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# A Single Amino Acid of the Human and Rat Neurotensin Receptors (Subtype 1) Determining the Pharmacological Profile of a Species-Selective Neurotensin Agonist

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**ABSTRACT.** The neurotensin (NT) receptor, subtype 1 (NTR1), is a 7-transmembrane-spanning receptor, forming 3 extracellular and 3 intracellular loops. Previously, we showed that the third outer loop (E3) is the binding site for NT and its analogs, several of which bind with higher affinity to rat NTR1 (rNTR1) than to human NTR1 (hNTR1). In particular, NT34 {[3,1′-naphthyl-l-Ala$^{11}$]NT(8–13)} has greater than 60-fold higher affinity for rNTR1 (46 and 60 pM for transiently- and stably-transfected cells, respectively) than for hNTR1 (2.8 and 5.8 nM for transiently- and stably-transfected cells, respectively) isolated from transfected cell membranes. Previously, our molecular modeling studies of rNTR1 and hNTR1 showed that the binding pocket in the human receptor for NT34 is smaller in volume from the bulky residue Tyr$^{339}$ in the pocket center, as compared with the corresponding residue Phe$^{344}$ in the rat binding pocket. Therefore, with site-directed mutagenesis, we derived mutant forms of rNTR1(F344Y) and hNTR1(Y339F). Examination of the mutant receptors from membranal preparations of transfected cells in radioligand binding assays and with intact cells in functional assays (phosphatidyl-4,5-bisphosphate turnover) showed that the human-like rat receptor and the rat-like human receptor bound NT34 with a predicted reverse of binding compared with its binding to the wild-type receptors. These results strongly affirm our molecular modeling studies and demonstrate the importance of the study of even minor structural variations in proteins to determine the basis of significantly different drug responses, an area of focus for pharmacological research in the 21st century. BIOCHEM PHARMACOL **60**;6:793–801, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** neurotensin receptor; subtype 1; binding site; site-directed mutagenesis; structure–activity studies; peptide analogs

NT¶ is an endogenous peptide neurotransmitter (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) [1]. It is distributed heterogeneously in nervous tissue and is present in the gastrointestinal tract at much higher concentrations than in the brain [2]. A large body of data obtained from whole animal studies, in which NT was injected directly into the brain, suggests that this peptide is an endogenous neuroleptic.

There are three subtypes of the NT receptor that have been molecularly cloned from several mammalian species, including human and rat [3]. The focus of this report is on the NTR1 [4–6]. The cDNA for this receptor subtype codes for a 424- and a 418-amino-acid protein expressed in the rat and human brain, respectively. This receptor is hypothesized to contain 7 transmembrane-spanning regions, with 3 extracellular and 3 cytoplasmic loops. The putative third intracellular loop couples to G-proteins to stimulate the release of inositol phosphates [7], while the third extracellular loop plays an important role in the binding of NT (8–13), a C-terminal fragment of NT [8, 9].

Because of the neuroleptic-like properties of NT, our laboratory has focused on the development of compounds that penetrate into the brain and activate NT receptors. Such a compound could be a novel neuroleptic. Since the activity of NT resides in the last six residues (8–13) [10–12], we have focused our research on the design and synthesis of novel analogs of NT (8–13). Of the many compounds that we have synthesized and tested, one

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¶ *Abbreviations*: NT, neurotensin; NTR1, neurotensin receptor subtype 1; rNTR1, rat NTR1; hNTR1, human NTR1; PCR, polymerase chain reaction; PI, phosphatidyl inositol; $IP_1$ inositol 1-phosphate; and E3, third outer loop of a 7-transmembrane-spanning receptor.

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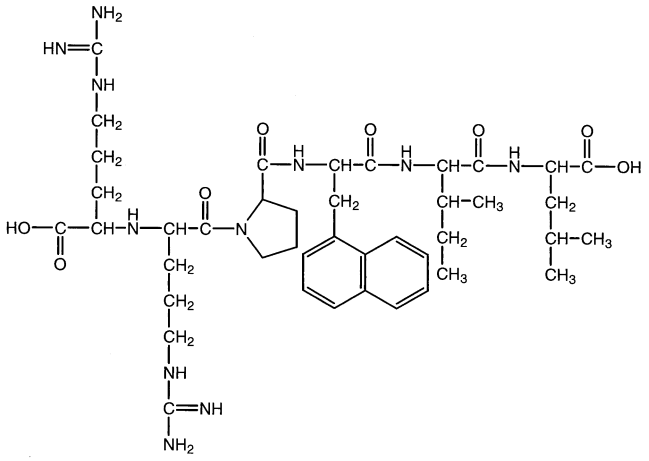


