Identification of Residues Involved in Neurotensin Binding and Modeling of the Agonist Binding Site in Neurotensin Receptor 1*

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The neurotensin receptor 1 (NTR1) subtype belongs to the family of G protein-coupled receptors and mediates most of the known effects of the neuropeptide including modulation of central dopaminergic transmission. This suggested that nonpeptide agonist mimetics acting at the NTR1 might be helpful in the treatment of Parkinson's disease and schizophrenia. Here, we attempted to define the molecular interactions between neurotensin-(8–13), the pharmacophore of neurotensin, and the rat NTR1. Mutagenesis of the NTR1 identified residues that interact with neurotensin. Structure-activity studies with neurotensin-(8–13) analogs identified the peptide residues that interact with the mutated amino acids in the receptor. By taking these data into account, computer-assisted modeling techniques were used to build a tridimensional model of the neurotensin-(8–13)-binding site in which the N-terminal tetrapeptide of neurotensin-(8–13) fits in the third extracellular loop and the C-terminal dipeptide binds to residues at the junction between the extracellular and transmembrane domains of the receptor. Interestingly, the agonist binding site lies on top of the previously described NTR1-binding site for the nonpeptide neurotensin antagonist SR 48692. Our data provide a basis for understanding at the molecular level the agonist and antagonist binding modes and may help design nonpeptide agonist mimetics of the NTR1.

Most neuropeptides and peptide hormones exert their effects through binding to receptors that belong to the family of G protein-coupled receptors (GPCR) with seven transmembrane (TMs) helices. In general, several GPCR subtypes have been identified for a given neuropeptide. Over the past decade, a number of nonpeptide antagonist ligands of neuropeptide GPCRs have been discovered, most often through random screening of large numbers of compounds (1, 2). As a rule, approaches have been useful for understanding the molecular basis of subtype or species selectivity of GPCRs for agonist and antagonist ligands. They have shown that most of the time peptide agonist and nonpeptide antagonist binding sites for a given receptor are topologically distinct (7–10) and sometimes have provided indications as to the molecular mechanisms by which an agonist may activate its receptor (11–15). Finally, they may assist in the rational design of selective nonpeptide ligands with agonist or antagonist properties.

Neurotensin (NT) is a 13-amino acid peptide that exerts neuromodulatory functions in the central nervous system and endocrine/paracrine actions in the periphery. Three NT receptors, termed NTR1, NTR2, and NTR3 according to the order in which they were cloned, have been identified so far (16–21). The NTR1 and NTR2 are GPCRs and share 60% homology, whereas the NTR3 belongs to an entirely different family of proteins (21). All three receptors bind NT through its C-terminal heptapeptide sequence -Arg-Arg-Pro-Tyr-Ile-Leu-OH (22). The NTR1 has high affinity for NT, whereas the NTR2 has lower affinity for the peptide and is selectively recognized by the anti-histamine H1 receptor antagonist levocetabamine. The nonpeptide NT antagonist SR 48692 preferentially binds to the NTR1. Many of the known central and peripheral effects of NT are blocked by SR 48692 and can therefore be attributed to the NTR1 (22–24). Recently, we provided evidence that the NTR2 mediates the SR 48692-insensitive central antinocisponsive effect of NT (25). The functions associated with NT binding to the NTR3 have yet to be elucidated.

In a recent study, using mutagenesis approaches combined with computer-assisted molecular modeling, we established a tridimensional model of the SR 48692-binding site in the rat NTR1 (26). Mutational analysis identified several residues in the receptor TMs that interact with the nonpeptide antagonist as follows: Met²⁰⁸ in TM4, Tyr²³⁴, Arg²³⁷, and Phe³¹¹ in TM6, and Tyr³⁶¹, Thr³⁶⁵, Phe³⁸⁵, and Tyr³⁹⁵ in TM7 (Fig. 1). A model of the rNTR1 was constructed using rhodopsin as a template. SR 48692 was then docked in the receptor model, taking into account the mutagenesis data. The antagonist binding site was found to lie within the first two helical turns of the TMs, facing the extracellular side of the membrane (26). These studies were facilitated by the fact that SR 48692 has a rather rigid structure in solution that has been elucidated by x-ray crystallography (27). For many purposes, it would be interesting to identify the agonist binding site in the NTR1 at the molecular level. This is rendered somewhat more difficult by the fact that the structure of NT-(8–13) is highly flexible and can adopt many conformations in solution (28, 29).

