

# Pivotal Role of an Aspartate Residue in Sodium Sensitivity and Coupling to G Proteins of Neurotensin Receptors

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## ABSTRACT

The highly conserved aspartate residue in the second transmembrane domain of G protein-coupled receptors is present in position 113 in the type 1 neurotensin receptor (NTR1) but is replaced by an Ala residue in position 79 in the type 2 neurotensin receptor (NTR2). NTR1 couples to Gαq to stimulate phospholipase C and its binding affinity for neurotensin is decreased by sodium ions and GTP analogs. By contrast, NTR2 does not seem to couple to any G protein in eukaryotic cells, and its binding of neurotensin is insensitive to sodium and GTP analogs. By using site-directed mutagenesis, we substituted Asp113 of the NTR1 by alanine and the homologous residue Ala79 of NTR2 by aspartate. Both mutant receptors display

similar affinity for neurotensin as compared with their respective wild type. We demonstrate that the presence of the Asp residue determines by itself the occurrence of the sodium effect on neurotensin affinity for both wild-type and mutated NTR1 and -2. The introduction of an Asp in the second transmembrane domain of NTR2 is not enough to restore a functional coupling to G proteins. In contrast, replacement of Asp113 by Ala residue in NTR1 strongly decreases its ability to activate inositol turnover, indicating that the functionally active conformation of NTR1 is maintained by interaction of sodium ions with aspartate 113.

Several guanine nucleotide-binding protein (G protein)-coupled receptors are sensitive to Na<sup>+</sup> ions that reduce their affinity for agonists (Ceresa and Limbird, 1994). Site-directed mutagenesis studies have identified a highly conserved Asp residue in the second transmembrane (TM) spanning domain as the site of Na<sup>+</sup> regulation of agonist binding (Neve et al., 1991; Kong et al., 1993a,b; Quintana et al., 1993; Ceresa and Limbird, 1994). Mutant receptors obtained from these studies can be classified into two categories. For the first class of Asp mutant receptors, such as those of *alpha*-2 and *beta* adrenergic receptors (Neve et al., 1991; Ceresa and Limbird, 1994), agonist binding was no longer regulated by GTP analogs, indicating that mutation of the Asp residue disturbs receptor-G protein interactions. A second class of Asp mutant receptors such as the sst2 receptor remained sensitive to GTP analogs (Kong et al., 1993a), indicating a different mode of interaction of these receptors with G proteins.

The two neurotensin receptors (NTR) cloned to date (Tanaka et al., 1990; Vita et al., 1993; Mazella et al., 1996; Chalon et al., 1996) represent an excellent model to study NT binding regulation by Na<sup>+</sup>. Indeed, the type 1 NT receptor

(NTR1) bears the highly conserved Asp residue in the second TM domain and binding to NT is sensitive to Na<sup>+</sup> ions and GTP analogs. Moreover, the NTR1 is functionally coupled to phospholipase C when stably expressed in CHO or LTK cells (Hermans et al., 1992; Watson et al., 1992; Chabry et al., 1994). By contrast, the NTR2 (Mazella et al., 1996; Chalon et al., 1996) is characterized by the absence in its sequence of the Asp residue in TM II, the corresponding position being occupied by an alanine. Interestingly, the binding of NT to this receptor type is insensitive to physiological concentrations of Na<sup>+</sup> (IC<sub>50</sub> ≥ 200 mM) and to GTP analogs (Mazella et al., 1996). The NTR2 does not seem to interact with classical G proteins, because no coupling was observed in HEK cells stably transfected with the mouse NTR2 cDNA (Botto et al. 1998). The purpose of the present work was to assess the importance of the conserved Asp residue in the regulation of NT binding to its receptors by Na<sup>+</sup> ions and GTP. Using site-directed mutagenesis, we replaced the Asp113 residue of the rat NTR1 by the corresponding Ala residue of the NTR2 and incorporated an aspartate instead of alanine in position 79 in the NTR2 sequence. The effect of Na<sup>+</sup> ions and guanosine-5'-O-(γ-thio)triphosphate (GTPγS) on NT binding properties were studied for mutant receptors and compared with those of native receptors. We show that the presence of

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**ABBREVIATIONS:** NT, neurotensin; NTR1, neurotensin receptor type 1; NTR2, neurotensin receptor type 2; PI, phosphoinositide(s); GTPγS, guanosine-5'-O-(γ-thio)triphosphate; G protein, guanine nucleotide-binding protein; TM, transmembrane domain.

an aspartate in the TM II is an absolute requirement for the effect of  $\text{Na}^+$  but not for the effect of the GTP analog.