**FIG. 1. Structure of NT34, Arg[8]-Arg[9]-Pro[10]-Nal[11]-Ile[12]-Leu[13], or [3,1-naphthyl-l-Ala[11]]NT(8–13).**

analog, designated NT34 (Fig. 1), has been demonstrated to possess a high affinity for the rNTR1, and a 60- to 100-fold lower affinity for the hNTR1. With this compound, we found that the binding site of NT (8–13)-like ligands is located in the third extracellular loop (E3) of the NTR1 [8]. Independent molecular modeling studies suggest that the third outer loop forms the binding site of NT(8–13)-like ligands [9]. This computer modeling-derived NT(8–13) binding site is primarily composed of eight residues (i.e. Phe[326], Ile[329], Trp[334], Phe[337], Tyr[339], Phe[341], Tyr[342], and Tyr[344] in the hNTR1; Phe[331], Ile[334], Trp[339], Phe[342], Phe[344], Phe[346], Tyr[347], and Tyr[349] in the rNTR1) (Fig. 2). Seven aromatic residues form a hydrophobic pocket on the extracellular surface of the NT receptor to accommodate its ligand by cation–pi, van der Waals (pi–pi), and hydrogen bonding interactions. In the present study, we report that, with NT34, site-directed mutagenesis, and computer modeling, we can make, for the first time, the rat NT receptor human-like and the human NT receptor rat-like by alternating one single amino acid (Tyr[339] ↔ Phe[344]) that is located in the center of the putative NT(8–13) binding site. We can alternate the pharmacological profile of NT34 at human and rat NT receptors by simply exchanging the H- and OH-groups at the binding site at the atomic level. These results affirm our reported computer modeling studies [9]. Alternating Tyr and Phe is a common protein mutation among species. These results demonstrate the importance of the study of structural variations, even minor structural variations in proteins, to determine significantly different drug responses. Such studies will be a focus of pharmacological research in the 21st century.

## MATERIALS AND METHODS

### *Materials*

The NT and NT34 peptides used in these studies were synthesized by the Mayo Protein Core Facility (D. J. McCormick, Director; Mayo Clinic) using solid-phase methods described previously [13]. Briefly, Fmoc chemistry was used to synthesize peptides with Arg PMC-protected side chains on an automated peptide synthesizer (433A, Applied Biosystems). Protocols concerning activation, coupling times, amino acid dissolution, coupling solvents at 100 μmol, and synthesis scale were followed according to the protocols developed by the manufacturer, Perkin-Elmer/ABI. The NT peptides were purified by reverse-phase HPLC using a semi-preparative $C_{18}$ column (2.2 × 25 cm, Vydac) in aqueous solutions of 0.1% trifluoroacetic acid and an aqueous gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid. A combination of analytical HPLC and electrospray ionization mass spectrometry (PE-Sciex API 1658) was used to analyze peptide purity and to confirm molecular weight.

### *Site-Directed Mutagenesis and Stable Transfections*

*In vitro* mutagenesis was performed using the Sculptor *in vitro* Mutagenesis System from Amersham according to the manufacturer's instructions. The templates for mutagenesis were PCR products H74 and R74 from a previous study that were cloned into M13mp18. Mutagenic oligonucleotides were obtained from Applied Biosystems. Oligonucleotide sequences were as follows:

*Y339F*, 5′-*d*(*TGGACTCCGTTCCTCTTTG*
*ACTTCTACCACTAC*)-3′.

*F334Y*, 5′-*d*(*TGGACTACGTTCCTCTACGA*
*TTTCTACCACTAT*)-3′.

Putative mutants were screened by sequencing on an ABI DNA Sequencer (Applied Biosystems) using the AutoRead Sequencing Kit (Pharmacia Biotech, Inc.) or by the Molecular Biology Core Facility (Mayo Clinic). Mutants were sequenced completely, and subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen). The PCR products H18 or R18 were subcloned into the partial mutant clones to assemble the full-length constructs (H18 + H74 and R18 + R74). Transfections into HEK-293 or Chinese hamster ovary (CHO-K1) cells were done using the $CaPO_4$ method. Stable transformants were selected from CHO-K1 cells with Geneticin® (antibiotic G-418 sulfate, 1 mg/mL, GIBCO), screened for NT binding activity, and expanded into stable cell lines.

### *Cell Culture*

CHO-K1 cells that had been stably transfected with the hNTR1(Leu) [6] and rNTR1 genes, and HEK-293 cells that had been transiently transfected with mutant receptors were cultured in 150-mm petri plates containing 35 mL of Dulbecco's modified Eagle's medium containing 1x nonessential amino acid solution (GIBCO) supplemented with 5% (v/v) FetalClone II bovine serum product (HyClone Laboratories). hNTR1(Leu) is a genetic variant of hNTR1