In a mutational study of the NT-NTR1 interaction in which
the charged residues in the extracellular domains and the TMs of the rNTR1 were substituted for glycyl residues, it was proposed that Asp\(^{339}\) in E1 might interact with the positive charges on the side chains of Arg\(^{8}\) and Arg\(^{9}\) in the NT sequence and that Arg\(^{221}\) in the upper part of TM3 might make an ionic link with the C-terminal carboxylate of NT (30). However, in the course of studying the SR 48692/rNTR1-binding site, we found that mutating Arg\(^{143}\) in Gln or Met did not alter the affinity of NT for the NTR1, precluding an ionic interaction of this residue with the C terminus of NT. We further observed that some of the residues that participate in the antagonist-receptor interaction (Met\(^{208}\), Arg\(^{327}\), and Phe\(^{331}\)) were also involved in NT binding. In addition, Tyr\(^{347}\) in the third extracellular loop (E3) connecting TM6 and TM7 appeared to be essential for NT binding (26). Others proposed that the binding site of NT in the rNTR1 entirely lies in E3, based on computer-assisted modeling of both the receptor and the ligand (31). A number of residues in E3, mainly aromatic (Phe\(^{331}\), Trp\(^{333}\), Phe\(^{344}\), Phe\(^{346}\), and Tyr\(^{349}\)), were depicted as interacting with the C-terminal hexapeptide sequence of NT. However, there were no mutagenesis data to support these findings. Actually, our previous work showed that mutating Phe\(^{346}\) or Tyr\(^{349}\) did not affect the affinity of NT for the rNTR1 and, conversely, that mutation of Tyr\(^{347}\) greatly reduced the peptide affinity (26).

In the present study, we combined mutational analysis of the rNTR1 and structure-activity studies with NT-(8–13) analogs in order to identify which residues in the NT pharmacophore might interact with the receptor residues that were found by mutagenesis to be important for NT binding. Thus, the pharmacological properties of NT on rNTR1 bearing mutations on residues Met\(^{208}\) (TM4), Arg\(^{327}\) (TM6), Phe\(^{331}\) (TM6), and Tyr\(^{347}\) (E3) as well as other residues in E3 (Trp\(^{338}\), Thr\(^{341}\), and Phe\(^{344}\)) were analyzed (Fig. 1). The binding or biological potency of NT-(8–13) analogs substituted on each of the 6 residues in the hexapeptide sequence or amidated at the C terminus was determined on the mutant receptors that showed decreased affinity for NT. These data were then used to dock NT-(8–13) in a model of the rNTR1 constructed as described previously (26) with the additional representation of extracellular loops. Our model predicts that the C-terminal dipeptide of NT-(8–13) interacts with residues in TMs 4 and 6 that lie at or near the junction with the extracellular domain of the rNTR1 and that the rest of the molecule interacts with extracellular residues in E3. Interestingly, the NT-binding site rests on top of the SR 48692 binding pocket that penetrates deeper in the TM core, and both ligands share common points of anchorage at the junction between the TMs and the extracellular domain of the receptor.
RESULTS

W339A and F344A Mutations in E3—Trp<sup>339</sup> and Phe<sup>344</sup> in the E3 loop of the rNTR1 were mutated in alanine, and saturation experiments with both <sup>125</sup>I-NT and [<sup>3</sup>H]SR 48692 were performed on the wild type and mutant receptors (Table I). All receptors were well expressed in COS M6 cells with SR 48692 <i>B</i><sub>max</i> values ranging from 3 to 20 pmol/mg protein. None of the mutations affected antagonist affinity, consistent with previous data showing that the SR 48692 binding pocket lies below E3 in the TM s of the rNTR1 (26). In contrast, the W339A and F344A mutations resulted in a 10- and 5-fold decrease, respectively, in agonist affinity as compared with the wild type rNTR1.