## Materials and Methods

**Materials.** NT was purchased from Peninsula Laboratories (Belmont, CA) and  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT was prepared and purified as previously described (Sadoul et al., 1984). The pcDNA3 expression vector was purchased from Invitrogen (San Diego, CA), Dulbecco's modified Eagle's medium and gentamycin were purchased from Life Technologies (Gaithersburg, MD). Fetal calf serum was purchased from Boehringer Mannheim (Indianapolis, IN), and oligodeoxynucleotides and restriction or modification endonucleases were from Eurogentec (Seraing, Belgium). *Taq* polymerase was from Appligene (Appligene Oncor, Illkirch, France).

**Mutant NT Receptor Construction and Expression.** The *Hind*III-*Not*I fragment (1.45 kb) of the rat NTR1 cDNA and the *Hind*III-*Apa*I fragment (1.5 kb) of the mouse NTR2 cDNA were subcloned into the pcDNA3 expression vector. Site-directed mutagenesis was performed by polymerase chain reaction according to the method of Jones et al. (1990) using oligodeoxynucleotides bearing the point mutation. The introduction of mutations in the receptor cDNAs was confirmed by sequencing with the dye terminator ABI PRISM sequencing kit (Perkin-Elmer, Norwalk, CT) using appropriate oligodeoxynucleotide primers.

All cDNA constructs obtained by polymerase chain reaction were subcloned into the eukaryotic expression vector pcDNA3 containing the cytomegalovirus promoter. Transient transfections were performed with 1 to 4  $\mu\text{g}$  of recombinant pcDNA3 plasmid by the diethylaminoethyl-dextran precipitation method (Cullen, 1987) onto semiconfluent COS-7 cells grown in 100-mm cell culture dishes. Binding assays were performed approximately 60 h after transfection. Membranes from nontransfected COS-7 cells were totally devoid of specific  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT binding.

**Binding Studies.** Binding experiments were carried out on freshly prepared cell homogenates as previously described (Chabry et al., 1994). Competition experiments were initiated by incubation of cell homogenates (10  $\mu\text{g}$  for NTR1-transfected cells and 50  $\mu\text{g}$  for NTR2-transfected cells) in 250  $\mu\text{l}$  of binding buffer: 50 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin, 1 mM  $\text{MgCl}_2$ , and 0.8 mM 1-10 phenanthroline (metallopeptidase inhibitor) with 0.4 nM  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT (2000 Ci/mmol) (Sadoul et al., 1984) and increasing concentrations of unlabeled NT or GTP $\gamma\text{S}$  (from  $10^{-10}$  to  $10^{-6}$  M). Saturation experiments were performed by competition assay in which the binding of 0.4 nM  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT (2000 Ci/mmol) was inhibited by increasing concentrations of unlabeled NT

