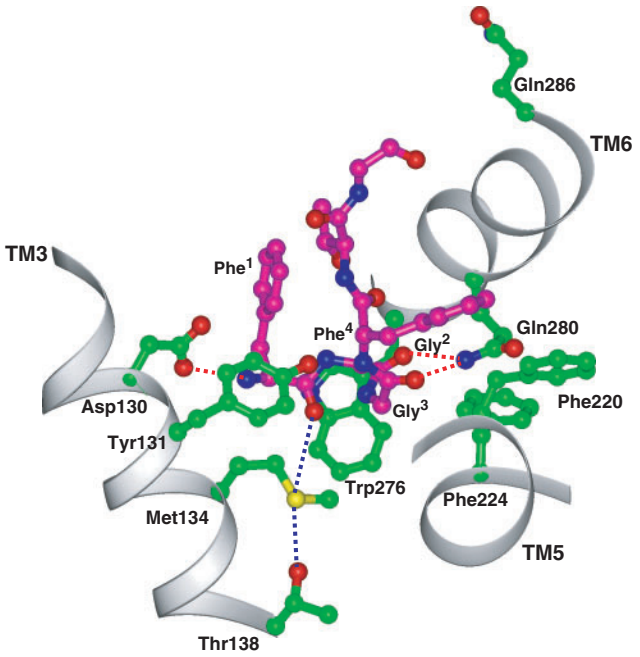

Fig. 2. **Hydrogen bonds between Gln280 and the backbone amide carbonyls of the Gly^2-Gly^3-Phe^4 portion of nociceptin were observed in the resulting form III-NR-nociceptin complex structure.** The blue broken lines indicate sulphur–oxygen interactions in the backbone carbonyl of the Phe^1-Gly^2 portion, Met134 and Thr138. Since Tyr131 is also close to Met134 and indirectly contributes to the nociceptin binding, a Tyr131Ala mutation caused a decrease in nociceptin-binding affinity (*18*). Phe^1 and Phe^4 residues of nociceptin form an aromatic cluster with Phe220, Phe224 and Trp276 in NR. The absence of one of the aromatic residues resulted in the reduction of nociceptin-binding affinity (*18*).

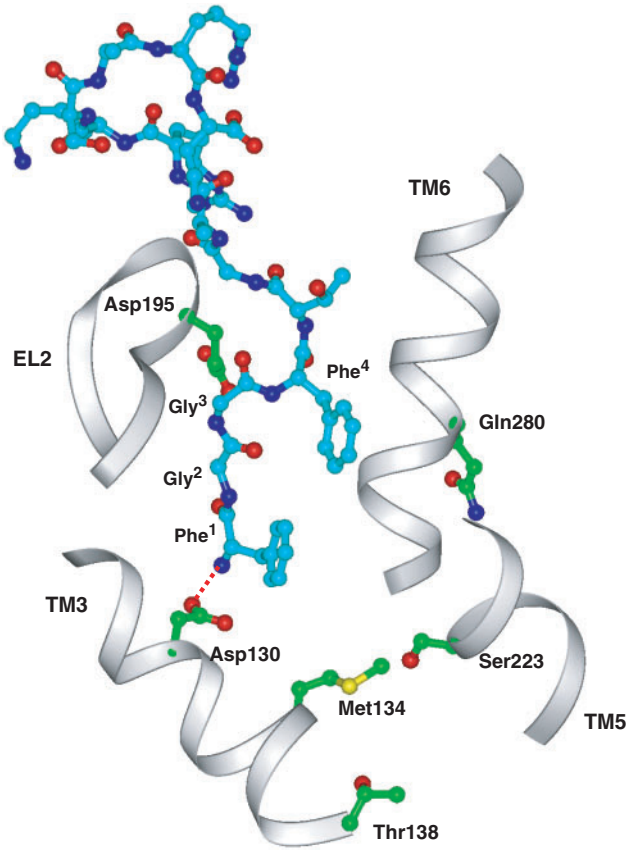

Fig. 3. **A molecular model of the nociceptin–receptor complex in form IV.** The conformation of nociceptin (Phe^1-Gln^{13}) corresponds to that of mode 1 in form III.

residue in TM3 as observed in form III (Fig. 3). But in contrast to form III, no hydrogen bonds were found between the residues in TM6 and the backbone structure of the Phe^1-Gly^2-Gly^3-Phe^4 portion because the Gln280 residue was located outside the ligand-binding site due to the rigid-body rotation of TM6.