Comparison of the <i>B</i><sub>max</i> values obtained with either radiolabeled ligand shows for the wild type rNTR1 a 2-3-fold higher value for SR 48692 as compared with NT. We have previously shown that in membranes from cells transfected with the NTR1, three populations of binding site with high (<i>K</i><sub>d</sub> ~ 0.1–0.2 nM), low (<i>K</i><sub>d</sub> ~ 50–100 nM), and very low (<i>K</i><sub>d</sub> ~ 10 μM) affinities for NT are present (34). [<sup>3</sup>H]SR 48692 binds to all three sites with the same affinity, whereas <sup>125</sup>I-NT, over the concentration range used, measurably binds only to the high affinity site (34). In contrast to the wild type receptor, both the W339A and F344A mutants showed much higher (>10-fold) SR 48692 than NT <i>B</i><sub>max</sub> values (Table I). Competition experiments were performed for the three receptors using [<sup>3</sup>H]SR 48692 as the labeled ligand and NT as the competitor (Fig. 2A). A similar proportion of binding (20–25%) could not be competed for by NT, indicating that the proportion of very low affinity NT-binding site was the same for the wild type and mutant receptors. IC<sub>50</sub> values for NT on the three receptors are given in Table II. The values for the mutant receptors were 50 to 150 times lower than that for the wild type receptor. These data can be interpreted as indicating that both the W339A and F344A mutations increased the proportion of low affinity NT-binding site in addition to decreasing agonist affinity for the high affinity binding site.

This suggests that mutations in the E3 loop modified the conformational equilibrium of the NTR1. Therefore, it was of interest to compare the ability of NT to stimulate IP production in COS cells transfected with the wild type and mutant receptors (Fig. 2B). Wild type NTR1-transfected cells responded to NT with an EC<sub>50</sub> value of 0.77 ± 0.18 nM (mean ± S.E. from three independent experiments) that was closed to its high affinity <i>K</i><sub>d</sub> value (Table I). The W339A and the F344A mutants receptors also responded to NT (Fig. 2B) with EC<sub>50</sub> values that were 28.0 ± 5.0 and 47.0 ± 4.0 nM, respectively (means ± S.E. from three independent experiments). These values were 1–2 orders of magnitude higher than corresponding high affinity <i>K</i><sub>d</sub> values (Table I). Thus, in addition to affecting the conformational equilibrium of the NTR1, the W339A and F344A mutations appear to modify the coupling of the receptor high affinity state to G protein(s).

Y347A, Y347M, and Y347F Mutations in E3—Our previous studies have shown that mutating Tyr<sup>347</sup> to alanine in the rNTR1 resulted in a loss of detectable <sup>125</sup>I-NT binding without modifying the affinity for SR 48692 (26). The data in Table I show in addition that the <i>B</i><sub>max</sub> values for [<sup>3</sup>H]SR 48692 were comparable for the wild type rNTR1 and Y347A mutant, indicating that the latter was well expressed in COS cells. In order to determine the decrease in NT affinity for the Y347A mutant, competition experiments were performed with [<sup>3</sup>H]SR 48692 as the labeled ligand, and IC<sub>50</sub> values for NT were derived from the competition curves (Table III). The data show that the loss of NT binding potency amounted to almost 4 orders of magnitude for the Y347A mutant as compared with the wild type receptor. Such a loss indicates a major role of the Tyr<sup>347</sup> side chain in NT binding. In order to determine the respective

![Fig. 2. Competitive inhibition of [<sup>3</sup>H]SR 48692-specific binding by NT and NT-stimulated IP production with the wild type and W339A and F344A receptors. A, competition binding experiments were performed with membrane homogenates of COS M6 cells transfected with the wild type receptor (open squares) and the W339A (closed diamonds) and F344A (open circles) mutant receptors. The values are the means ± S.E. of three independent experiments. B, NT-stimulated IP production (after subtraction of basal IP levels) was (in pmol/2×10<sup>5</sup> cells) as follows: 1738 ± 484, 937 ± 362, and 626 ± 206 for the wild type, W339A, and F344A receptors, respectively. The values are the means ± S.E. from three independent experiments.]
TABLE II

IC50 values for NT and NT analogs competitive binding inhibition of [125I]NT or [3H]SR 48692 to wild type and mutant rNTR1

| Peptide           | IC50, nM (125I-NT) | IC50, nM ([3H]SR 48692) |
|-------------------|--------------------|-------------------------|
| NT (8–13)         | 0.19 ± 0.06        | 1.42 ± 0.18             |
| NT (8–13)         | 0.01 ± 0.003       | 0.46 ± 0.13             |
| Citr1 mutant      | 0.34 ± 0.13        | 3.93 ± 3.28             |
| Citr2 mutant      | 1.05 ± 0.23        | 36.5 ± 7.3              |
| Ala12 mutant      | 0.54 ± 0.10        | 7.51 ± 4.07             |
| Ala11 mutant      | 283 ± 51           | 1133 ± 66               |
| Ala13 mutant      | 10.5 ± 2.3         | 10.9 ± 5.1              |
| Ala12 mutant      | 5.56 ± 1.04        | 14.6 ± 3.8              |
| WT                | 5.83 ± 0.74        | 312 ± 43                |
| W339A             | 1.01 ± 0.36        | 55.3 ± 24.7             |
| Y347M             | 12.5 ± 2.0         | 474 ± 374               |
| Y347F             | 49.1 ± 7.3         | 960 ± 333               |

