Mutagenesis and Modeling of the Neurotensin Receptor NTR1

IDENTIFICATION OF RESIDUES THAT ARE CRITICAL FOR BINDING SR 48692, A NONPEPTIDE NEUROTENSIN ANTAGONIST

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The two neurotensin receptor subtypes known to date, NTR1 and NTR2, belong to the family of G-protein-coupled receptors with seven putative transmembrane domains (TM). SR 48692, a nonpeptide neurotensin antagonist, is selective for the NTR1. In the present study we attempted, through mutagenesis and computer-assisted modeling, to identify residues in the rat NTR1 that are involved in antagonist binding and to provide a tentative molecular model of the SR 48692 binding site. The seven putative TMs of the NTR1 were defined by sequence comparison and alignment of bovine rhodopsin and G-protein-coupled receptors. Thirty-five amino acid residues within or flanking the TMs were mutated to alanine. Additional mutations were performed for basic residues. The wild type and mutant receptors were expressed in COS M6 cells and tested for their ability to bind (125I-NT and [3H]SR 48692. A tridimensional model of the SR 48692 binding site was constructed using frog rhodopsin as a template. SR 48692 was docked into the receptor, taking into account the mutagenesis data for orienting the antagonist. The model shows that the antagonist binding pocket lies near the extracellular side of the transmembrane helices within the first two helical turns. The data identify one residue in TM 4, three in TM 6, and four in TM 7 that are involved in SR 48692 binding. Two of these residues, Arg327 in TM 6 and Tyr351 in TM 7, play a key role in antagonist/receptor interactions. The former appears to form an ionic link with the carboxylic group of SR 48692, as further supported by structure-activity studies using SR 48692 analogs. The data also show that the agonist and antagonist binding sites in the rNTR1 are different and help formulate hypotheses as to the structural basis for the selectivity of SR 48692 toward the NTR1 and NTR2.

Many neuropeptide receptors belong to the family of GTP-binding protein-coupled receptors (GPCRs) with seven putative transmembrane domains (TMs). The past decade has witnessed the discovery of a number of nonpeptide antagonist ligands of GPCRs for neuropeptides such as cholecystokinin, tachykinins, or angiotensin (1–3). Most often, these compounds are selective for a given neuropeptide receptor subtype and, unlike peptides, cross the blood-brain barrier. As such, they have proven extremely useful for exploring the central and peripheral physiopathological functions associated with the receptor they antagonize.

It seems to be a general feature that peptide agonist and nonpeptide antagonist bind to distinct epitopes of GPCRs. Site-directed mutagenesis of GPCRs, like vasopressin, tachykinins, or angiotensin receptors, has allowed for the determination of several conserved or specific residues that are involved in agonist and/or antagonist binding (4–6). In some studies, mutagenesis data were complemented by computer-assisted modeling to construct tridimensional models of ligand-receptor complexes (7). This approach has led to the proposal of spatial models for the binding sites of a number of GPCRs that interact with small nonpeptide ligands (4, 8, 9). Multiplication of such tridimensional models of GPCRs, depicting the binding site for nonpeptide antagonists, would define a general binding epitope for these small nonpeptide ligands. This approach will be of great interest for the design of new selective high affinity molecules.

The neuropeptide neurotensin (NT) exerts central actions that include hypothermia (10), analgesia (11), and a number of effects that involve the modulation of nigrostriatal and mesocortico-limbic dopaminergic pathways (12, 13). Two NT receptors termed NTR1 and NTR2 (or NTRH and NTRL) have been cloned so far (14–17). They share 60% homology and both belong to the family of GPCRs. The NTR1 has high affinity for NT, whereas the NTR2 has lower affinity for the peptide and is selectively recognized by levocabastine, an anti-histamine H1 receptor antagonist (14–17). These receptors have widespread, though not identical, central and peripheral distributions and exhibit distinct ontogenic profiles (15, 16). SR 48692, a nonpeptide NT antagonist (18), preferentially binds to the NTR1 (16, 17) and has provided a useful tool to define the functions associated with this receptor (19). In particular, SR 48692 blocks many of the effects attributed to NT interaction with mesencephalic dopaminergic neurons (20). In contrast, it does not antagonize the hypothymic and analgesic responses to NT, suggesting that these effects are not initiated through the NTR1 (21).