The models of the complex of receptor with F/G-NC—a nociceptin analogue with a ψ-bond between Phe^1 and Gly^2—were constructed by modifying the peptide bond of Phe^1-Gly^2 in nociceptin in the nociceptin–receptor complex models. The overall structures were kept similar to those of the corresponding nociceptin–receptor complex models. No hydrogen bond was found between NR and the amino group in ψ-bond, which is characteristic of the peculiar chemical structure of F/G-NC. The backbone carbonyl groups of the Gly^2-Gly^3-Phe^4 portion formed hydrogen bonds with Gln280 in the complex model derived from mode 2 of form III. However, in the other complex structures (form III, mode 1; form IV, mode 1; and form IV, mode 2), no hydrogen bonds were formed in the Gly^2-Gly^3-Phe^4 portion (data not shown).

The synthetic analogue ligand Ac-RYYRIK-NH_2 has a completely different amino acid sequence; nevertheless, it exhibits potent agonist activity. Initially, we assumed that the N-terminal amino group of nociceptin is substituted with the cationic guanidium moiety of Arg^1 (Fig. 4). Subsequently, the long methylene chain of Arg^1 was found to occupy a similar space in the Gly^2-Gly^3 portion of nociceptin, and the N-terminal acetamide moiety exhibited two distinct conformations. In the first conformation (Fig. 4A), the nitrogen atom of the N-terminal acetamide was located proximal to the Ser223 residue in TM5, which is well conserved among the biogenic amine receptors. In the second conformation (Fig. 4B), the oxygen atom of acetamide formed an intramolecular hydrogen bond with one of the nitrogen atoms of the guanidium moiety of Arg^1. The backbone carbonyl of Arg^1 formed a hydrogen bond with Gln280 in TM6 in form III. The aromatic moiety of Tyr^2 was located at a similar position to Phe^4 of nociceptin, and the phenol group of Tyr^2 was proximal to the backbone carbonyl of Val283 in TM6. The aromatic moiety of Tyr^3 bound at a similar site to the aromatic moiety of Phe^1 of nociceptin in mode 2 of the nociceptin–receptor complex model. The side chain of Arg^4 bound Glu194 in the second extracellular loop of NR.

The Ac-RYYRIK-NH_2–receptor complex models for form IV were also constructed under the assumption of an ionic interaction between the guanidium moiety of Arg^1 and Asp130 in TM3. However, these models showed no significant interactions between Ac-RYYRIK-NH_2 and the residues in TM6 (data not shown), suggesting that the rotated conformation of TM6 is unsuitable for ligand binding.