TABLE III

IC50 values for NT competitive binding inhibition of [3H]SR 48692 to wild type and mutant rNTR1

| Receptor          | IC50, nm ([3H]SR 48692) |
|-------------------|-------------------------|
| WT                | 34,000 ± 14,000         |
| Y347A             | 5,000 ± 500             |
| Y347M             | 120 ± 15                |

IC50 values were derived from curves of competitive binding inhibition experiments, to wild type (WT) and mutant receptors, with [3H]SR 48692 as labeled ligand and NT as competitor. Values are the means ± S.E. from three independent determinations.

Neurotensin Receptor Agonist Binding Site

Neurotensin Structure-Activity Relationships with the W339A, F344A, and Y347A Mutant Receptors—In an attempt to determine which residue in the NT-(8–13) sequence might interact with aromatic residues in E3, competition binding experiments on the wild type and W339A, F344A, and Y347F mutant receptors were performed with [3H]SR 48692 as the labeled ligand and the six NT-(8–13) analogs described above. The Y347F mutant had to be used for these experiments because the Y347A and Y347M mutants do not retain sufficient NT affinity. IC50 values for NT, NT-(8–13), and NT-(8–13) analogs were derived from the competition experiments (Table II). The ratio of the IC50 value for a mutant receptor over that for the wild type receptor was then calculated for NT-(8–13) and each of its analogs. We reasoned that if mutating an amino acid in the receptor affects the interaction of a residue in the NT-(8–13) sequence with the peptide-binding site, then modifying the side chain of that NT-(8–13) residue should not greatly affect the analog binding potency and the IC50 value ratio should tend to one. Plots of the ratio values thus obtained for each mutant receptor versus the position of the substituted residue in NT-(8–13) are represented in Fig. 3. The data show that with all three mutant receptors the potency of [Ala12]NT-(8–13) was significantly less affected than that of NT-(8–13) by the mutations. We interpret these results as indicating that Tyr11 in the NT-(8–13) sequence is likely to interact through π-π contacts with Trp339, Phe344, and Tyr347 in the E3 loop of the rNTR1. The data further suggest that the hydroxyl group of Tyr347 might form a hydrogen bond with the hydroxyl group of Tyr11 in the NT sequence. It was also observed that the potency of [Ala10]NT-(8–13) was less decreased than that of NT-(8–13) by the W339A receptor mutation, suggesting that Trp339 is either interacting with Pro10 or necessary for the correct positioning of this residue in the NT-binding site.

Neurotensin Structure-Activity Relationships with the M208A and F331A Mutant Receptors—Similar studies as above were conducted with the M208A and F331A mutant receptors, except that in this case competition experiments were performed with 125I-NT as the labeled ligand because, as already mentioned, [3H]SR 48692 does not retain sufficient affinity for these receptors to permit binding studies. IC50 values are given in Table II, and plots of the IC50 ratio values are shown in Fig. 3. Interestingly, the binding potency of [Ala12]NT-(8–13) was identical for the wild type NTR1 and the M208A mutant receptor. This result suggests that Met208 may form hydrophobic interactions with the side chain methyl
groups of Ile\textsuperscript{12}. In addition, the potencies of NT-(8–13) analogs modified on either side of Ile\textsuperscript{12}, i.e. [Ala\textsubscript{13}]NT-(8–13) and [Ala\textsubscript{13}]NT-(8–13), were significantly less perturbed than that of NT-(8–13) by the M208A mutation, suggesting that the fitting of Tyr\textsuperscript{11} and Leu\textsuperscript{13} in the NT-binding site is sensitive to the disruption of the Met\textsuperscript{208}–Ile\textsuperscript{12} interaction. With regard to the F331A mutant, two analogs, [Cit\textsubscript{9}]NT-(8–13) and [Ala\textsubscript{13}]NT-(8–13)–NH\textsubscript{2}, exhibited significantly smaller decreases in potency than NT-(8–13). This suggests that Phe\textsuperscript{331} might be engaged in cation–π interactions with the side chain of Arg\textsuperscript{9} and hydrophobic interactions with the side chain methyl groups of Leu\textsuperscript{13}.

