Chimeric Rat/Human Neurotensin Receptors Localize a Region of the Receptor Sensitive to Binding of a Novel, Species-specific, Picomolar Affinity Peptide

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Recently, we reported the development of a species-specific neurotensin analog that displays selective binding affinity at the rat and human neurotensin (NT) receptor, L-[3,2\(^3\)H]Nal\(^{11}\)NT(8–13) (where Nal is naphthylalanine) (NT19). We have developed another neurotensin analog, L-[3,1\(^3\)H]NT(8–13), (NT34), that exhibits a 126-fold difference in binding affinities between the rat and human receptors. This compound differs from our previous reported species-specific ligand in the steric positioning of the naphthyl ring on the L-alanine side chain. For NT34, the observed K\(_d\) values at the rat and human neurotensin receptors were 0.046 and 5.8 nM, respectively. In stimulating phosphatidylinositol turnover, the observed EC\(_{50}\) values were 2.8 nM and 130 nM in rat and human, respectively. We constructed a series of chimeric rat/human neurotensin receptor genes and expressed them by transient transfection into human embryonic kidney (HEK-293) cells. Radioligand binding assays were then performed using neurotensin and NT34. Our results led us to propose a region of the neurotensin receptor that may be involved in determining species specificity, i.e. the transmembrane VI, the third extracellular loop, and transmembrane VII regions of the neurotensin receptor.

Neurotensin (NT)\(^\dagger\) is a tridecapeptide that was originally isolated from the bovine hypothalamus (1). Since its discovery, NT has been found to be important in the mammalian central nervous system with effects that include hypothermia (2), anorexia and weight loss (3), nociception (3), sedation and muscle relaxation (4), and decreased locomotor activity (5). Initial studies at the cellular level linked NT with the production of cGMP (6), phosphatidylinositol (7), calcium mobilization (8), and the stimulation of adenyl cyclase (9). Neurotensin has been found to be important in the mammalian central nervous system with effects that include hypothermia (2), anorexia and weight loss (3), nociception (3), sedation and muscle relaxation (4), and decreased locomotor activity (5). Initial studies at the cellular level linked NT with the production of cGMP (6), phosphatidylinositol (7), calcium mobilization (8), and the stimulation of adenyl cyclase (9).

Our work has focused on developing novel NT analogs of NT(8–13), both nonpeptide (14) as well as peptide forms (15). Recently, we reported the development of many new NT(8–13) analogs, including a species-specific one, NT19, with a \(\beta\) naphthylalanine substituted into position 11 of the native NT(8–13). This compound displays much higher binding affinity at the rat NT receptor (NTR) compared to the binding at the human NTR (see above).

At the molecular level, there are several differences between the rat (16) and human (17, 18) neurotensin receptors in their primary sequences. The rat receptor comprises 424 amino acids, while the human receptor consists of 6 fewer amino acids. In addition, the proteins share an 84% homology at the amino acid level, and both proteins display a transmembrane topology similar to that of other G-protein coupled receptors. Thus, the original placement of the NTR proteins in the membrane was based on the bacteriorhodopsin model for G-protein coupled receptors (19). Bacteriorhodopsin is the only seven-transmembrane domain protein for which a detailed experimental structure of the helical bundle is available. It has been used as a template to construct three-dimensional models for other proteins and receptors in this large group.

In order to design rationally drugs that bind to neurotensin receptors, we have been interested in the structural basis for the ligand-receptor interaction at this receptor. We thought that we had an excellent tool to do so, with NT19. However, subsequently, we developed another NT(8–13) compound, namely L-[3,1\(^3\)H]NT(8–13) (NT34), that exhibits an even greater difference in binding affinities between the rat and human NT receptors. We present the biochemical and pharmacological properties of NT34 at both the rat and human receptors. We show that it is the most potent compound ever reported at the rat NTR.

We used NT34 together with a proven, valuable approach in examining ligand-receptor interactions of other G-protein coupled receptors, namely the construction of chimeric receptors. The construction of chimeric receptors from functionally different subtypes was first used by Kobilka et al. (20). Subsequently, the use of chimeric receptors has also been used in defining interspecies variations in agonist and antagonist binding (21–24).