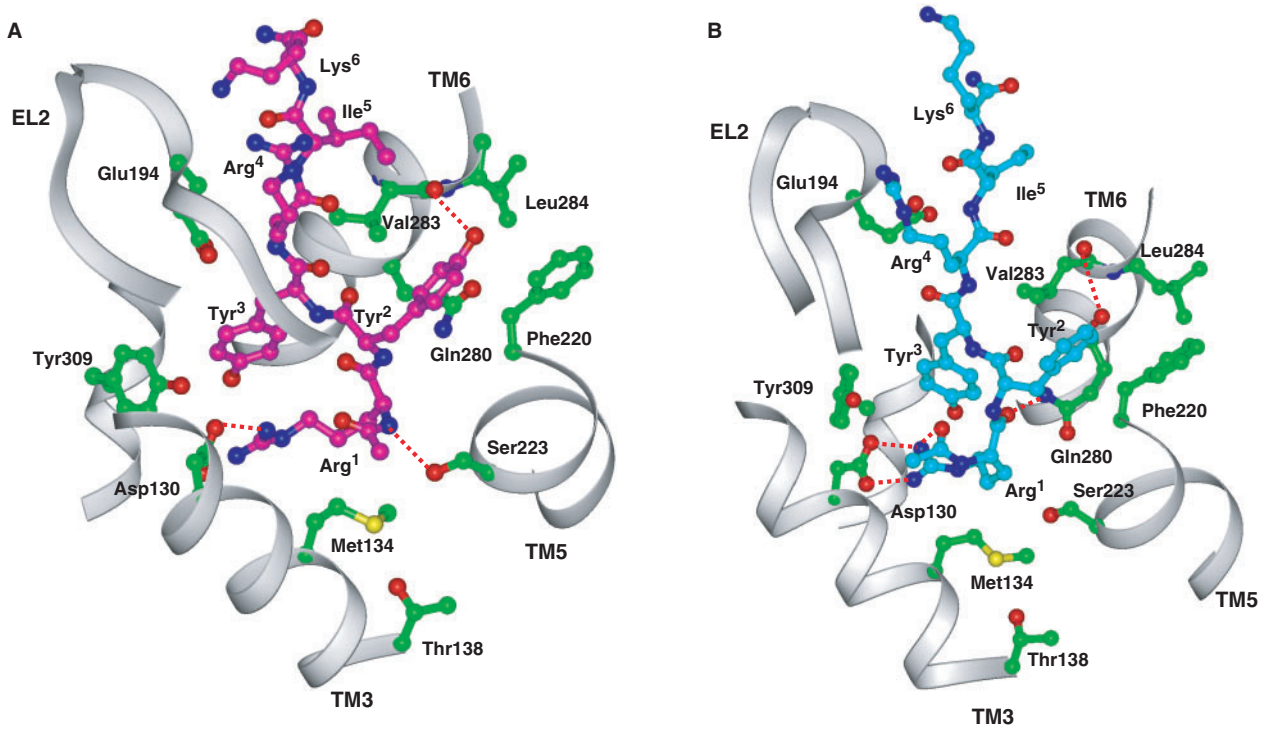

Fig. 4. **Two conformations of Ac-RYYRIK-NH$_2$ in the ligand-receptor complex models**. The carbon atoms of the peptide ligand are coloured cyan in (A) and magenta in (B).

Table 1. **Potency (EC_{50}), efficacy (% of maximum), and affinity (IC_{50}) for nociceptin, Ac-RYYRIK-NH$_2$, and F/G-NC of the wild-type and mutant nociceptin receptors measured using NR-Gα_i fusion proteins.** The EC_{50}s and efficacies were determined from [^{35}S]GTPγS-binding assay as well as from Fig. 6. To determine IC_{50}, membrane preparation (20 μg) was incubated in 200 μl of a solution containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.14 mg/ml bacitracin, 0.2 mg/ml bovine serum albumin, 20 nM of [^{3}H]nociceptin and various concentrations of cold ligands at 30°C for 90 min. After incubation, the membranes were collected on a filter and washed with 20 mM potassium phosphate buffer (pH 7.2). The radioactivity was measured using a liquid scintillation counter. In a Gln280Ala mutant, no specific binding was observed even in the presence of 20 nM [^{3}H]nociceptin (ND, not determined). All data were calculated by curve-fitting analysis using KaleidaGraph (Synergy Software).

Ligand	Wild			T138A			S223A			Q280A		
	EC_{50} (nM)	Max (%)	IC_{50} (nM)	EC_{50} (nM)	Max (%)	IC_{50} (nM)	EC_{50} (nM)	Max (%)	IC_{50} (nM)	EC_{50} (nM)	Max (%)	IC_{50} (nM)
Nociceptin	12	100	1.5	240	100	180	32	100	0.73	770	100	ND
Ac-RYYRIK-NH$_2$	19	88	0.76	78	75	2.8	13	89	1.3	74	105	ND
F/G-NC	100	92	1200	300	55	1900	140	81	1700	1400	57	ND