Neurotensin Structure-Activity Relationships with the R327M Mutant Receptor—Arg\textsuperscript{327} is located within one helical turn of TM6 near the E3 loop connecting TM6 and TM7. We have previously shown that mutating this residue into Met resulted in a loss of detectable SR 48692 and NT binding (26). However, this mutant receptor retained the ability to respond to NT by an increase in inositol phosphate (IP) production with the same basal and NT maximal responses than the wild type rNTR1 but with a potency for NT that was decreased by 4 orders of magnitude (26). We have shown that Arg\textsuperscript{327} is involved in an ionic interaction with the carboxylic acid group of SR 48692 (26). It is known from previous structure-activity studies that the C-terminal COOH function of NT is essential for NT binding. In order to see if the large decrease in NT potency toward the R327M mutant receptor could be due to the disruption of an ionic interaction between Arg\textsuperscript{327} and the C-terminal acidic function of NT, we determined the potencies of NT, NT-(8–13), and NT-(8–13)–NH\textsubscript{2} for their ability to stimulate IP production in the wild type rNTR1 and the R327M mutant. NT-(8–13)–NH\textsubscript{2} being amidated at the C terminus would be expected to have the same potency for the wild type and mutant receptor, should our hypothesis of an ionic link be correct. The data presented in Fig. 4 and Table IV show that NT-(8–13) was three times more potent than NT for stimulating IP production in COS cells transfected with the wild type rNTR1. Amidating the C terminus of NT-(8–13) resulted in an almost 1000-fold loss in potency for NT-(8–13)–NH\textsubscript{2}. The potency of both NT and NT-(8–13) was decreased by 4 orders of magnitude in cells transfected with the R327M mutant as compared with the wild type receptor. In sharp contrast, NT-(8–13)–NH\textsubscript{2} retained the same potency in both transfected cell systems. Actually, the amidated analog was 10 times more potent than its parent peptide NT-(8–13) in stimulating IP production in R327M receptor-transfected cells (Fig. 4 and Table IV). These data provide strong evidence that Arg\textsuperscript{327} forms an ionic bond with the C-terminal carboxylate of NT.

**Table IV**

| Receptor          | EC<sub>50</sub> nM |
|-------------------|-------------------|
| WT                | 0.57 ± 0.20       |
| NT                | 0.19 ± 0.12       |
| R327M             | 130 ± 40          |
| NT-(8–13)         | 12,000 ± 3,000    |
| NT-(8–13)–NH\textsubscript{2} | 3,800 ± 1,400 |
|                   | 230 ± 90          |

**FIG. 4.** Effect of NT and NT analogs on IP production in COS M6 cells transfected with wild type or R327M receptors. Concentration-response curves for NT (square), NT-(8–13) (circle), and NT-(8–13)–NH\textsubscript{2} (triangle)-stimulated IP production were performed with the wild type receptor (open symbols) or the R327M mutant (closed symbols). The values are the means ± S.E. from three independent experiments.
observed only at very high NT concentrations (100 μM). The very low potency of NT in this system precluded further structure-activity studies with NT analogs for testing the hypothesis that Asp139 might interact with Arg8 or Arg9.

**Model of the NT-(8–13)-rNTR1 Complex**—We have previously described a model of the rNTR1-SR 48692 complex (26). In this model, only the seven TMs were taken into account, and their positions relative to one another as well as their orientations in the membrane were determined using rhodopsin as a template (35). In order to construct a model of the rNTR1-NT-(8–13)-binding site, the TMs orientation was kept identical to that of our previous model, and the E3 loop sequence was entered into the modeling program. The above mutagenesis and structure-activity data were taken into account for manually docking the NT-(8–13) sequence in its binding site thought to lie between the E3 loop and the extracellular side of TM4, -6, and -7. Energy minimization was then performed while constraining the TMs and assigning distances between rNTR1 and NT-(8–13) residues according to the interactions described in the preceding sections. This led to the model of the rNTR1-NT-(8–13) complex represented in Fig. 5A (side view) and B (top view). In this model, the backbone of NT-(8–13) adopts a rather linear conformation with the C-terminal COOH group in close proximity to the guanidinium function of Arg327 in TM6 of the receptor and the N terminus pointing upward near the upper region of the E3 loop. The side chain methyl groups of Leu13 are facing the aromatic ring of Phe331 (TM6), whereas those of Ile12 are close to Met208 (TM4). The side chain of Tyr11 occupies a
central position in the E3 loop, close to Tyr<sup>347</sup> and to a lesser extent to Trp<sup>339</sup> and Phe<sup>344</sup>. The side chain of Arg<sup>6</sup> is oriented so that the guanidinium function lies near Phe<sup>331</sup>. Finally, the side chain of Arg<sup>6</sup> stretches outside the E3 loop.