Preliminary data have indicated that NT and SR 48692 bind to different regions of the NTR1 (22). In the present study we attempted, through mutagenesis and computer-assisted modeling, to identify residues in the NTR1 that are involved in antagonist binding and to provide a tentative molecular model of the SR 48692 binding site. Because of the hydrophobic and aromatic nature of SR 48692 (Fig. 1), we initially chose to...
Structure of SR 48692, SR 48527, and SR 49711. The main chemical features of these compounds are an adamantane moiety (A) in SR 48692 replaced by a cyclohexane ring (A') in SR 48527 and SR 49711, and pyrazole (B), dimethoxyphenyl (C), and quinolyl (D) structures found in all three antagonists. Note that the carbon atom that bears the COOH acidic function becomes asymmetrical in the enantiomers SR 48527 and SR 49711.

SR 48692: R1R2 = A
SR 48527: R1 = ● A', R2 = H
SR 49711: R1 = H, R2 = ● A'

Fig. 1. Structure of SR 48692, SR 48527, and SR 49711. The main chemical features of these compounds are an adamantane moiety (A) in SR 48692 replaced by a cyclohexane ring (A') in SR 48527 and SR 49711, and pyrazole (B), dimethoxyphenyl (C), and quinolyl (D) structures found in all three antagonists. Note that the carbon atom that bears the COOH acidic function becomes asymmetrical in the enantiomers SR 48527 and SR 49711.

mutate hydrophilic and aromatic residues present in or close to the putative TMs of the NTR1 (Fig. 2). The wild type and mutant receptors were expressed in COS M6 cells and tested for their ability to bind [125I]-NT and [3H]SR 48692. A first tridimensional model of the SR 48692 binding site in the NTR1 was constructed using bacteriorhodopsin as a template (23). SR 48692 was docked into the receptor, taking into account the mutagenesis data for orienting the antagonist in the transmembrane region. This initial model pointed to a number of hydrophilic residues (Met, Thr, His, Glu, Lys, and Arg) that might also interact with SR 48692 (Fig. 2), thus leading us to construct and test a second series of mutant receptors.

Bacteriorhodopsin, though not a GPCR, was at the time we initiated these studies the only protein with seven transmembrane α-helices whose tridimensional structure was known with some resolution (23). Recently, structural data on the arrangement of the transmembrane helices of rhodopsin, the prototype GPCR, has become available (24, 25). Therefore, the representation of the SR 48692-NTR1 complex reported here was achieved by using rhodopsin instead of bacteriorhodopsin as a template for modeling the NTR1 and by positioning SR 48692 into the core of the seven helices so as to fit the mutagenesis data. Our model identifies a number of residues in TMs 6 and 7 that are involved in SR 48692 binding. Two of these residues, Arg

SR 48692: R1R2 = A
SR 48527: R1 = ● A', R2 = H
SR 49711: R1 = H, R2 = ● A'

Computer-assisted Modeling—The nTR1 sequence was aligned by means of the “Viseur” software engine with those of the hNTR1, the nNTR2, and mNTR2, other GPCRs, and bovine rhodopsin (25) to define by sequence comparison the regions in the nTR1 that would most likely correspond to the seven putative TMs thought to exist in all such receptors. These domains are shown in Fig. 2. A three-dimensional model of the TMs of the nTR1 was built using the Sybyl program by the Powel method with a dielectric constant value of 4 and with fixed Kollman charges and essential hydrogen atoms, while freezing the helical backbone.
RESULTS