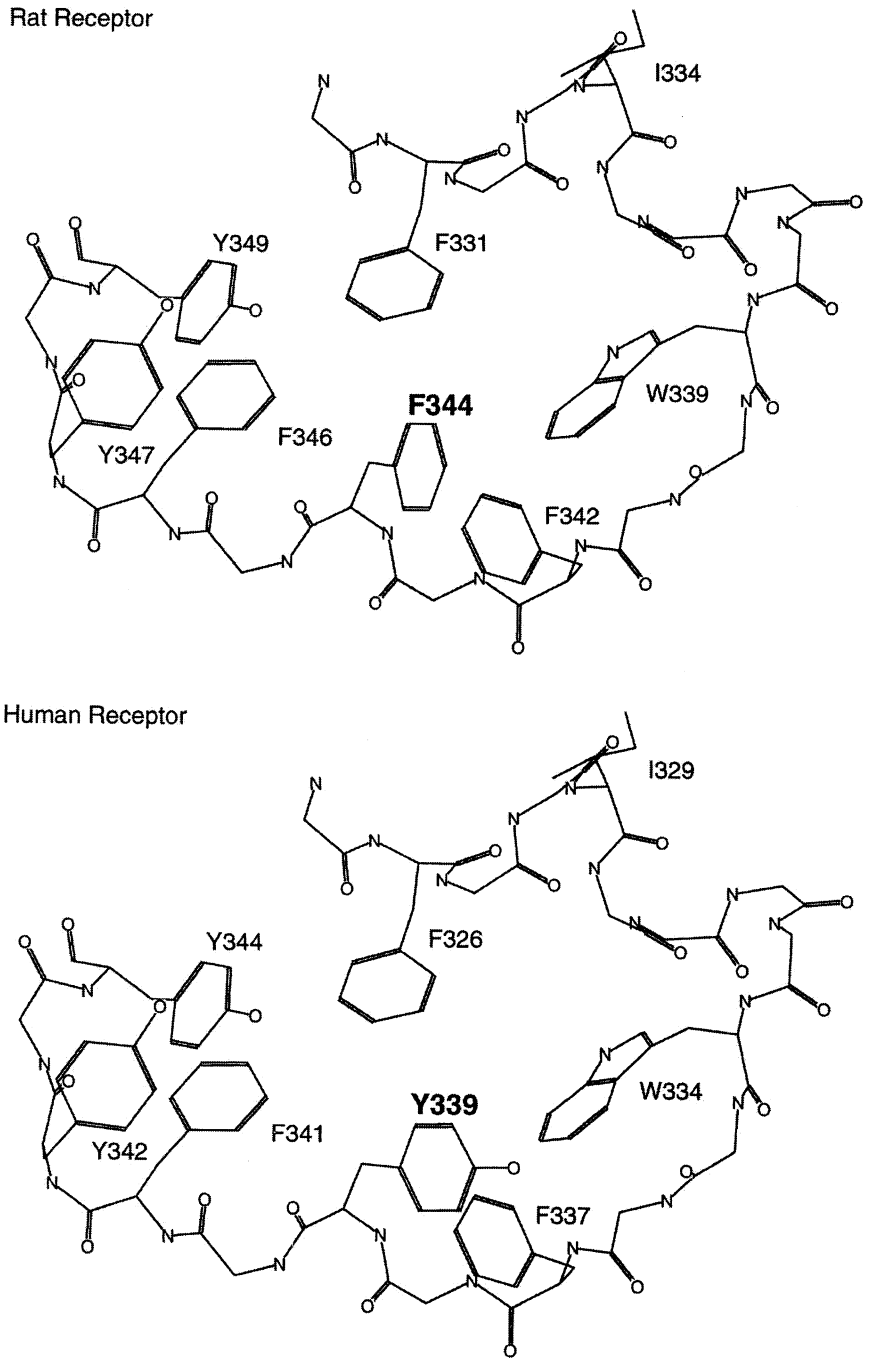



**FIG. 2. Pseudo-three-dimensional structures of the third extracellular (E3) loop in human and rat receptors as a putative NT(8–13) binding site. Reproduced with permission from *J Biol Chem* 271: 15060–15068, 1996. Copyright (1996) The American Society for Biochemistry & Molecular Biology. [Ref. 9].**

that has Leu instead of Phe at position 194 [6]. CHO-K1 cells (subculture 9–19) were harvested at confluency, whereas HEK-293 cells were harvested 48 hr after transfection. Cells were removed by aspiration of the medium, followed by a wash with 50 mM Tris–HCl, pH 7.4 (determined at 25°) (6 mL), which was discarded, resuspension in 5–10 mL of Tris–HCl, scraping the cells with a rubber spatula into a centrifuge tube, and collection of cells by centrifugation at 300 *g* for 5 min at 4°, in a GPR centrifuge (Beckman Instruments). The cellular pellet (in 50 mM Tris–HCl, 1 mM EDTA, pH 7.4) was stored in liquid nitrogen until radioligand binding was performed.

For use in binding assays, crude membrane preparations were obtained by centrifugation of the cellular pellet at 35,600 *g* for 10 min. The supernatant was decanted and discarded, and the cellular pellet was resuspended in 2 mL of 50 mM Tris–HCl, 1 mM EDTA (pH 7.4) followed by homogenization with a Brinkmann Polytron (Brinkmann)

at setting 6 for 10 sec. Centrifugation was repeated as above, the supernatant was decanted and discarded, and the final cellular pellet was resuspended in 50 mM Tris–HCl, 1 mM EDTA, 0.1% bovine serum albumin, and 0.2 mM bacitracin. The protein concentration of the membrane preparation of CHO-K1 cells was estimated by the method of Lowry *et al.* [14], using BSA as a standard.

### *Radioligand Binding Assays*

We used a Biomek 1000 robotic workstation (Beckman Instruments) for all pipetting steps in the radioligand binding assays as previously described by our group [15]. Competition binding assays with [$^3$H]NT (1 nM) and various concentrations of unlabeled NT and peptide analogs were carried out with membrane preparations from the appropriate cell lines. Nonspecific binding was determined with 1 μM unlabeled NT in assay tubes with a total volume of 1 mL. Incubation was at 20° for 30 min. The assay was routinely terminated by the addition of cold 0.9% NaCl (5 × 1.5 mL), followed by rapid filtration through a GF/B filter strip that had been pretreated with 0.2% polyethylenimine. Details of binding assays have been described before [15]. The data were analyzed using the LIGAND program [16].

### *PI Turnover Assays*

Intact CHO-K1 cells were harvested for PI turnover analysis at about 80% confluency. Cells were detached from the petri plates by removal of culture medium, followed by incubation of the cellular monolayer for 20 min at 37° with gentle shaking in a modified Puck's $D_1$ solution containing 2 mM EGTA. We have described elsewhere the details of assaying in intact cells the relative changes in PI turnover by using a radioactively labeled precursor [17]. Briefly, intact CHO-K1 cells were prelabeled with D-*myo*-[$^3$H]inositol (18.3 Ci/mmol) in the presence of lithium chloride (final concentration, 10 mM). Then cells were stimulated with NT or the appropriate analog. The amount of [$^3$H]$IP_1$ produced by the cells was isolated chromatographically on Dowex 1-X8 (200–400 mesh). For the experiments described here the stimulation time was 30 min. The number of CHO-K1 cells per assay tube was $8 \times 10^4$ to $2.25 \times 10^5$.