**DISCUSSION**

In the present study, we used a combination of mutagenesis, pharmacological, and molecular modeling approaches in order to provide a tridimensional representation of the NT-binding site in the rNTR1. Our strategy was similar to that previously employed for modeling the SR 48692-rNTR1 complex (26). It entailed determining the receptor residues that are important for ligand binding by mutagenesis, modeling the rNTR1, and docking the ligand in the receptor taking into account the mutagenesis data. The latter two steps are easier to perform for SR 48692 than for NT for two reasons as follows: (i) unlike NT or NT-(8–13) which are highly flexible in solution (28, 29), SR 48692 has a rigid structure that has been determined by x-ray crystallography (27); (ii) the SR 48692 binding site lies entirely within the TMs (26), whereas that of NT-(8–13) is partly extracellular, which makes it more difficult to model the NT-binding site as rhodopsin provides a good template for orienting the TMs of GPCRs but cannot be used for modeling extracellular domains. For this reason, it was necessary to establish which residues in the NT-(8–13) sequence are interacting with the receptor residues that are important for agonist binding as determined by mutagenesis. This was achieved by performing systematic structure-activity studies with NT-(8–13) analogs and the mutant receptors that showed decreased NT potency.

This approach led us to identify two residues in the receptor, Arg<sup>327</sup> in TM6 and Tyr<sup>347</sup> in the E3 loop, that play critical roles in binding NT-(8–13) and to determine the structural elements in the NT-(8–13) sequence with which these residues interact. Thus, we propose that Arg<sup>327</sup> makes an ionic link with the C-terminal COOH group of NT-(8–13) and that Tyr<sup>347</sup> lies close to Tyr<sup>11</sup> with which it forms n-n interactions and hydrogen bonding. The similar large loss (3–4 orders of magnitude) in agonist affinity observed following reciprocal modification of the interacting partners (Arg<sup>327</sup> to Met and Leu<sup>31</sup>-COOH to Leu<sup>13</sup>-NH<sub>2</sub>; Tyr<sup>347</sup> to Ala and Tyr<sup>11</sup> to Ala) is consistent with our proposal. Furthermore, the dramatic decrease of potency (20,000-fold) of the [Ala<sup>11</sup>]NT-(8–13) analog could be accounted for by additional weaker n-n interactions between Tyr<sup>347</sup> in NT and Trp<sup>339</sup> and Phe<sup>344</sup> in the E3 loop of the receptor. We also propose that the side chains of Ile<sup>12</sup> and Leu<sup>13</sup> in the NT-(8–13) sequence form hydrophobic interactions with Met<sup>208</sup> and Phe<sup>331</sup>, respectively. In these cases, the decrease in agonist potency (30-fold) that results from the M208A and F331A mutations is 10–20 times smaller than that (500-fold) brought about by the corresponding Ile<sup>12</sup>A and L13A modifications in the NT sequence. This could mean that the side chain methyl groups of Ile<sup>12</sup> and Leu<sup>13</sup> are involved in other interactions with receptor residues not identified here or, alternatively, that they are important for correctly positioning the C-terminal -Ile-Leu-COOH sequence of NT in the receptor binding site. Finally, we suggest that the guanidinium group of Arg<sup>6</sup> in NT forms cation-n interactions with Phe<sup>344</sup>, the 70-fold loss of potency of [Cit<sup>8</sup>]NT-(8–13) being consistent with this proposal.

Altogether, our mutagenesis data support the proposal that the side chain of Arg<sup>6</sup> forms cation-n interactions with Arg<sup>6</sup>. Previous studies have shown that mutating Asp<sup>139</sup> to glycine led to a loss of NT binding, and it was proposed that this residue might form ionic interactions with Arg<sup>6</sup> (30). Here, we show that the D139A mutant receptor is devoid of measurable NT and SR 48692 binding. It can be stimulated to produce IPs at very high NT concentrations (100 μM), which represents at least a 5 order of magnitude loss of potency as compared with the wild type rNTR1. However, it seems unlikely that Asp<sup>139</sup> could form an ionic link with Arg<sup>6</sup> because [Cit<sup>8</sup>]NT-(8–13) would be expected to exhibit a greater loss of potency than that observed here. Rather, we think it more likely that the D139A mutation produces a major change in receptor conformation that affects both agonist and antagonist binding. In particular, Asp<sup>139</sup> lies close to Cys<sup>142</sup> which, by analogy with other GPCRs (36, 37), is thought to make a disulfide bridge with Cys<sup>225</sup> in the E2 loop. Mutating Asp<sup>139</sup> might possibly affect the formation of the disulfide bond that has been shown for other GPCRs (38, 39) to be essential for maintaining receptor conformation.