Mutation of Aromatic and Hydrophobic Residues in the rNTR1—Sixteen aromatic and 6 hydrophobic residues located within TMs 1–7 or near the junction between the TMs and the extracellular domains of the rNTR1 were mutated into Ala (Fig. 2 and Table I). The wild type and mutant receptors were expressed in COS M6 cells, and saturation experiments with 125I-NT or [3H]SR 48692 were performed with membranes prepared from each transfectant. Kd values for both ligands are shown in Table I. The Y351A mutant, the Y324A and F331A mutants, and the Y359A mutant exhibited 200-fold, 20-fold, and 5-fold increases in agonist affinity of the F331A and Y351A mutants observed in saturation experiments.

Mutation of Hydrophilic Residues—An initial model of the rNTR1 was constructed using bacteriorhodopsin as a template. The model also predicted that a number of hydrophilic residues might interact with the antagonist. In particular, it suggested that the carboxylate function of the antagonist might form an ionic link with either one of four basic residues in the extracellular domains of the rNTR1, i.e. Arg124 in TM 3, Lys238 in TM 4, His243 in TM 5, His348 and Thr354 in TM 7), which are conserved between the rNTR1 and the hNTR1, rNTR2, mNTR2, and bovine rhodopsin (66). The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.

The residues conserved between the rNTR1, hNTR1, rNTR2, mNTR2, and bovine rhodopsin (66). The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.

The residues conserved between the rNTR1, hNTR1, rNTR2, mNTR2, and bovine rhodopsin (66). The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.

![Fig. 2. Alignments of the sequences in the seven transmembrane domains of rNTR1, hNTR1, rNTR2, mNTR2, and bovine rhodopsin (66). The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.](image)

**TABLE I**

| TM | Mutation | Kd (nM) | Neurtensin | SR 48692 |
|----|----------|---------|------------|----------|
| 6  | Wild type | 0.12 ± 0.02 | 2.60 ± 0.20 |
|    | TM1      | 0.11 ± 0.01  | 2.21 ± 0.12  | 19  |
|    | TM3      | 0.16 ± 0.03  | 4.42 ± 1.12  | 124 |
|    | TM5      | 0.08 ± 0.02  | 2.36 ± 0.25  | 237 |
|    | TM6      | 0.12 ± 0.01  | 4.21 ± 0.66  | 238 |
|    | TM7      | 0.12 ± 0.01  | 3.14 ± 0.55  | 243 |
|    | F331A    | 0.28 ± 0.03  | 106 ± 2.0  |
|    | F333A    | 0.20 ± 0.05  | 2.86 ± 0.61  | 331 |
|    | F346A    | 0.22 ± 0.02  | 3.12 ± 0.42  | 347 |
|    | F349A    | 0.45 ± 0.10  | 3.02 ± 0.77  | 350 |
|    | F350A    | 0.23 ± 0.03  | 5.37 ± 1.19  | 351 |
|    | F358A    | 0.13 ± 0.04  | 8.94 ± 1.19  | 352 |
|    | F359A    | 0.41 ± 0.13  | 29.1 ± 1.6  |
|    | F359A    | 0.40 ± 0.09  | 29.2 ± 2.2  |

* Kd values in nm were derived from competition binding experiments with unlabelled NT and SR 48692 as the competitors and 125I-NT as the labeled ligand. The values are the means ± S.E. of three to five experiments. ND, not determinable.

and the Y359A mutant exhibited 200-fold, 20-fold, and 5-fold increases in Kd values for SR 48692, respectively, as compared with the wild type rNTR1. The data also confirmed the decreased antagonist affinity of the F358A mutant and the decreased agonist affinity of the F331A and Y351A mutants observed in saturation experiments.