### *Statistical Analysis*

The values presented for $K_d$ and $EC_{50}$ are expressed as geometric means ± SEM [18, 19].

## RESULTS

### *Transient Transfections in HEK-293 Cells*

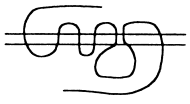
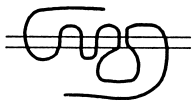
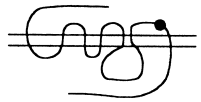
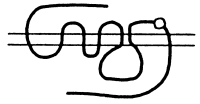
Our initial studies with NT34 {L-[3,1′-Nal$^{11}$NT(8–13)}, Fig. 1 and the site-mutants were done with the use of transiently transfected HEK-293 cells. Site-directed mutagenesis converted Tyr$^{339}$ of the hNTR1 to Phe$^{339}$ (human Y339F), and in the rNTR1 Phe$^{344}$ (the rat receptor is 5 amino acids longer at this point in the structure) was changed to Tyr$^{344}$ (F344Y). In Table 1, the $K_d$ values derived from membrane preparations of transiently transfected HEK-293 cells containing the wild-type hNTR1, the mutant hNTR1(Y339F), the wild-type rNTR1, and the mutant rNTR1(F344Y) are presented. For all receptors tested, the $K_d$ values for NT were essentially identical. However, for the species-specific NT34, the $K_d$ values were remarkably different. NT34 bound with 61-fold higher affinity to the wild-type rat receptor ($K_d$ = 0.046 ± 0.005 nM) than to the wild-type human receptor ($K_d$ = 2.8 ± 0.3 nM) in membrane preparations from transfected HEK-293 cells. However, NT34 had a 25-fold increased affinity at the human Y339F receptor, giving a $K_d$ of 0.110 ± 0.008 nM, similar to that for the wild-type rNTR1. Conversely, NT34 had a 35-fold decreased affinity for the rat F344Y receptor ($K_d$ = 1.6 ± 0.2 nM), to give a binding affinity similar to that for its binding to the wild-type hNTR1 ($K_d$ = 2.8 ± 0.3 nM).

### *Stable Transfections in CHO-K1 Cells*

To study the effect of these site mutations further, we stably transfected CHO-K1 cells with these mutants. In Table 1, we present the results of competitive binding assays with NT and NT34 in membrane preparations with each of the cell lines expressing the indicated receptors. Again, for results of binding with NT at each of the four cell lines, the $K_d$ values were almost indistinguishable from one another, with values in the same range of those derived for NT at the receptors expressed by HEK-293 cells. For the species-specific NT34, the results again demonstrated that NT34 bound with 97-fold higher affinity to the wild-type rat receptor ($K_d$ = 0.060 ± 0.005 nM) than to the wild-type human receptor ($K_d$ = 5.8 ± 0.6 nM) in membrane preparations from transfected CHO-K1 cells. However, mutation of the hNTR1 to produce the human Y339F mutant receptor increased the binding affinity of NT34 for the human receptor by 145-fold, giving a $K_d$ of 0.040 ± 0.002 nM, similar to that for the wild-type rNTR1. Conversely, mutation of wild-type rNTR1 to the F344Y mutant decreased the binding affinity of NT34 by 28-fold ($K_d$ = 1.65 ± 0.05 nM), producing a binding affinity similar to NT34 binding to wild-type hNTR1.

To evaluate the effects of the reciprocal mutations further, we established binding assays of NT34 with receptors expressed on the surface of intact CHO-K1 cells (Table 2). The $K_d$ value for NT binding to surface receptors in all cell types was almost identical, indicating that there was little difference in the affinity of NT for any of the expressed receptor forms. However, for the NT34 analog there were marked differences in the binding affinity between the human and rat receptors. Thus, in the intact cell binding assay, NT34 bound with 230-fold higher affinity to the wild-type rNTR1 ($K_d$ = 0.22 ± 0.01 nM), compared with its binding for the wild-type hNTR1 ($K_d$ =

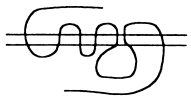
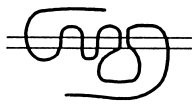
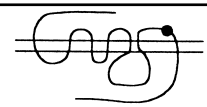
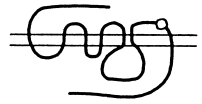
**TABLE 1. Binding results in HEK-293 and CHO-K1 membrane preparations of the NTR1**

| | Wild-type human NTR1 | | Wild-type rat NTR1 | | Mutant human NTR1 (Y339F) | | Mutant rat NTR1 (F344Y) | |
|---|---|---|---|---|---|---|---|---|
| | $K_d$ [nM] | | | | | | | |
| Cell line | HEK | CHO | HEK | CHO | HEK | CHO | HEK | CHO |
| Neurotensin | 1.7 ± 0.2 (8) | 1.54 ± 0.07 (78) | 1.8 ± 0.1 (11) | 1.80 ± 0.09 (43) | 1.3 ± 0.1 (4) | 0.74 ± 0.08 (4) | 1.80 ± 0.09 (3) | 3.4 ± 0.2 (3) |
| NT34 | 2.8 ± 0.3 (3) | 5.8 ± 0.6 (4) | 0.046 ± 0.005 (3) | 0.060 ± 0.005 (5) | 0.110 ± 0.008 (4) | 0.040 ± 0.002 (4) | 1.6 ± 0.2 (3) | 1.65 ± 0.05 (4) |

Values are geometric means ± SEM; each value is the mean of duplicate determinations made in independent experiments; values in parentheses indicate the number of independent experiments. In the schematic diagrams of the receptors, the circles indicate the position of site-directed mutagenesis. HEK-293 cells were transiently transfected. CHO-K1 cells were stably transfected.