Characterization of NR-Gα_i—Wild-type and mutant receptors were used to construct NR-Gα_i fusion proteins for measuring the activity and affinity for NR ligands, nociceptin, F/G-NC and Ac-RYYRIK-NH$_2$. Using these fusion proteins, the receptor activity was measured based on the stimulation of [^{35}S]GTPγS-binding activities that depended on ligand concentrations (Fig. 6). The wild-type NR-Gα_i fusion protein was activated by nociceptin, Ac-RYYRIK-NH$_2$, and F/G-NC with EC_{50} value of 12, 19 and 100 nM, respectively. Specific [^{3}H]nociceptin binding was also detected in membrane preparations expressing the wild-type NR-Gα_i. To compare the affinities for the three ligands, a displacement assay with cold ligands was performed in the presence of 20 nM [^{3}H]nociceptin. The IC_{50} values of the wild-type NR-Gα_i for nociceptin, Ac-RYYRIK-NH$_2$ and F/G-NC were 1.5, 0.76 and 1200 nM, respectively (Table 1).

The replacement of Thr138 in TM3 with alanine resulted in more serious effect for nociceptin than for the other two ligands. The EC_{50} for nociceptin increased 20-folds (240 nM) in the [^{35}S]GTPγS-binding assay, and those for Ac-RYYRIK-NH$_2$ and F/G-NC increased 4- (78 nM), and 3-folds (300 nM), respectively (Table 1). Eventually, EC_{50}s for nociceptin and F/G-NC were essentially the same for Thr138Ala. IC_{50}s for [^{3}H]nociceptin displacement experiments also exhibited almost the same tendency (Table 1). These results indicated that the Thr138 mutation was effective but not essential for ligand binding. The Ser223Ala mutation in TM5 did not significantly affect the pharmacological properties

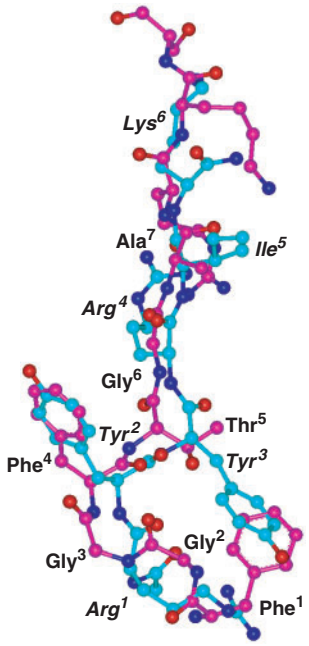

Fig. 5. **Superimposed structures of the receptor-bound conformations of nociceptin and Ac-RYYRIK-NH_2 in form III.** The carbon atoms of nociceptin and Ac-RYYRIK-NH_2 are coloured magenta and cyan, respectively. The amino acid residues of Ac-RYYRIK-NH_2 are indicated by italicized three-letter codes.