To assess the above predictions, additional mutant receptors were constructed and tested in binding and pharmacological experiments. The M204A mutation did not significantly affect the binding affinity of either SR 48692 or NT (Table II). In contrast, the M208A mutant exhibited a 10-fold increase in Kd for the antagonist and a 6-fold increase in Kd for NT, as compared with the wild type rNTR1 (Table II). The T354A mutation of the rNTR1 complex predicted that a number of hydrophilic residues might interact with the antagonist. In particular, it suggested that the carboxylate function of the antagonist might form an ionic link with either one of four basic residues in the extracellular domains of the rNTR1, i.e. Arg124 in TM 3, Lys238 in TM 4, His243 in TM 5, His348 and Thr354 in TM 7). These residues are conserved between the rNTR1 and the hNTR1, rNTR2, mNTR2, and bovine rhodopsin (66). The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.

The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.

The residues conserved between the rNTR1 and the other proteins are represented by periods. The TMs are numbered in circles on the right. They are oriented so that their extracellular side face the left of the figure. Shaded residues are those that were mutated in the present study.
mutant showed a 5–6-fold decrease in SR 48692 affinity and no change in NT affinity. The T68A, T156A, Q236A, T242A, H325A, and H348A mutants bound SR 48692 and NT with the same affinity as the wild type rNTR1.

Mutating Arg143 to Lys, Gln, or Met; Lys 235 to Ala, Gln, or Arg; and Arg328 to Met did not affect the affinity of the rNTR1 for either SR 48692 or NT (Table II). In contrast, the R327M and R327E mutants were devoid of both antagonist and agonist binding (Table II). The latter observation could mean either that Arg327 is essential for antagonist and agonist binding or that the mutated receptors were not expressed in COS M6 cells.

Effect of SR 48692, SR 48527, and SR 49711 on NT-stimulated IP production in COS M6 cells expressing either the wild type rNTR1 or the R327M mutant. Concentration-response curves for NT-stimulated IP production (A) and inhibition of NT-stimulated IP production by SR 48692 (B), SR 48527 (C), and SR 49711 (D) in cells transfected with the wild type rNTR1 (open symbols) and the R327M mutant (closed symbols). In B–D, cells were stimulated with 1 nM and 30 μM NT for the wild type and the mutant receptor, respectively, and basal IP production has been subtracted from the data. The values are the means ± S.E. of three to five independent experiments.

### Table II

| TM  | Mutation | Kᵣ, Kᵢ* (nM) | Neurotensin | SR 48692 |
|-----|----------|---------------|-------------|----------|
|     | Wild type | 0.12 ± 0.02   | 2.60 ± 0.20 |          |
| TM1 | T68A     | 0.18 ± 0.01*  | 5.88 ± 0.11 |          |
| TM3 | R143K    | 0.11 ± 0.01   | 1.55 ± 0.29 |          |
|    | R143Q    | 0.13 ± 0.03   | 1.77 ± 0.32 |          |
|    | R143M    | 0.09 ± 0.04   | 2.75 ± 0.62 |          |
|    | T156A    | 0.22 ± 0.05   | 2.52 ± 0.27 |          |
| TM4 | M204A    | 0.20 ± 0.06   | 4.56 ± 0.45 |          |
|    | M208A    | 1.26 ± 0.26   | ND          |          |
|    | 1.91 ± 0.59* | 56.0 ± 17.0 |          |          |
| TM5 | K235A    | 0.18 ± 0.04   | 2.70 ± 0.52 |          |
|    | K235Q    | 0.32 ± 0.11   | 4.11 ± 0.77 |          |
|    | K235R    | 0.18 ± 0.07   | 2.91 ± 0.35 |          |
|    | Q239A    | 0.25 ± 0.09   | 4.85 ± 0.63 |          |
|    | T242A    | 0.26 ± 0.15   | 4.32 ± 1.29 |          |
| TM6 | H325A    | 0.10 ± 0.01   | 1.84 ± 0.16 |          |
|    | R327M    | ND            | ND          |          |
|    | R327E    | ND            | ND          |          |
|    | R328M    | 0.28 ± 0.04   | 5.00 ± 0.80 |          |
| TM7 | H348A    | 0.22 ± 0.02   | 1.71 ± 0.34 |          |
|    | T354A    | 0.33 ± 0.11   | 13.1 ± 1.4  |          |