**TABLE 2. Pharmacological and functional results in intact CHO-K1 cells**

| | Wild-type human NTR1 | | Wild-type rat NTR1 | | Mutant human NTR1 (Y339F) | | Mutant rat NTR1 (F344Y) | |
|---|---|---|---|---|---|---|---|---|
| | $K_d$ [nM] | $EC_{50}$ [nM] | $K_d$ [nM] | $EC_{50}$ [nM] | $K_d$ [nM] | $EC_{50}$ [nM] | $K_d$ [nM] | $EC_{50}$ [nM] |
| Neurotensin | 5.4 ± 0.4 (15) | 4.5 ± 0.3 (30) | 6.4 ± 0.7 (4) | 5.0 ± 0.3 (4) | 7.9 ± 0.4 (5) | 4.6 ± 0.8 (3) | 5.8 ± 0.4 (3) | 8 ± 1 (3) |
| NT34 | 51 ± 3 (3) | 61 ± 9 (7) | 0.22 ± 0.01 (4) | 3.42 ± 0.03 (9) | 0.46 ± 0.02 (4) | 3.28 ± 0.07 (3) | 5.9 ± 0.3 (4) | 59 ± 2 (3) |

$K_d$ = equilibrium dissociation constant. $EC_{50}$ = amount needed to stimulate 50% of maximal PI response. Values are geometric means ± SEM; each value is the mean of duplicate determinations made in independent experiments; values in parentheses indicate the number of independent experiments. In the schematic diagrams of the receptors, the circles indicate the position of site-directed mutagenesis.

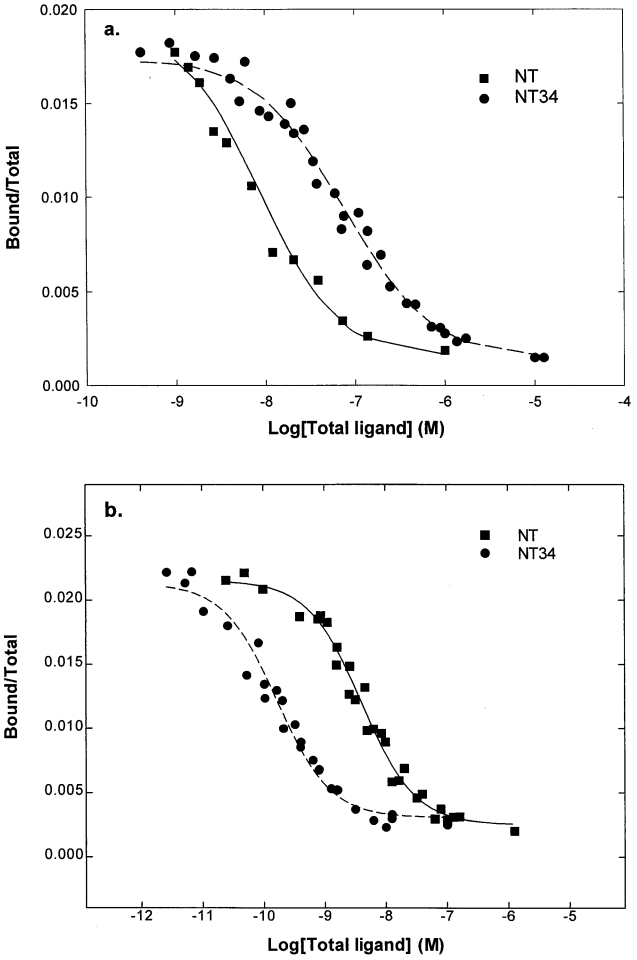



**FIG. 3. Competition binding between [$^3$H]NT and NT34 at the human (a) and rat (b) NTR1, expressed by CHO-K1 cells. Assays were performed with intact cells stably expressing either human or rat NTR1, 1 nM [$^3$H]NT, and various concentrations of compounds as described in the text. Curves were generated using the LIGAND program [16]. Data are the means of duplicate determinations and are representative results from one of at least three independent experiments.**