By using quite a different approach than ours for modeling the NT-(8–13)-rNTR1 complex, Pang et al. (31) proposed that the NT-(8–13)-binding site lies entirely in the E3 loop. A number of residues in E3 were predicted to interact with NT-(8–13). However, these predictions were not directly tested by mutagenesis of the rNTR1 (31). Our previous data (26) and those presented here do confirm some of the interactions proposed by Pang et al. (31). However, they do not support others and predict points of interaction for NT-(8–13) outside the E3 loop that were not described in the model of Pang et al. (31). Thus, we agree with Pang et al. (31) on the proposed n-n interactions between Phe<sup>344</sup> and Trp<sup>339</sup> in the E3 loop of the rNTR1 and Tyr<sup>11</sup> in NT-(8–13). However, we provide strong evidence that the major anchoring point for Tyr<sup>11</sup> in the E3 loop is Tyr<sup>347</sup> through n-n interactions and hydrogen bonding. Phe<sup>331</sup> was reported to make cation-n interactions with Arg<sup>6</sup> and hydrophobic interactions with Ile<sup>12</sup> (31). Our data support the former proposal but not the latter. Rather, they are consistent with Phe<sup>331</sup> interacting with the side chain of Leu<sup>13</sup> and Met<sup>208</sup> in TM4 with that of Ile<sup>12</sup>. The model of Pang et al. (31) suggested that Phe<sup>346</sup> and Tyr<sup>349</sup> interact with Arg<sup>6</sup> and Arg<sup>9</sup>. This is unlikely in view of our previous findings showing that both the F346A and V349A mutant receptors retained the same affinity for NT as the wild type rNTR1 (26). Finally, we provide evidence for a strong ionic link between Arg<sup>327</sup> in TM6 and the C-terminal carboxyl group of NT-(8–13). This crucial interaction was not accounted for in the model of Pang et al. (31). Thus, although Pang et al. (31) correctly predicted by means of conformational studies that the E3 loop of the rNTR1 is an important part of the NT-binding site, the present combined mutational, structure-activity, and modeling approach has allowed us to define the residues, both in the NT and E3 loop sequences, that are involved in agonist binding and to describe other points of NT-receptor interaction outside the E3 loop.

Comparison between the NT-(8–13)-binding site in the rNTR1 as described here and that of SR 48692 as previously reported (26) provides interesting information (Fig. 5C). Both ligands share common points of anchorage in the receptor that are Met<sup>208</sup> at the junction between TM4 and the E2 loop, Phe<sup>331</sup> at the junction between TM6 and the E3 loop, and Arg<sup>327</sup> located in TM6 one helical turn from the E3 loop. Met<sup>208</sup> and Phe<sup>331</sup> form hydrophobic interactions with the adamantane cage of the antagonist or with the side chain methyl groups of Ile<sup>12</sup> and Leu<sup>13</sup> in the agonist. Arg<sup>327</sup> forms an ionic link with the carboxyl function attached to the carbon atom that also bears the adamantane moiety in SR 48692 or with the C-terminal COOH group of NT. Thus, the (adamantane)-CH(COOH)- structure of SR 48692 occupies the same position in the receptor as the C-terminal dipeptide, -Ile-Leu-COOH, of NT. The N-terminal 8–11 sequence of NT-(8–13) fits in the E3 loop that connects TMs 6 and 7 with Tyr<sup>11</sup> occupying a central position and interacting closely with Tyr<sup>347</sup> in the receptor,
whereas the rest of the SR 48692 molecule points toward the intracellular side of the membrane, making interactions with residues that are located within the first two helical turns of TMs 6 and 7 (Fig. 5C). Therefore, the agonist and antagonist binding sites are distinct but partially overlap at the junction between TMs and extracellular domains, sharing strong anchoring points in the receptor such as Arg227. This would clearly account for the competitive antagonist behavior observed for SR 48692.