* Kᵢ values in nM were derived from competition binding experiments with unlabeled NT and SR 48692 as the competitors and ¹²⁵I-NT as the labeled ligand. The values are the means ± S.E. of three to five experiments. ND, not determinable.
COS M6 cells were transfected with the wild type rNTR1 and the R327M mutant and NT-stimulated IP production was measured as described under “Experimental Procedures.” EC₅₀ values for NT are derived from Fig. 4, respectively. The values are the means ± S.E. of three to five experiments.

|          | Wild type | R327M   |
|----------|-----------|---------|
| NT, EC₅₀ | 0.57 ± 0.20 | 11,900 ± 3300 |
| SR 48692, Kᵢ | 2.45 ± 0.60 | 1570 ± 470 |
| SR 48527, Kᵢ | 24.4 ± 6.3 | 1670 ± 610 |
| SR 49711, Kᵢ | 11,200 ± 2900 | 14,900 ± 7600 |

The large decrease in potency of SR 48692 observed with the R327M mutant receptor is compatible with the side chain of Arg³²⁷ making an ionic link with the carboxylic function of the antagonist. To assess this hypothesis, two enantiomeric SR 48692 analogs, SR 48527 and SR 49711, were tested for their ability to inhibit NT-stimulated IP production in COS M6 transfected with either the wild type rNTR1 or the R327M mutant receptor. SR 48527 and SR 49711 are identical to SR 48692 except for the adamantane moiety, which is substituted by a cyclohexane group, thus rendering asymmetrical the carbon atom that bears the carboxylic function (Fig. 1). Previous studies have shown that SR 48527 and SR 49711 bind with 10- and 1000-fold lower affinity than SR 48692 to the rNTR1, respectively (22). Recent x-ray crystallographic studies have revealed that the spatial disposition of all the main chemical groups in SR 48692 and SR 48527 is similar whereas SR 49711 differs from its two analogs by the carboxylic function, which points in opposite direction (29). Therefore, we reasoned that the R327M mutation should much more greatly affect the interaction of SR 48527 than that of SR 49711 with the rNTR1. The results presented in Fig. 4 and Table III are fully consistent with this assumption. Both enantiomers antagonized NT-stimulated IP production in wild type rNTR1-expressing COS M6 cells with potencies that closely matched their reported binding affinity (22). In cells expressing the R327M mutant, the potency of SR 48527 to antagonize NT-stimulated IP production was reduced by a 100-fold, whereas that of SR 49711 was barely affected.

**Model of the SR 48692-rNTR1 Complex**—A model of the rNTR1 was constructed using the recently published tridimensional structure of rhodopsin as a template (24, 25). SR 48692 was then docked into the receptor, taking into account the mutagenesis data described above. Our current representation of the SR 48692/rNTR1 complex is depicted in Fig. 5. The antagonist binding pocket is located in the upper part of the transmembrane region with the adamantane group of SR 48692 being at the level of the cell surface and the rest of the molecule lying no deeper than two helical turns inside the core of the TMs. Most of the residues identified as participating to the binding of SR 48692 are found within TMs 6 and 7. Taking into account the loss in antagonist affinity caused by mutating these residues, interactions between the antagonist and the receptor can be described as follows: 1) the adamantane cage of the antagonist is oriented toward the extracellular side of the membrane and makes hydrophobic interactions with the side chain of Met³⁰⁸ and Phe³³ⁱ; 2) the carboxylic function of SR 48692 makes an ionic interaction with the guanidinium group of Arg³²⁷ in TM6; 3) the pyrazole group makes π–π interactions with Tyr³⁵¹; 4) the chloroquinolinyl moiety resides in the plane of the membrane and makes π–π interactions with Tyr³³⁴; and 5) the dimethoxyphenyl moiety points toward the intracellular side of the membrane and makes π–π interactions with the side chain of Tyr³⁵¹ oriented perpendicular to it, a hydrogen bond between one of its methoxy groups and Tyr³⁵¹, a hydrogen bond between the other methoxy group and both Tyr³³⁴ and Tyr³³⁵, and hydrophobic and/or π–π interactions with the side chains of Phe³⁵⁸ and possibly Tyr³⁵⁹.