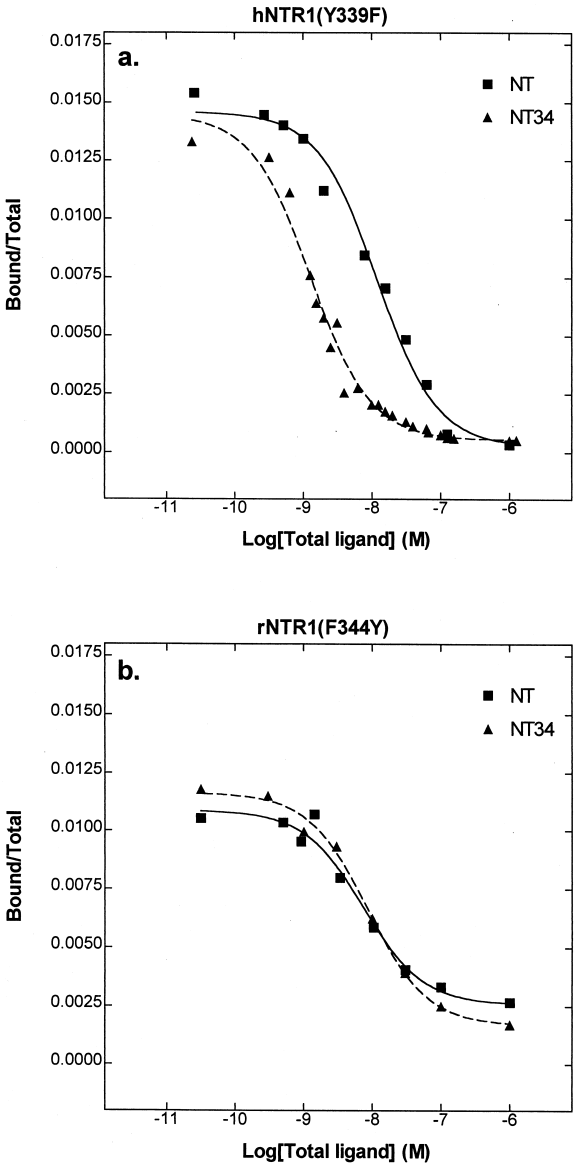



**FIG. 4. Competition binding between [$^3$H]NT and NT34 at the mutant hNTR1(Y339F) (a) and the mutant rNTR1(F344Y) (b) expressed by intact CHO-K1 cells. Assays were performed with intact cells stably expressing either human or rat mutant NTR1, 1 nM [$^3$H]NT, and various concentrations of compounds as described in the text. Curves were generated using the LIGAND program [16]. Data are the means of duplicate determinations and are representative results from one of at least three independent experiments.**

51 ± 3 nM). With the Y339F mutant human receptor, the affinity of NT34 was increased 110-fold, giving a $K_d$ value of 0.46 ± 0.02 nM, again similar to that for the wild-type rNTR1. Conversely, with the F344Y mutant rat receptor, the affinity of NT34 for binding was decreased by 27-fold ($K_d$ = 5.9 ± 0.3 nM), to give a binding affinity closer to that for its binding to the wild-type hNTR1. We present representative competitive binding curves in Figs. 3 and 4. For all these cell lines, there was no difference in the number of receptors expressed per cell (Table 3), which could have affected these binding results.

To determine the effects of the site mutations on receptor physiological function, we performed PI turnover analysis on both wild-type receptors and the two site mutants. For all cell lines tested, the $EC_{50}$ values for NT were in the same range (Table 2). However, for the species-specific NT34 analog, the results of PI turnover were remarkably different. NT34 was 18-fold more potent at the wild-type rat receptor ($EC_{50}$ = 3.42 ± 0.03 nM) compared with its potency at the wild-type human receptor ($EC_{50}$ = 61 ± 9 nM). However, in the cell line expressing the human Y339F mutant receptor, the potency of NT34 for stimulation of PI turnover increased 18-fold, giving an $EC_{50}$ (3.28 ± 0.07 nM) that was identical to its potency for the wild-type rat receptor. Conversely, for the cells expressing the rat F344Y mutant receptor, the potency of NT34 decreased 17-fold, producing an $EC_{50}$ value (59 ± 2 nM) that was identical to its potency for the wild-type human

**TABLE 3. Comparison of $B_{max}$ (sites/cell) in intact CHO-K1 cell lines**

| Receptor expressed in cell line | Sites/cell* |
|---|---|
| Wild-type human NTR1 | 138,000 ± 8,000 (4) |
| Wild-type rat NTR1 | 140,000 ± 40,000 (4) |
| Mutant rat(F344Y) NTR1 | 150,000 ± 60,000 (3) |
| Mutant human(Y339F) NTR1 | 260,000 ± 70,000 (3) |

*There was no significant difference for the mean values among the four groups analyzed (ANOVA, $P = 0.264$). Values are means ± SEM; the number of experiments is given in parentheses.

receptor. Representative dose–response curves of PI turnover for the mutant receptors with NT and NT34 are shown in Figs. 5 and 6.

## DISCUSSION

Of the eight amino acids that are included in E3, two amino acids differ between the human and rat receptors. From our proposed binding site model [9], it was predicted that $Tyr^{339}$ in the hNTR1 is the pivotal residue for binding, with its counterpart in the rat receptor being $Phe^{344}$ (the rat receptor is 5 amino acids longer at this point in the structure). Our present studies were designed to test the hypothesis that this single amino acid residue was responsible for the difference in binding affinity of NT34 between the human and rat receptors. Predictions were made that the affinity for binding of NT34 to these receptors could be changed by changing $Phe^{344}$ to $Tyr^{344}$ (F344Y) in the rat receptor and, likewise, changing $Tyr^{339}$ to $Phe^{339}$ (Y339F) in the hNTR1. However, since in this part of the receptor (at least for the rat), $Tyr^{347}$ is the important residue for the binding of NT [20], no changes in the binding of the parent peptide would be expected.