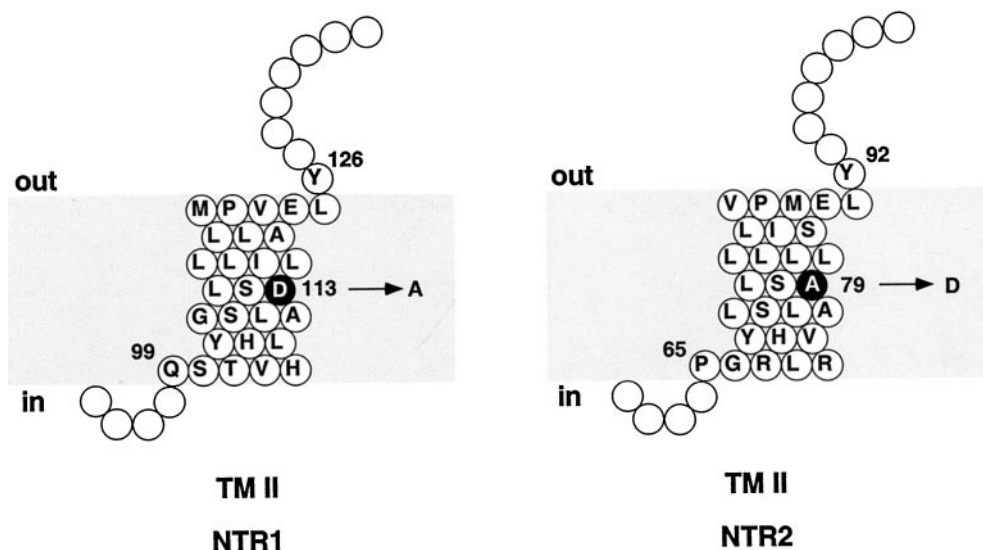
(0.2–50 nM). We have previously demonstrated that iodinated and unlabeled peptides bound NT receptors with the same affinity (Sadoul et al., 1984). The nonspecific binding was determined in parallel experiments in the presence of 1  $\mu\text{M}$  unlabeled NT. After 20 min at 25°C, incubation media were filtered through cellulose acetate filters (Sartorius, Bohemia, NY). Filters were rinsed twice with 2 ml of ice-cold binding buffer and counted in a Packard gamma counter. The effect of  $\text{Na}^+$  ions on  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT binding was measured with NaCl concentrations ranging from 3 to 300 mM. In some cases, competition experiments with unlabeled NT were performed in the presence of 15 or 50 mM  $\text{Na}^+$ .

**Phosphoinositides (PI) Determination.** Twenty four hours after transfection with different NT receptor forms, cells were grown in 12-well plates for 15 to 18 h in the presence of 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]myo-inositol (ICN Biomedicals, Ovsay, France) in a serum-free Ham's-F10 medium. Cells were washed with Earle's buffer, pH 7.5, (25 mM HEPES, 25 mM Tris, 140 mM NaCl, 5 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , and 5 mM glucose) containing 0.1% bovine serum albumin, and incubated for 15 min at 37°C in 900  $\mu\text{l}$  of 30 mM LiCl in Earle's buffer. NT was then added at the indicated concentrations for 15 min. The reaction was stopped by 750  $\mu\text{l}$  of ice-cold 10 mM HCOOH, pH 5.5. After 30 min at 4°C, the supernatant was collected and neutralized by 2.5 ml of 5 mM  $\text{NH}_4\text{OH}$ . Total [ $^3\text{H}$ ]PI were separated from free [ $^3\text{H}$ ]inositol on Dowex AG-X8 (Bio-Rad, Hercules, CA) (Van Renterghem et al., 1988) chromatography by eluting successively with 5 ml of water and 4 ml of 40 mM and 1 M ammonium formate, pH 5.5. The radioactivity contained in the 1 M fraction was counted after addition of 5 ml of Ecolume (ICN Biomedicals).

## Results

The TM II Asp residue conserved in most of the G protein-coupled receptors is present in position 113 in the sequence of the NTR1 and substituted by Ala79 in the NTR2 (Fig. 1). This amino acid was replaced on each receptor by the homologous residue of the other. We then compared the binding properties and sensitivity to  $\text{Na}^+$  ions and GTP $\gamma\text{S}$  as well as the coupling efficiency of the mutant receptors to those of the wild-type receptors.

**Compared Affinities of Mutant and Wild-Type NT Receptors Toward NT.** Competition binding experiments between  $^{125}\text{I}$ -labeled Tyr<sub>3</sub>-NT and unlabeled NT were performed on membrane homogenates from COS-7 cells transiently transfected with recombinant plasmids of the wild-type and mutated NTR1 and NTR2. Figure 2 shows that the



**Fig. 1.** Schematic representation of predicted second TM spanning domain of NTR1 and NTR2. Homologous position of Asp113 in NTR1 is shown to be Ala79 in NTR2.