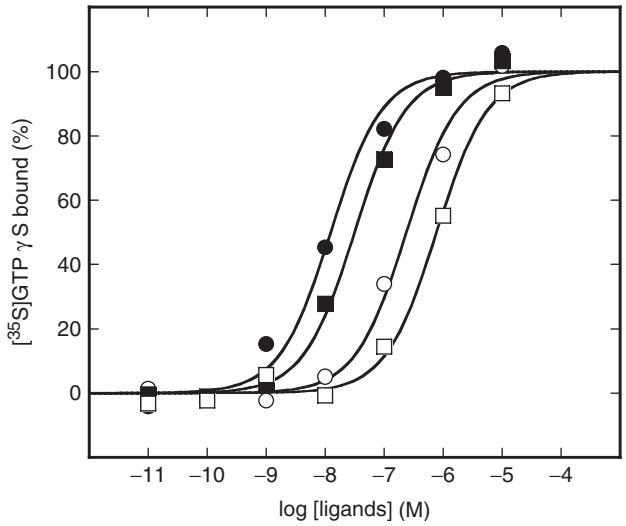

Fig. 6. **Nociceptin-dependent stimulation of [^{35}S]GTPγS binding to the wild-type and mutant NR-Gα_i.** Wild type (filled circles), Thr138Ala (open circles), Ser223Ala (filled squares) and Gln280Ala (open squares). Ten micrograms of membrane fractions expressing NR-Gα_i fusion proteins were incubated in 100 µl of 20 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 160 mM NaCl, 1 mM dithiothreitol, 100 pM [^{35}S]GTPγS, 10 µM GDP, and 10 mM $MgCl_2$ at 30°C for 30 min. [^{35}S]GTPγS bound to the membrane was trapped on a GF/B glass fiber filter, washed three times with 300 µl of cold 20 mM potassium phosphate buffer (pH 7.2), and counted with a liquid scintillation counter. The maximum activated level of [^{35}S]GTPγS binding is defined as 100%. The representative data of three separate experiments are shown.

of the three ligands. In contrast, the Gln280Ala mutation in TM6 dramatically decreased the affinity for the three ligands (Table 1). The EC_{50}s for nociceptin, Ac-RYYRIK-NH_2, and F/G-NC increased 64- (770 nM), 4- (74 nM) and 14-folds (1400 nM), respectively. In a Gln280Ala mutant, no specific binding was observed by using 20 nM [^{3}H]nociceptin. Therefore, Gln280 was essentially important for binding to these ligands (Table 1).

DISCUSSION

At present, computer modelling of GPCRs and ligand-bound structures is becoming useful to understand the molecular events during receptor recognition and for designing new pharmacological ligands. However, its use is still limited due to the lack of sufficient structural information of receptors. The available crystal structure of rhodopsin is applicable only for modelling inactive (inverse agonist bound) receptor structures. Thus, the structures of activated receptor are deduced from the rhodopsin structure based on biochemical and biophysical experimental observations. Several classical biogenic amine receptors such as muscarinic acetylcholine, dopamine and adrenergic receptors show a few functionally different properties depended on a bound ligand as same as rhodopsin photointermediates. We previously reported that NR also shows three stepwise activation stages, namely, an antagonist bound form, a partial agonist bound form, and a full agonist bound form (*16*). It is reasonable to consider that each functionally different state corresponds to a peculiar structure and this stepwise activation theory is accepted not only to biogenic amine receptors but also to almost of all GPCRs. In another words, conformational changes of rhodopsin during its activation should be general transformation scheme for many GPCRs. Thus, rhodopsin structure and its conformers during activation are suitable base models for construction of every NR structural models. During the building of ligand–receptor complex models, we often find a few candidate conformations of ligands that have almost the same energy levels. Site-directed mutagenesis is frequently applied to evaluate the predicted candidates.

In this study, we constructed activated NR models and ligand–receptor complexes with three agonists. Forms III and IV corresponded to the partial agonist-bound form and the full agonist-bound form, respectively, of the biogenic amine receptors reported previously. Modes 1 and 2 of the nociceptin–receptor complexes corresponded to two types of ligand conformation and differed mainly in terms of three residues at the N-terminal. Since the peptide ligands are larger than non-peptide ligands, limited positions and angles were allowed for peptide fragments at the site estimated as a ligand-binding site. Systematic conformational search for the N-terminus Phe^1 for nociceptin by placing the positively charged group proximal to the negatively charged Asp130 in TM3 afforded only two candidate conformations. The Gly^2- Arg^8 portion was placed in a narrow cave, where the ligand-binding site was estimated and allowed highly limited possibilities for initial conformations. Eventually, these initial conformations of the

fragment was optimized by molecular dynamics calculations and reached to the essentially two models that corresponded to modes 1 and 2, because of the highly limited space for the binding site. The remaining portion of nociceptin was localized at the surface of the receptor and thus had no sterical limitations with no influence on the conformation of the N-terminal Phe^1- Arg^8 portion that was surprisingly found to form a similar 3D shape with Ac-RYYRIK-NH_2 in this study (Fig. 5)