To elucidate the structural basis for the pharmacological distinctions between the rat and human NT receptors, we constructed a series of chimeric rat/human NT receptor genes and expressed them by transient transfection into human embryonic kidney cells (HEK-293). The pharmacological properties of the expressed chimeric receptor proteins were assessed by radioligand binding utilizing the newly developed species-specific ligand, NT34. We present here the results of our work.

* This work was supported by the Mayo Foundation and by United States Public Health Service Grant MH27692 (to E. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: NT, neurotensin; CHO-K1, Chinese hamster ovary cells; PI, phosphatidylinositol; HEK-293, human embryonic kidney cells; NTR, neurotensin receptor; HPLC, high pressure liquid chromatography.
which represents the first report to identify regions of the NTRs that are important for determining species specificity of binding.

**Experimental Procedures**

Materials—The NT peptides and NT peptide analogs in this study are listed in Table I. Doctor Daniel J. McCormick of the Mayo Protein Core Facility (Mayo Clinic, Rochester, MN) synthesized the peptides presented here by previously described methods (25). Briefly, Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry was used to synthesize peptides with t-buty1-protected side chains, either individually on automated peptide synthesizers (Applied Biosystems 430A or 431A) or simultaneously on a multiple peptide synthesizer (ACT350, Advanced Chemtech, Louisville, KY). Protocols concerning activation coupling times, amino acid dissolution, coupling solvents, and synthesis scale were followed according to the protocols developed in the Protein Core Facility. All peptides were purified by reverse-phase HPLC using a C18 column (2.2 × 25 cm, Vydac, Hesperia, CA) in 0.1% trifluoroacetic acid/water and a gradient of 10%-60% acetonitrile in 0.1% trifluoroacetic acid/water. A combination of analytical HPLC and mass spectrometry was used to analyze peptide purity.

Taq DNA polymerase was obtained from Promega (Madison, WI), and Pwo DNA polymerase was obtained from Boehringer Mannheim, while the PCR Optimizer kit was obtained from Invitrogen. Oligonucleotides were obtained from the Molecular Biology Core Facility (Mayo Clinic, Rochester, MN) or from Keystone Laboratories (Menlo Park, CA). The pGEM-T Vector system was obtained from Promega, and the pGEMScript SK (+) cloning kit from Stratagene (LaJolla, CA). Sequencing was performed by Stratagene (La Jolla, CA), and sequencing was done on an ALF DNA sequencer using the AutoRead Sequencing Kits (Pharmacia Biotech Inc.) or by the Molecular Biology Core Facility (Mayo Clinic, Rochester, MN). Restriction enzymes were obtained from Promega (HindII, BstEI, XbaI) and New England Biolabs (Beverly, MA) (BstWII). T4 DNA Ligase was obtained from Promega. pCDNA3 eukaryotic expression vector was obtained from Invitrogen.

Construction of Rat Human Chimeras—Human and rat neurotensin receptor cDNAs were cloned as described previously (16, 17). Oligonucleotides were designed to introduce restriction enzyme sites into PCR products of human and rat neurotensin receptors by silent mutations. The oligonucleotide sequences are as follows.

| Ref. | Peptide | Sequence | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|------|---------|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1    | NT(8–13)| p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| 9    | Neurtensin | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| 34   | L-[Ile-Nal]4 NT(8–13) | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| 19   | L-[13-Ile-Nal]4 NT(8–13) | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |

*Na1, (naphthyl)alanine.

a See Cusack et al. (15).

**Species-specific NT Ligand and Receptor Chimeras**

Structural comparison of NT peptide analogs

| Sequence | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|----------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| NT(8–13) | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| Neurtensin | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| L-[Ile-Nal]4 NT(8–13) | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |
| L-[13-Ile-Nal]4 NT(8–13) | p-Glu L-Leu L-Tyr L-Glu L-Asn L-Lys L-Pro L-Arg L-Arg L-Pro L-Tyr L-Ile L-Leu |

**Results**

The structures of the analogs tested are listed in Table I. We have numbered the compounds for easy reference.