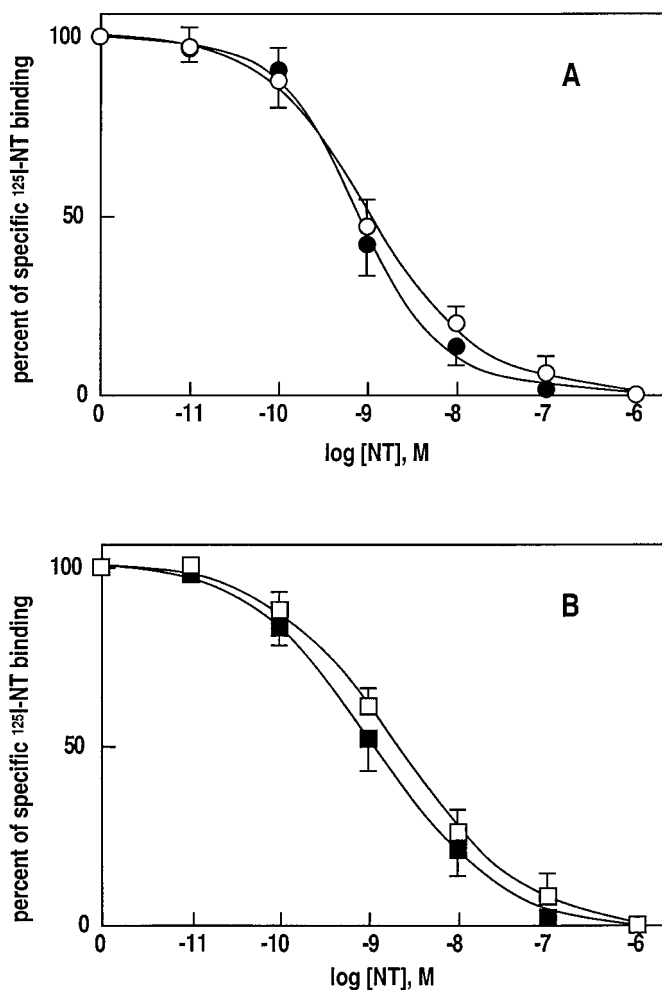
affinity of the D113A NTR1 mutant for NT ( $IC_{50} = 0.8 \pm 0.15$  nM,  $n = 3$ ) was not significantly altered when compared with that of the wild-type receptor ( $IC_{50} = 0.6 \pm 0.2$  nM,  $n = 4$ ) (Fig. 2A). The  $IC_{50}$  value of the A79D NTR2 mutant was only slightly higher ( $IC_{50} = 2 \pm 0.3$  nM,  $n = 3$ ) than that of the wild-type NTR2 ( $IC_{50} = 1.5 \pm 0.4$  nM,  $n = 4$ ) (Fig. 2B), indicating that the affinity of the A79D-NTR2 mutant was not significantly different from that of the native NTR2.

**Effect of Sodium Ions on Binding of Mutant and Wild-Type NT Receptors.** The binding of 0.4 nM  $^{125}I$ -labeled Tyr<sub>3</sub>-NT to homogenates of cells transfected with the wild-type NTR1 receptor was inhibited in a concentration-dependent manner by Na<sup>+</sup> (Fig. 3A) with a half-maximal inhibiting concentration ( $IC_{50}$ ) of  $17.7 \pm 2.2$  mM ( $n = 4$ ). Substitution of the Asp113 residue by an alanine decreased the sensitivity of the mutated NTR1 to Na<sup>+</sup> ions by a factor of 10 ( $IC_{50} = 144 \pm 11$  mM,  $n = 4$ ) (Fig. 3A).