To investigate the difference between forms III and IV, we mutated Gln280 of NR to alanine. Our receptor–ligand-complex models suggest that Gln280 in TM6 participated in the recognition of the backbone amides of the three agonists in form III but not in form IV. Because of the rigid-body rotation of TM6 in form IV, Gln280 could not locate the binding site in form IV. Therefore, a mutation at Gln280 should have critical effects on the recognition of a ligand, only if the receptor–ligand complexes had a form III conformation. A previous mutational experiment showed that Gln280 mutation to histidine weakly affected nociceptin binding (*20*). This result indicated that Gln280 contributed to interactions with nociceptin and that form III is a nociceptin-bound form of NR. However, this could not be conclusively decided by the Gln280His mutation, since histidine side chain could work as a proton donor as same as an amide group of glutamine. The present mutation experiment with Gln280Ala clearly shows that Gln280 is crucial for the binding of all the three ligands. Taking into account previous data, a proton donor is required to bind nociceptin; thus, this is in accordance with the present complex model in form III. Therefore, the present binding experiments of the three ligands indicate that the three peptide ligands bind form III and that the activation of NR does not accompany the rigid-body rotation of TM6 in agonist binding. This fact is unique because form III corresponds to the partial agonist-bound receptor structure of the biogenic amine receptors; further, a κ-opioid receptor was reported to have the rigid-body rotation of TM6 during agonist binding and receptor activation (*7*).

Fortunately, several mutation results have already been reported for NR. We, therefore, also referred to previous point-mutation results for NR to distinguish the resulting structures and confirm the predicted models. Two phenylalanine residues of nociceptin (Phe^1 and Phe^4) form an aromatic cluster with Phe220, Phe224 and Trp276 of NR in the model (Fig. 2), suggesting that the absence of one of the aromatic residues may lead to a reduction in nociceptin-binding affinity (*18*). The mutation of Tyr309Phe is also believed to reduce nociceptin-binding affinity (*21*), since Tyr309 forms a hydrogen bond with Asp130, which is essential to nociceptin binding as described earlier. It is reasonable to assume that other mutations such as Thr277Ala and Arg302Asp (*21*) indirectly influence the nociceptin-binding affinity in the model because these residues are in contact with the ligand-binding residues. The resulting form III-NR-nociceptin complex structure clearly explains the previous point-mutation experiments for NR.

We obtained two conformations for Ac-RYYRIK-NH_2 from our structural modelling. It was assumed that Ser223 in our model was involved in the recognition of the N-terminal acetamide moiety of Ac-RYYRIK-NH_2 in the first conformation (Fig. 4A) in form III but not in the second conformation (Fig. 4B). The analysis of a Ser223Ala mutant was decisive in comparing these two conformations and indicated that the Ser223 mutation did not influence NR activity. Therefore, the acetamide moiety is considered not to interact with Ser223 but to form a hydrogen-bond network that connects the acetamide carbonyl, Arg^1 side chain and Asp130 (Fig. 4B). The lack of the acetyl group would impede the network due to the unfavourable positive charge of the N-terminal amine. This consideration is consistent with a previous report that the lack of acetamide at the N-terminal position (*i.e.* NH_2-RYYRIK-NH_2) led to a 90-fold decrease in affinity (*22*). It is interesting that Ser223 is not involved in the ligand recognition of NR although in TM5, it is located at an analogous position of the highly conserved serine in the biogenic amine receptors that recognize the catechol ring of adrenergic ligands.