**DISCUSSION**

In the present study, we have made use of two complementary approaches in an attempt to visualize at the molecular level the interaction of SR 48692, a nonpeptide NT antagonist, with the rNTR1. The first approach entailed computer-assisted modeling of the rNTR1 according to a method that has been described for a number of GPCRs (7). One important step in the
method consists in attributing to GPCRs the known tridimensional structure of bacteriorhodopsin (23). However, this has been criticized, because bacteriorhodopsin, although a protein with seven transmembrane helices, is not a GPCR. Quite recently, the tridimensional helical packing of rhodopsin, a true GPCR, was reported (24, 25). We therefore used rhodopsin as a template in constructing our model of the rNTR1. The second approach involved mutagenesis of the rNTR1 to find out residues that might interact with SR 48692. This helped docking the antagonist into the receptor, which led to the model proposed in Fig. 5 for the antagonist-receptor complex. It should be pointed out that our model was constructed based on the assumption that the residues for which mutation decreases SR 48692 potency are involved in molecular interactions with the antagonist. Although there is strong evidence that this is so for Arg327, the data to support the other interactions are more indirect. Some of these aspects will be further discussed below.

All the residues identified here as participating to the binding of the antagonist, except Met208, were found within TM6 and 7 or lying at the junction between these TM and the third extracellular loop. This appears to be a common feature of peptide receptors with respect to nonpeptide antagonist binding. Thus, previous studies have shown that nonpeptide antagonists of the neurokinin NK1, cholecystokinin CCK-B, and angiotensin AT1 receptors bind to epitopes that are located within and/or at the junction between extracellular loops and transmembrane helices (5, 30–34). Furthermore, TM6 and 7 are often involved in the binding of the small nonpeptide ligands (5, 30–34). Another common feature of peptide GPCRs lies in the different binding epitopes found for peptide agonists and nonpeptide antagonists. This has been demonstrated for the NK1, NK2, CCK-B, AT1, and vasopressin V1a receptors (4, 5, 30–36). Our present data conform with this property. Thus, a number of mutations that affected SR 48692 binding (Y324A, Y351A, T354A, F358A, and Y359A) had no or little effect on NT binding. Interestingly, mutating Tyr347, which lies close to Tyr351 in the third extracellular loop, had the opposite effect. Thus, in competition binding experiments with [3H]SR 48692, NT exhibited a Ki value of 34 μM. This shows that the third loop-TM7 junction contains two tyrosine residues in close spatial proximity that are essential for and discriminate between antagonist and agonist binding. These data suggest that the decreased SR 48692 affinity for the Y351A mutant reflects a direct interaction of Tyr351 with the antagonist rather than a perturbation of the receptor conformation. Indeed, a change in conformation in the vicinity of Tyr351 might be expected to modify the side chain orientation of the nearby Tyr347 residue, which is essential for NT binding and, hence, to markedly alter agonist affinity. The 300-fold decrease in SR 48692 affinity of the Y351A mutant receptor is compatible with the proposed π-π and hydrogen bond interactions of Tyr351 with the dimethoxyphenyl and pyrazole groups of the antagonist. Structure-activity studies using SR 48692 analogs bearing structural modifications of these chemical moieties should prove useful to confirm the role of Tyr351 in antagonist binding.