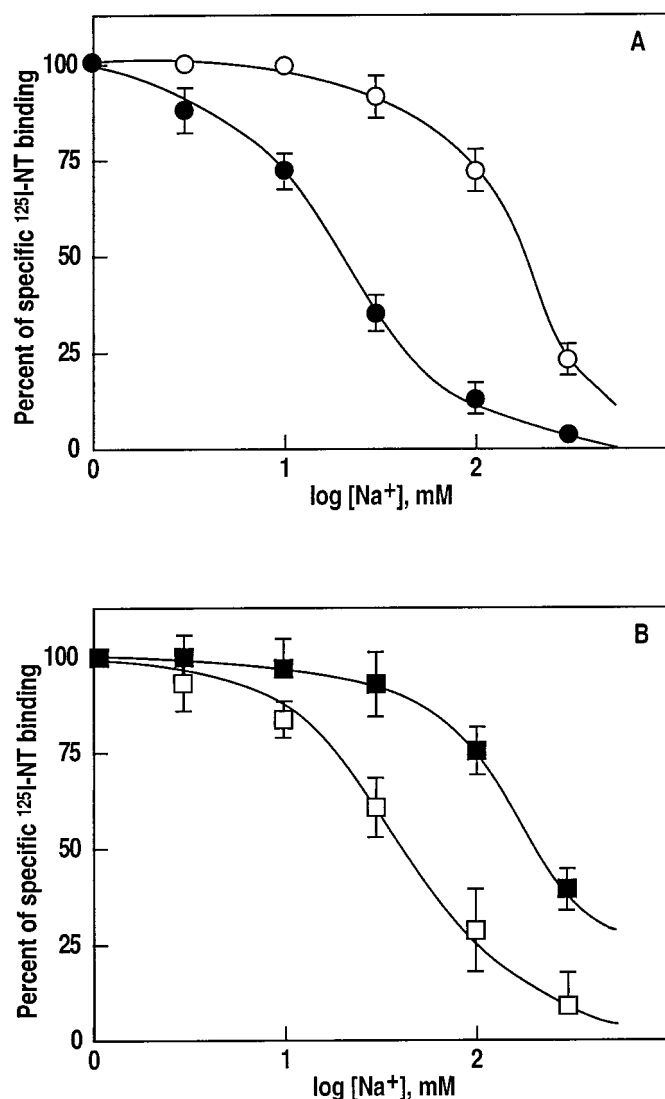
The very weak effect of Na<sup>+</sup> ions on the binding of  $^{125}I$ -labeled Tyr<sub>3</sub>-NT to the wild-type mouse NTR2 ( $IC_{50} = 225 \pm 17$  mM,  $n = 4$ ) was in agreement with the absence of an

aspartate in the TM II of the receptor molecule (Fig. 3B). Interestingly, when Ala79 was replaced by an Asp residue, the binding of  $^{125}I$ -labeled Tyr<sub>3</sub>-NT to the expressed mutant NTR2 became more sensitive to sodium with an  $IC_{50}$  of  $55 \pm 5$  mM ( $n = 4$ ) (Fig. 3B). These results demonstrated that receptors bearing an Asp in their TM II, i.e., the wild-type NTR1 and the A79D mutant NTR2, were much more sensitive to sodium ions than those lacking this Asp, i.e., the D113A NTR1 and the wild-type NTR2.

To determine the NT binding parameter affected by Na<sup>+</sup> ions, both maximal binding capacities ( $B_{max}$ ) and affinities ( $K_d$ ) of each wild-type and mutant NT receptor were determined in saturation experiments carried out in the absence or in the presence of various Na<sup>+</sup> concentrations. Figure 4 shows that  $B_{max}$  values of the mutant or wild-type NTR1 and NTR2 were not modified by Na<sup>+</sup> ions. However, the  $K_d$  value



**Fig. 2.** Competition between  $^{125}I$ -labeled Tyr<sub>3</sub>-NT and neurotensin for binding to wild-type and mutated NTR1 (A) and NTR2 (B). Binding of  $^{125}I$ -Tyr<sub>3</sub>-NT was measured in presence of increasing concentrations of unlabeled NT to homogenates prepared from COS-7 cells transfected with wild-type (●) or D113A (○) NTR1 (A) and with wild-type (■) or A79D (□) NTR2 (B). Results are expressed as percentage of specific binding measured in absence of unlabeled peptide. Each point is mean  $\pm$  S.E.M. calculated from three or four experiments.



**Fig. 3.** Effect of Na<sup>+</sup> on specific  $^{125}I$ -labeled Tyr<sub>3</sub>-NT binding to wild-type and mutated NTR1 (A) and NTR2 (B). Specific binding of  $^{125}I$ -labeled Tyr<sub>3</sub>-NT (0.4 nM) was measured in presence of increasing concentrations of NaCl on homogenates prepared from COS-7 cells transfected with wild-type (●) or D113A (○) NTR1 in A and with wild-type (■) or A79D (□) NTR2 in B. Results are expressed as percentage of specific binding measured in absence of sodium. Each point represents mean of duplicate determinations from four different experiments.

of the wild-type NTR1 rised from  $0.45 \pm 0.05$  nM ( $n = 5$ ) in the absence of  $\text{Na}^+$  to  $1.23 \pm 0.18$  nM ( $n = 3$ ) in the presence of 15 mM  $\text{Na}^+$  ( $p < .05$ ) (Fig. 4A). The affinity of the D113A mutant NTR1 for NT ( $K_d = 0.53 \pm 0.07$  nM,  $n = 3$ ) was significantly affected ( $p < .1$ ) by the presence of 145 mM  $\text{Na}^+$  in the incubation medium ( $K_d = 2.25 \pm 0.75$  nM,  $n = 2$ ) (Fig. 4B).