**Radioligand Binding Studies**—The results from radioligand binding assays with the analogs studied are presented in Table II (34). The compounds are listed in rank order of potency at the hNTR in CHO-K1 cells. All the peptides tested had Hill coefficients close to unity (data not included), indicating that binding was to a single class of receptors. NT(8–13) was the most potent at the hNTR, with a Kd of 0.14 ± 0.01 nM. At the nNTR,
NT34 was the most potent compound, exhibiting a picomolar affinity ($K_d = 0.046 \pm 0.003 \text{ nM}$), while at the hNTR the $K_d$ was $5.8 \pm 0.6 \text{ nM}$. Comparing NT34 and NT19, a simple repositioning of the naphthyl ring on the L-alanine side chain (Fig. 1) increased binding affinity at the rNTR 33-fold and at the hNTR 13-fold. Additionally, the human/rat $K_d$ ratio for NT34 became 126 as compared to the $K_d$ ratio for NT19 of 51 (see Table II).

PI Turnover—We tested the ability of these compounds to stimulate wild-type neurotensin receptors by measuring PI turnover in intact CHO-K1 cells incubated with these compounds. All peptides tested were full agonists (Table II). For PI turnover in intact CHO-K1 cells incubated with these compounds, $K_d$ values were determined as geometric mean values obtained in the stably transfected CHO cells (Table II). In Fig. 2 (A and B), we present representative NT34 dose-response curves for PI turnover in intact CHO-K1 cells expressing the hNTR and rNTR, respectively. The human/rat $EC_{50}$ ratio for NT34 was 46, while for NT19 was 15 (Table II).

**Table II**

| Ref. | Analog | hNTR $K_d$ (nM) | rNTR $K_d$ (nM) | h/r ratio |
|------|--------|----------------|----------------|-----------|
| 1    | NT(8–13) | 0.14 ± 0.01 (4) | 1.5 ± 0.1 (3) | 0.005     |
| 19   | $\alpha$-[3.2-Nal]11NT(8–13) | 76 ± 7 (6) | 320 ± 60 (4) | 0.1(3)    |
| 13   | Neurotensin | 1.60 ± 0.07 (72) | 4.5 ± 0.3 (30) | 0.3(3)    |
| 34   | $\alpha$-[3.1-Nal]11NT(8–13) | 5.8 ± 0.6 (4) | 130 ± 20 (3) | 0.88      |
| 36   | [3H]NT | 1.7 ± 0.2 (20) | 20(3) | 0.046(4) |

In Fig. 2 (A and B), we present representative competitive binding curves with NT and NT34 in HEK-293 cells transiently transfected with hNTR and rNTR. When untransfected HEK-293 cells were tested for NT binding, we found no significant competition at NT concentrations up to 1 $\mu$M (data not included). Additionally, we transfected the HEK-293 cells with the vector (pcDNA 3) and tested for binding with NT and NT 34. No competition for $[3H]$NT binding could be detected in these cells at peptide concentrations up to 20 $\mu$M.

In the first set of constructs, C1 RH, we substituted the human/rat ligand and receptor chimeras for NT ligands and receptor chimeras. We tested the ability of these compounds to stimulate wild-type neurotensin receptors by measuring PI turnover in intact CHO-K1 cells incubated with these compounds. All peptides tested were full agonists (Table II).
amino extracellular region of the rNTR in the hNTR. Binding assays with NT and NT34 revealed Kd values that were more similar to results obtained with the hNTR (Table III). The binding of NT and NT34 to the C1 RH chimera were in the same Kd range, i.e. 0.61 nM for NT and 0.87 nM for NT34, suggesting a binding affinity more closely resembling that found for the human, wild-type gene product.

Next we constructed a chimera, C2, with substitutions made at the junction of TM V and the third intracellular loop (Fig. 3). Our initial site of substitution was made based on the membrane model proposed for the amino acid sequence based on Tanaka's results with the rNTR (16). Subsequently, we re-aligned our positioning of the receptor in the membrane after conducting modeling studies (36). Thus, the difference in the placement of the membrane position as shown in Fig. 3 reflects these changes. In the first C2 chimera, C2 RH, we substituted the rat sequence up to the junction of TM V and i-3 loop of the human receptor. Radioligand binding at the C2 RH revealed a Kd for NT of 1.7 ± 0.6 nM and for NT34, a Kd of 1.79 ± 0.06 nM (Table III). In this case the Kd values for both compounds are almost identical to each other and in the same nanomolar range as seen with these compounds in the wild-type human NTR.
Species-specific NT Ligand and Receptor Chimeras

To clarify further these findings, we prepared the mirror image construct of the C2 chimera, i.e. the replacement of the rat receptor sequence from the TM V to the carboxyl-terminal tail into the human receptor. Results with this C2 RH chimeric receptor exhibited a $K_d$ for NT of $2.9 \pm 0.1$ nM and for NT34, a $K_d$ of $0.067 \pm 0.003$ nM (Table III). Interestingly, these results resembled the $K_d$ values for the wild-type rat receptor in HEK-293 cells, as opposed to those for the hNTR.