The nNTR1 has previously been shown to exist in a high and a low affinity state for NT, both states being recognized with the same affinity by SR 48692 (34). The present findings that the W339A and F344A mutations markedly increase the proportion of the low affinity state suggest that the E3 loop may have flexibility and adopt conformations that confer either high or low affinity to the agonist binding site. The antagonist binding site lying below the E3 loop within the TMs would not be affected by the conformational change of the loop, and this would explain the observation that SR 48692 binds with the same affinity to the high and low affinity NT receptor states. The location of the NT-binding site is particularly interesting in view of the fact that agonist-induced G protein coupling and internalization have been shown to involve the third intracellular (I3) loop (40) and the C-terminal tail (41) of the nNTR1, respectively. The I3 loop and C-terminal domain both connect to the E3 loop through TMs 6 and 7, respectively. It may be suggested that upon binding of NT to the nNTR1, conformational changes of the E3 loop may transconform the I3 and C-terminal domains through connecting TMs 6 and 7, thereby promoting G protein coupling and internalization. SR 48692 that binds below the E3 loop to residues in TMs 6 and 7 would prevent transconformation of the intracellular domains and lock the receptor in an inactive state. If these hypotheses are correct, it might be predicted that mutations in the E3 loop or connecting TM domains could affect the transduction properties of the nNTR1. As shown here this is so for the W339A and F344A mutations, and we have obtained preliminary evidence that this is also the case for other mutations in the E3 loop and TM7.

A number of studies have attempted to delineate small neu- ropeptide-binding sites for GPCRs through receptor mutagenesis or chimeric construction approaches (3, 12, 42–45). However, few studies have provided molecular models of peptide agonist-receptor complex by combining mutagenesis, structure-activity studies, and computer-assisted modeling. To our knowledge, this was done for the thyrotropin-releasing hormone, the somatostatin SSTR2, and the vasopressin V1a recep-
tors (5, 6, 46). The binding pocket for these three peptides lies mainly within the core of the TMs, and it can be noted that in each case a number of residues in TMs 6 and 7 are involved in peptide-receptor interactions (5, 6, 46). Our own model of the NT-nNTR1 complex shows the peptide-binding site to lie between the surface of the membrane and the E3 loop. It differs in this respect with the TRH, somatostatin, and vasopressin receptor models, suggesting a different binding mode for these peptides on the one hand and NT on the other hand. Although the δ agonist-β opioid receptor complex has not been modeled, studies of chimeric δ-μ and mutant δ opioid receptors indicated that the binding site for δ agonists might be comprised of residues in the E3 loop and at the top of TMs 6 and 7 (47). This suggests that NT and δ opioid agonists may share common mechanisms of receptor binding and activation. Interestingly, sequence homology analysis indicates that, in the GPCR family, the opioid receptors are among the most closely related to the NTR1. Mutational analysis of neurokinin receptors have shown that residues that are important for peptide agonist binding are scattered throughout extracellular domains (42). Therefore, it appears that neuropeptide-binding sites in GPCRs may be located entirely inside or outside or partly inside and outside the TMs. This variability is in contrast with GPCRs of small neurotransmitter ligands like the biogenic amines for which the binding site has always been found to reside in a structurally conserved region inside the TMs (3, 4). It also contrasts with the observation that the binding pocket of nonpeptide antagonists of neuropeptide GPCRs always lie within the core of the seven TMs (10, 48–50).

The amino acid sequences of the E3 loop and TMs 6 and 7 are highly conserved in the nNTR1 and hNTR1. In particular, all the residues shown here to play a role in NT binding are conserved with the exception of Phe344 in the nNTR1 which is replaced by a Tyr in the hNTR1. It is therefore likely that the NT-binding site in the hNTR1 will be quite similar to that described here for the nNTR1. This conclusion was also reached by Pang et al. (31). Our proposed model of the NT-binding site might be of help for the design of nonpeptide agonist mimetics of the NTR1. Such compounds might be useful for the treatment of brain disorders such as Parkinson’s disease or schizophrenia (51). A nonpeptide agonist of the NTR1 should include among other features a COOH group linked to an aliphatic structure in order to mimic the -Ile-Leu-COOH sequence of NT, a phenol ring for fulfilling the role of Tyr11 in NT, and positively charged groups for mimicking the side chains of Arg23 and Arg5. The choice of the relative spatial disposition of these chemical moieties could then be guided by our tridimensional model of the NT-binding site.

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