The affinity of the wild-type NTR2 was also significantly decreased ( $p < .01$ ) by the presence of 225 mM  $\text{Na}^+$  ( $K_d = 7.2 \pm 0.7$  nM,  $n = 2$ ) when compared with experiments performed in the absence of  $\text{Na}^+$  ( $K_d = 2.6 \pm 0.3$  nM,  $n = 4$ ) (Fig. 4C). For the A79D mutant NTR2, although the  $K_d$  value was increased to  $3.4 \pm 0.9$  nM ( $n = 3$ ) in the presence of 50 mM  $\text{Na}^+$ , the difference observed with the  $K_d$  value obtained in the absence of  $\text{Na}^+$  ions ( $2.5 \pm 0.8$  nM,  $n = 3$ ) was not significant (Fig. 4D). These data indicate that physiologically relevant concentrations of  $\text{Na}^+$  ions can modulate the affinity of both the NTR1 and NTR2 for NT provided an Asp residue is present in the conserved position of the TM II.

**Effect of GTP $\gamma$ S on Binding of Mutant and Wild-Type NT Receptors.** As previously shown, GTP analogs modulate the affinity of NT for the rat and human NTR1 stably transfected in LTK or CHO cells (Chabry et al., 1994; Watson et al., 1992). This effect was correlated with the coupling efficiency of this receptor type to phospholipase C and adenylate cyclase. By contrast, the absence of coupling observed for the mouse NTR2 stably expressed in HEK cells was associated with the absence of GTP $\gamma$ S effect on NT binding (Botto et al., 1998).

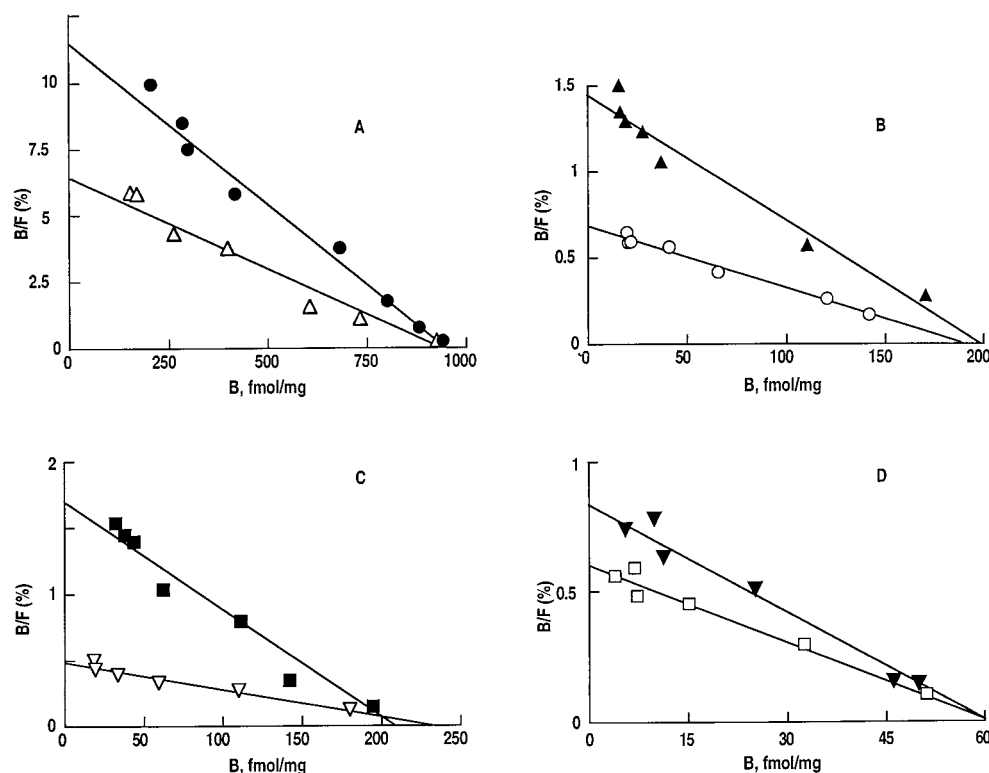
In an attempt to study the possible link between the  $\text{Na}^+$  sensitivity and the coupling efficiency, we measured the effect of GTP $\gamma$ S on NT binding to membrane homogenates from cells transfected with mutant and wild-type NT receptors (Fig. 5). The binding of  $^{125}\text{I}$ -labeled Tyr $_3$ -NT to D113A mu-