The next construct, C3 RH, involved substituting the rat sequence from the amino-terminal extracellular region through most of the i-2 loop (Fig. 3). Thus the difference between C2 RH and C3 RH is the region including the TM IV, o-2 loop, and TM V. An examination of the values for NT and NT34 at the C3 RH chimera revealed results that were almost identical to each other, and did not exhibit the large $K_d$ difference demonstrated with the wild-type rat receptor (Table III).

The final chimera, C4 RH, contained the human receptor sequence from the beginning of TM V through the carboxyl-terminal intracellular tail (Fig. 3). Competitive binding with NT and NT34 revealed $K_d$ values at this chimera of $1.5 \pm 0.3$ nM and $1.00 \pm 0.03$ nM, respectively. The observed $K_d$ values for NT and NT34 were similar to the $K_d$ values obtained at the wild-type hNTR in these cells.

DISCUSSION

We have previously reported the development of a species-specific compound, NT19, which exhibits a significant difference in binding affinity between the rat and human NT receptor (15). This compound has a substitution of $\gamma$-3,2-naphthylalanine for tyrosine in position 11 of the parent peptide, NT (8–13). We explored changes in the position of the aromatic ring of the naphthylalanine and developed a compound, $\gamma$-{[3,1-\text{Na}]NT(8–13)} or NT34, which, surprisingly, exhibited a more substantial difference in observed $K_d$ values for the human versus rat NTR. NT34 revealed a greater than 100-fold difference in binding between the rat and human receptor. The $K_d$ at the rat receptor was in the picomolar range (0.046 $\pm$ 0.003 nM), ranking it as the most potent NT agonist reported to date.

Our previous findings with position 11 substitutions of NT (8–13) suggested that the human receptor was less tolerant of changes in steric bulk at this position, i.e. changes in steric bulk at position 11 causes marked increases in the $K_d$ values observed for these compounds at the hNTR and much smaller increases for these compounds at the rNTR, as compared to the respective $K_d$ values for NT (8–13). Comparing NT34 with NT19, the position of the naphthyl ring on the i-alanine side chain makes the volume of NT34 relatively smaller than that of NT19 (Fig. 1). This structural change increased the binding affinity of NT34 13-fold and 33-fold over NT19 at the human and rat receptor, respectively (Table II). The large increase in binding affinity at the rat receptor suggests a very good “fit” of the smaller side chain of NT34’s residue 11 into the binding site. Our results strongly support the hypothesis that the NT(8–13) binding site of hNTR is relatively smaller than that of hNTR (36). Interestingly, an examination of the functional response, i.e. PI turnover, showed a 2.5-fold increase in potency at the hNTR of NT34 versus NT19, while at the rNTR the increase was 7.6-fold (Table II). Therefore, although binding and function increased with NT34 over NT19, the increase in potency of PI response was of a different order of magnitude from that for the binding affinity.

Our strategy was to use NT34 as a tool to elucidate the structural basis for the pharmacological distinctions between the human and rat receptor species. Accordingly, we constructed a series of chimeric rat/human NT receptor genes and expressed them by transient transfection in HEK-293 cells. Initially, we observed the $K_d$ for the wild-type human and rat receptors in HEK-293 cells. The values for these results compared favorably with the results we had obtained in stably transfected CHO-K1 cells (Table III and Table II). These results are strong evidence that the receptors have pharmacologically identical binding characteristics in both cell lines. Additionally, the comparable $K_d$ values for both NT and NT34 in both cell lines suggest that the membrane environment and/or cellular processing of the receptor proteins are similar in both cases.