The conformation of the receptor-bound Ac-RYY fragment showed a quite intriguing structural correlation with that of the N-terminal FGGF fragment of nociceptin, although their sequences are completely different from each other as shown in Fig. 5. In their conformations, the guanidium and the remaining side chain of Arg^1 of Ac-RYYRIK-NH_2 correspond to the N-terminal cationic amine and the Gly^2-Gly^3 fragment of nociceptin, respectively. Further, two aromatic groups of Tyr^2 and Tyr^3 of Ac-RYYRIK-NH_2 occupied spaces similar to those of the aromatic groups of Phe^4 and Phe^1 of nociceptin, respectively. The phenol hydroxyl group of Tyr^2 of Ac-RYYRIK-NH_2 was hydrogen bonded to the backbone carbonyl of Val283 at the C-terminus of TM6. While Gln280 forms two hydrogen bonds with the backbone carbonyls of Gly^2 and Gly^3 of nociceptin, the backbone carbonyl of Arg^1 of Ac-RYYRIK-NH_2 is only a hydrogen bond acceptor of Gln280. Thus, the hydrogen bond between Tyr^2 of Ac-RYYRIK-NH_2 and the backbone carbonyl of Val283 would be important for the ligand-receptor recognition. This structure may be consistent with the previous mutation results that Phe^1 is convertible to Tyr^1 of nociceptin (*23*), but Tyr^2 of Ac-RYYRIK-NH_2 is not convertible to Phe (*24*). There is another glutamine residue, Gln286, at the C-terminal end of TM6. It is reported that an alanine mutation at Gln286 does not influence the nociceptin-binding activity but is crucial for the activation of the receptor (*18*). Since the present receptor model suggests that Gln286 is located far from the ligand-binding site, the residue may not be involved in the conformation of the proximal N-terminal portion of TM3 that constructs the ligand-binding site. On the other hand, C-terminal end of TM6 would affect for an interaction and signal transduction to a G protein. Therefore, we surmise that the mutation from bulky Gln286 to a small alanine at the C-terminal end of TM6 has a serious effect for an expression of a receptor activation, but not for a ligand-binding activity.

Based on our analysis, we conclude that complex form III is a suitable structure for ligand–receptor complexes and that mode 2 is a suitable conformation of nociceptin in the ligand–receptor complex. From these structural models, we successfully explain that nociceptin and

Ac-RYYRIK-NH_2 can form similar 3D shapes in receptor-bound conformations, although their amino acid sequences are completely different. This result clarifies why both nociceptin and Ac-RYYRIK-NH_2 can potently activate NR.

Finally, we attempted to predict mutation effects at an indirect position for the ligand binding in the nociceptin–receptor complex. It would be important to investigate the mutation effects in the surrounding region of the ligand-binding pocket to evaluate a predicted structure. Thus, we focused on residues that would indirectly affect ligand binding and found that the sulphur atom of Met134 in TM3 is proximal to the carbonyl oxygen of the Phe^1-Gly^2 peptide bond in nociceptin and Thr138 (Fig. 2). The alanine mutation of Thr138 was thought to affect the conformation of Met134, thereby reducing the sulphur–oxygen interaction (*25*) between Met134 and the carbonyl oxygen of Phe^1. In addition, F/G-NC does not have this Phe^1 carbonyl oxygen, and the Thr138Ala mutation was expected to affect the binding of F/G-NC to a lesser extent than that of nociceptin. In fact, this mutation extensively affected nociceptin binding but weakly influenced F/G-NC binding (Table 1). These evidences suggest that the backbone carbonyl oxygen of Phe^1 is involved in nociceptin binding, probably through the binding of Met134 and Thr138 that indirectly contributes to keep nociceptin bound conformation. Tyr131 is also close to Met134 and indirectly contributes to the nociceptin binding in the model. The previous results of mutation at Tyr131 are interpreted in the same manner as the mutation effect at Thr138, and support the model (*18*).

In summary, we proposed a few structural models of a nociceptin–receptor complex by molecular modelling. Based on site-directed mutagenesis studies, we conclude that form III—a partial agonist-bound form of the biogenic amine receptors—is the most plausible receptor structure among all the structures, and that nociceptin binds with mode 2 conformation to form III of NR. The previous and present mutation experiments clearly indicate that the form III-NR-nociceptin bound structure is reasonable. Thus, we consider that NR is activated without the rigid-body rotation of TM6, in contrast to the κ-opioid receptor that is homologous to NR in the primary structure. We hope that the successful modelling for receptor bound 3D structures of nociceptin and Ac-RYYRIK-NH_2 will lead us to design novel NR ligands.

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