The R327M mutant receptor was devoid of measurable SR 48692 and NT binding. However, when expressed in COS M6 cells, it retained the ability to respond to NT by an increased IP production. Although the potency of NT was reduced by more than 4 orders of magnitude with the mutant as compared with the wild type receptor, the maximal NT stimulation was similar for both receptors, suggesting that they were expressed at comparable levels. Indeed, we have observed with the wild type rNTR1 that maximally stimulated IP production is directly related to the amount of transfected receptor. In addition, this shows that the R327M mutant is accessible to NT and functional, and, therefore, provides indirect evidence that its conformation was not dramatically changed by the mutation. SR 48692 inhibited NT-stimulated IP production with a 600-fold decreased potency in COS M6 cells transfected with the R327M mutant as compared with the wild type receptor. Such a loss in potency is compatible with Arg327, making an ionic link with the carboxylic function of the antagonist, as proposed in our model. This proposal is further supported by the results obtained with the enantiomers SR 48527 and SR 49711. Both enantiomers differ mainly in the orientation of the COOH group borne by the asymmetric carbon, as shown by crystallographic studies (29). The acidic function is oriented similarly for SR 48692 and the active S isomer, SR 48527, whereas it points in opposite direction in the much less active R compound, SR 49711 (22, 29). The fact that the inhibitory potency of SR 49711 was not affected by the R327M mutation, in contrast to that of SR 48692 and SR 48527, strongly argues in favor of the existence of an ionic link between Arg327 and the carboxylic function of the antagonist. These data also point to a major role of Arg327 in the NT binding site. It is possible that Arg327 makes an ionic interaction with the C-terminal COOH group of NT, as this acidic function has been shown to be essential for NT binding (37). Studies are in progress to test this hypothesis.

In a recent study, the binding site of NT in the rNTR1 was proposed to lie mainly in the third extracellular loop, based on computer-assisted modeling of both the receptor and the peptide ligand (38). Furthermore, chimeric rat/human NTR1 constructs identified the third extracellular loop and the connecting TM6 and TM7 as being involved in determining species specificity toward NT analogs (39). It is noteworthy that TM6 and TM7 also contain most of the residues that are important for SR 48692 binding. This suggests that the binding sites of NT and SR 48692 in the rNTR1 are close to each other and possibly overlap, which might explain the observation that SR 48692 behaves as an apparent competitive antagonist of NT binding and NT-mediated effects (18).

Recently, a second NT receptor subtype designated NTR2 was cloned from rat and mouse brain (16, 17). This receptor belongs to the family of GPCRs and shares 60% homology with the rNTR1. The NT functions associated with this receptor are as yet unknown. SR 48692 binds with 10- and 100-fold lower affinity to the rNTR2 and mNTR2, respectively, than to the rNTR1 (16, 17). Therefore, SR 48692 shows selectivity for the NTR1. Interestingly, all the residues that were found in the present study to play a role in SR 48692 binding to the rNTR1

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3 C. Labbé-Jullié, unpublished observation.
4 S. Barroso, unpublished observations.
are conserved in the rNTR2 and mNTR2 sequences, except Phe331, which is substituted by a tyrosine residue in both NTR2 sequences. The F331A mutation in the rNTR1 results in a 20-fold decrease in SR 48692 affinity. In our model, Phe331 makes hydrophobic interactions with the aliphatic adamantane moiety of the antagonist. It is possible that adding a hydroxyl group to the aromatic ring of Phe331 might decrease these hydrophobic interactions and account, at least in part, for antagonist selectivity. This could be tested directly by making the F331Y mutation in the rNTR1 or the reciprocal Y320F mutation in the rNTR2. In addition, other residues yet to be identified might contribute to the affinity and selectivity of SR 48692 for the NTR1. The present model should be helpful to direct further mutagenesis studies of both the NTR1 and NTR2 and hopefully to develop new antagonists with high selectivity for each of these receptors. This in turn should greatly help in determining their respective contributions to the biological effects of NT.

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