We reasoned that similar to the model for ligand binding to adrenergic receptors (35), the amino extracellular and carboxyl cytoplasmic sequences of the rat and human NT receptors were unlikely to affect significantly ligand binding specificities. However, there are many amino acids in the amino extracellular region of NT34 that are different between the rat and human NT receptors (Fig. 3). For example, we constructed a chimeric, C1 RH, that was designed to test our hypothesis. The ligand binding results with NT and NT34 indicated that the amino extracellular region was not contributing to the species specificity we observed. The $K_d$ values for NT and NT34 at C1 RH were almost identical (0.61 and 0.87 nM, respectively) and did not show the differential binding values characteristic of NT and NT34 at the rat receptor (Table III).

In chimera C2 RH, the rat receptor sequence up to the junction of TM V and the i-3 loop were substituted into the human NTR (Fig. 3). Binding results with this construct revealed $K_d$ values for NT and NT34 of 1.7 and 1.79 nM, respectively. Thus, the $K_d$ profile for NT and NT34 in this case more closely resembled binding in the wild-type human receptor rather than the wild-type rat receptor (Table III). This observation was reinforced by results with C2 HR. This next construct was the mirror image chimera of C2 RH, i.e. the human receptor sequence up to the junction of TM V and the i-3 loop (Fig. 3). $K_d$ values at this chimera for NT and NT34 were 2.9 and 0.067 nM, respectively. These results correspond most favorably to the binding affinities observed for NT and NT34 at the rat NTR (Table III). Taken together, the results from C2 RH and C2 HR support the location of the species defining region of NT34 to include the area from the i-3 loop through the carboxyl-terminal internal tail. Since it is highly unlikely that binding interactions occur intracellularly, i.e. in the i-3 loop and the carboxyl-terminal tail, this leaves TM VI, the o-3 loop, and TM VII regions as possible locations for the species defining binding of NT34.

We constructed another chimera that substituted the rat receptor into the human receptor up to the junction of the i-2 region and TM IV. This receptor, namely C3 RH, revealed binding parameters for NT and NT34 that were almost identical to each other, i.e. $K_d$ values = 0.32 and 0.504 nM, respectively, and also more comparable to results obtained in the wild-type hNTR expressed in HEK-293 cells (Table III). From these results it is clear that the o-2 loop is not involved in the species defining region for NT34. With the final chimera, C4 RH, the focus was on the TM V region of the NTR. The difference between the C4 RH and C2 RH is the TM V region, i.e. the TM V region in the C4 RH is derived from the human sequence, while in the C2 RH the TM V is from the rat sequence. In the C4 RH chimera, the observed $K_d$ values for NT and NT34 were 1.5 $\pm$ 0.3 and 1.00 $\pm$ 0.03 nM, respectively. These results with the C4 RH receptors indicate that the TM V region is not important to the species defining binding of NT34. These chimeric results must be viewed carefully, since the placement of residues could alter the spatial relationships between the extracellular domains of the receptors. Further experiments are
needed to elucidate completely the role of each segment in the probable species-specific binding of NT34. Experiments are currently in progress that should provide more evidence for this region.

Our results with NT34 and the rat/human chimeras presented here suggest a region on which to focus for possible NT binding, namely TM VI, α-3 loop, and TM VII of the NTR (Fig. 3). An examination of the amino acid sequence of the proposed NTR helical model indicates that there are only 5 amino acid residues that differ in the rat and human sequence in this region. Further studies using a combination of molecular techniques, e.g., site-directed mutagenesis, as well as the development of biochemical tools such as irreversible NT ligands and/or photoaffinity labels, may lead to a more inclusive and specific location of the NT binding site.

In summary, NT34 represents a new peptide ligand that exhibits picomolar affinity for the NTR. This increase in binding potency over its parent analog, NT19, is the result of the naphthyl ring placement on the L-alanine side chain at position 11 of the peptide. Second, our results with NT34 showed that the amino acid differences between the rat and human NTR sequences can confer dramatic pharmacological differences between homologs of the same receptor. This stresses the point that in evaluating binding affinities of new compounds as NT agonists or antagonists, pharmacological characteristics at the hNTR may be different from those observed at the rNTR. Finally, the differential binding of NT34 at the rat versus human NT receptor provides a valuable tool for defining the species-specific binding region on the NT receptor. These initial studies with NT34 and rat/human chimeric receptors have led us to propose a region of the NT receptor which is involved in determining species specificity and may imply the binding region of NT. This region includes TM VI, α-3 loop, and TM VII regions of the NT receptor.

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