The initial studies used membrane preparations of NTR1 from transiently transfected HEK-293 cells. The results of site-directed mutagenesis of the human and rat NT receptors strongly supported our predictions (Table 1). Although there was no difference in the binding affinity of NT across any of the cell lines tested, the binding affinity for NT34 was affected markedly by the Y339F mutation in the human receptor and the F344Y mutation in the rat receptor. Thus, for the human wild-type receptor and the Y339F mutant, the affinity of NT34 changed, respectively, from 2.8 to 0.11 nM with the single amino acid change in the receptor sequence. For the rat NTR1, the corresponding single amino acid change shifted the affinity of binding of NT34 from 0.046 nM for the wild-type receptor to 1.6 nM for the mutant F344Y receptor.

Continuing our studies using stably transfected CHO-K1 cells, we repeated experiments using membrane preparations of NTR1 from these cells. Again, it was found that the change of one amino acid in the human wild-type receptor from $Tyr^{339}$ to $Phe^{339}$ was sufficient to alter the binding affinity of NT34 from 5.8 nM (wild-type) to 0.040 nM (Y339F), representing a 145-fold increase in affinity, a

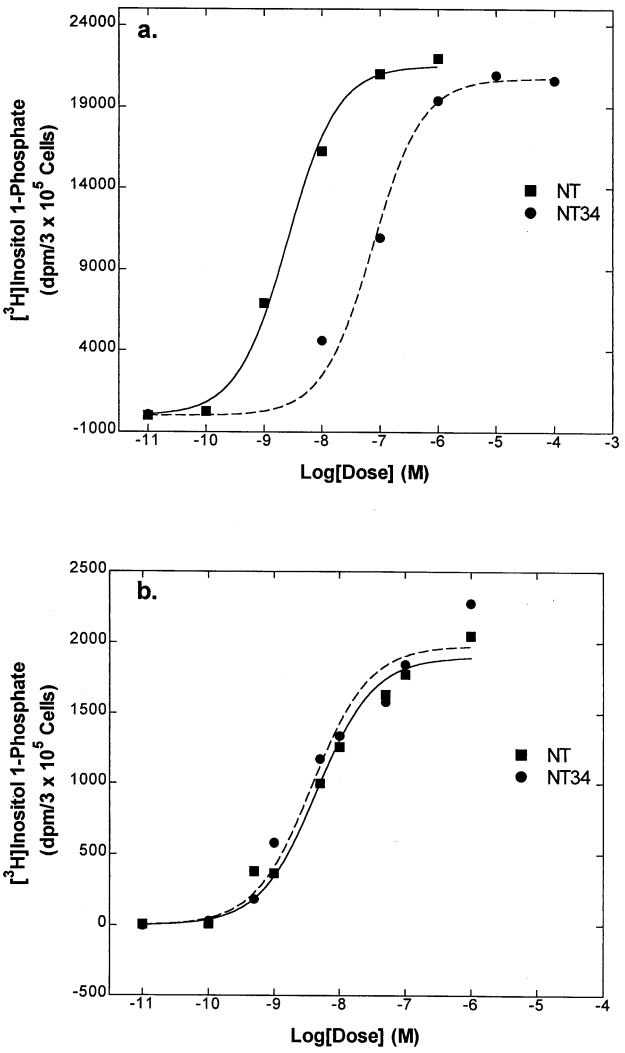



**FIG. 5. Dose–response curves for NT- and NT34-stimulated $[^3H]IP_1$ formation in intact cells containing hNTR1 (a) and rNTR1 (b). Assays were performed on intact CHO-K1 cells stably expressing either human or rat NTR1. Data are means of triplicate determinations from which the average of triplicate basal values has been subtracted (the basal level of $[^3H]IP_1$ at the hNTR1 in dpm/3 × $10^5$ cells was 310, whereas at the rNTR1 in dpm/3 × $10^5$ cells it was 420). The data presented are representative results from one of three independent experiments.**

value nearly identical to NT34 binding to the wild-type rat receptor. Conversely, with the rat wild-type receptor and its mutated form, i.e. $Phe^{344}$ to $Tyr^{344}$, NT34 bound with an affinity of 0.06 and 1.6 nM, respectively. The decrease in affinity of NT34 was about 28-fold with this mutation and is in the range seen with the wild-type human receptor.

A more important analysis was conducted using intact CHO-K1 cells. Binding results with the four forms of the expressed NT receptors again were without effect on NT binding activity (Table 2). However, with the mammalian-specific NT34, the results confirmed our prediction of the importance of residues $Tyr^{399}$ and $Phe^{344}$ at the NT binding site. For the human receptor mutant Y339F, NT34 exhib-

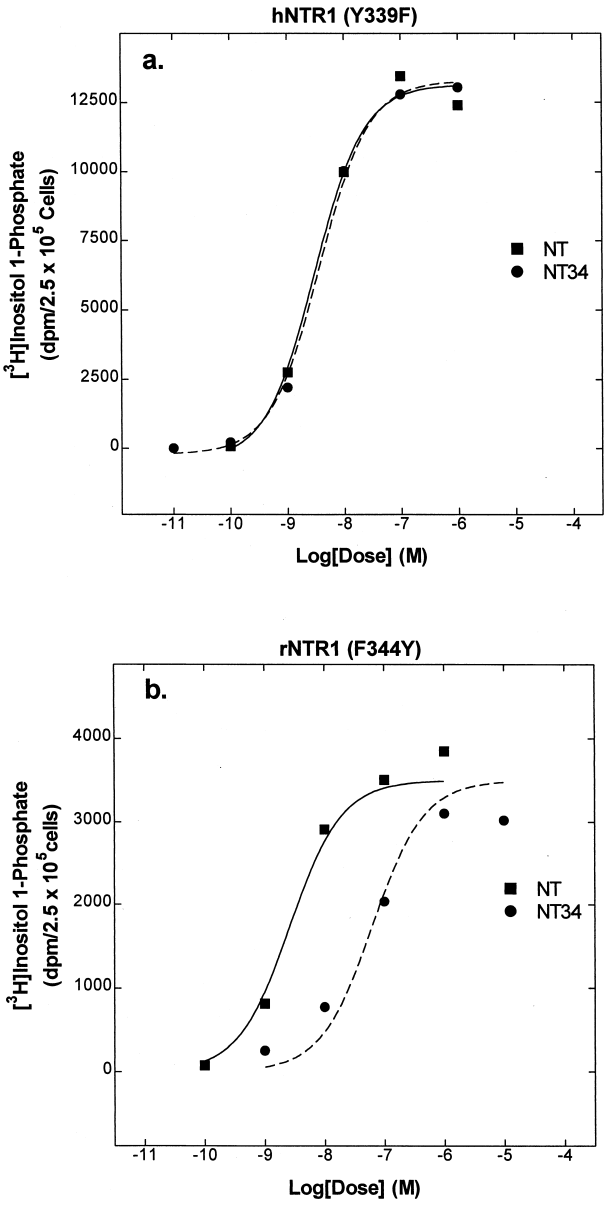



**FIG. 6. Dose–response curves for NT- and NT34-stimulated $[^3H]IP_1$ formation at the mutant hNTR1(Y339F) (a) and the mutant rNTR1(F344Y) (b) expressed by CHO-K1 cells. Assays were performed on intact CHO-K1 cells stably expressing the indicated human or rat NTR1. Data are means of triplicate determinations from which the average of triplicate basal values has been subtracted (the basal level of $[^3H]IP_1$ at the human Y339F mutant in dpm/2.5 × $10^5$ cells was 130, whereas at the rat F344Y mutant in dpm/2.5 × $10^5$ cells it was 590). The data presented are representative results from one of three independent experiments.**

ited a $K_d$ value similar to its binding to the wild-type rNTR1 (Table 2). In addition, the potency of NT34 at stimulating PI turnover in the Y339F mutants, $EC_{50}$ = 3.28 nM, was almost identical to the $EC_{50}$ for this analog at the wild-type rNTR1 ($EC_{50}$ = 3.42 nM). From our previous structure–activity studies, we proposed that hydrophobic and van der Waals pi–pi interactions were important for binding to the NT receptor.

From the present results with NT34 and the mutant rat and human receptors, we can propose that the OH group on the phenolic ring of the $Tyr^{339}$ of the human receptor is critical to the binding of NT34. Indeed, the difference from $Tyr^{339}$ to $Phe^{339}$ is merely the loss of a polar hydroxyl group (O + H). This change results in a substantial difference in the binding and function of NT34 at both wild-type and mutant receptors studied here. Thus, for the rNTR1, the mutant F344Y showed a decrease in binding affinity for NT34 of 0.22 nM for the wild-type rNTR1 versus 5.9 nM for the mutant. Similar to the human mutant, NT34 at the rat F344Y mutant showed an $EC_{50}$ = 59 nM, compared with the $EC_{50}$ for NT34 at the human receptor, which was 61 nM. The importance of the one critical amino acid change in both hNTR1 and rNTR1 is demonstrated by the dramatic change in the binding and potency of NT34. The large decrease in potency of NT34 observed with the F344Y mutant receptor is compatible with the species-specific NT34 not fitting as well into the binding pocket of the mutant rat receptor. As predicted, the hydroxyl group on Tyr in the F344Y rat mutant has decreased the volume of the binding site size sufficiently to effect a significant alteration in the affinity and potency for NT34. Additionally, it is important to point out that a functional effect on the hydrolysis of PI turnover is also changed by this point mutation. It could be proposed that the local conformational structure of the receptor has been altered with the change of a single amino acid, which significantly affects the potency toward PI turnover with NT34 but not with the natural ligand NT(1–13).

Other groups studying peptide receptors have also reported that agonist- and antagonist-binding sites are different [21, 22]. In a study describing mutations made to charged amino-acid residues of the rNTR1 [20], it was reported that residues $Arg^{327}$ and $Arg^{328}$ were important for NT binding. These residues are located in TM6 and are conserved in both the hNTR1 and rNTR1. This study also proposed that $Asp^{139}$ and $Arg^{143}$ (in the E1 loop) contributed to NT binding. Our findings, along with the results from the studies above [20–23], are useful in the development of a structural model for the NT antagonist- and agonist-binding site.

In summary, we have demonstrated a critical role for $Phe^{334}$ in the rNTR1 and its counterpart, $Tyr^{339}$, in the human receptor for affecting the binding affinity of a species-specific peptide, NT34. In support of our original hypothesis of a binding site for this agonist, site-directed mutagenesis of this residue revealed that significant changes to both binding affinity and potency of NT34 can be effected by reciprocal substitution of Tyr and Phe in rat and human NTR1, respectively. The present data provide strong evidence for a size difference in the binding pocket of the rat versus human NTR1, which can be distinguished with the use of the species-specific NT34. These results, along with observations from other groups, provide insights into the molecular interactions between the NTR1 and its ligands and the development of structural models of the NT

binding site on the NTR1 receptor. In addition, this work demonstrates the importance of studying even minor structural variations in proteins to determine the basis of significantly different drug responses, an area of focus for pharmacological research in the 21st century.

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