tant and wild-type NTR1 was inhibited in a concentration-dependent manner by GTP $\gamma$ S (Fig. 5). A maximal inhibition of about 65 to 75% was observed with an  $\text{IC}_{50}$  of  $3 \pm 0.7$  nM ( $n = 4$ ) for both type 1 NT receptors. By contrast, the binding of  $^{125}\text{I}$ -labeled Tyr $_3$ -NT to membranes from cells expressing either the A79D mutant or the wild-type NTR2 was not affected by GTP $\gamma$ S independently of the presence or the absence of the Asp residue in the TM II (Fig. 5).

**Agonist-Stimulated PI Turnover.** NT-stimulated PI production was measured in COS-7 cells expressing either the wild-type NTR1 and NTR2 or D113A-NTR1 and A79D-NTR2 mutants. Figure 6 shows that none of the NTR2 forms were able to increase PI production upon NT stimulation. In contrast, both the wild-type and the D113A mutant of NTR1 stimulated PI levels as a function of NT concentration (Fig. 6). However, although these two receptor forms displayed identical NT binding sensitivity to GTP $\gamma$ S (Fig. 5), NT was 100-fold less potent on the D113A-NTR1 ( $\text{EC}_{50} = 10.3 \pm 2.3$  nM,  $n = 3$ ) than on the wild-type NTR1 ( $\text{EC}_{50} = 0.09 \pm 0.01$  nM,  $n = 3$ ) (Fig. 6). This difference in the potency of NT to stimulate PI production is not due to the differential receptor expression between the wild-type NTR1 ( $B_{\text{max}} = 920 \pm 150$  fmol/mg,  $n = 5$ ) and the D113A-NTR1 ( $B_{\text{max}} = 200 \pm 23$  fmol/mg,  $n = 3$ ). Indeed, when the expression of the wild-type is lowered to the amount of mutant by transfection with 0.2  $\mu\text{g}$  of recombinant plasmid, the potency of NT remains quite identical, although the amount of [ $^3\text{H}$ ]PI accumulation is three times lower (Fig. 6).

## Discussion

This work demonstrates that the presence of the Asp residue in the second TM of NTR1 and NTR2 is critical for the modulation of their affinities by  $\text{Na}^+$  ions. This negatively

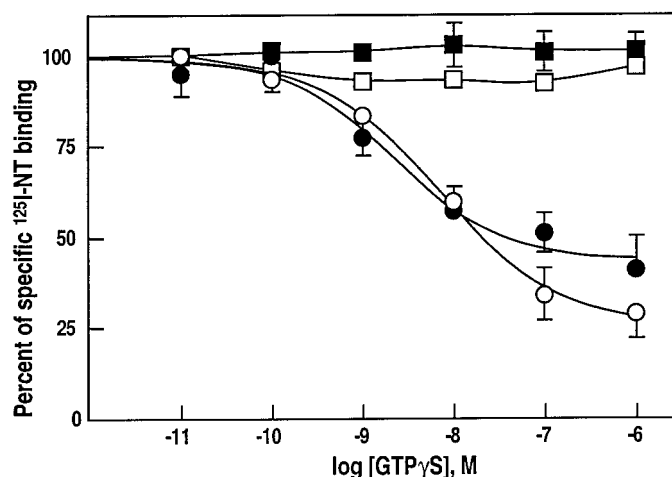


**Fig. 4.** Binding of  $^{125}\text{I}$ -labeled Tyr $_3$ -NT to wild-type and mutated NT receptors in presence or absence of sodium ions. Data are presented as Scatchard representations of specific  $^{125}\text{I}$ -labeled Tyr $_3$ -NT binding to homogenates from cells transfected with wild-type NTR1 (A), D113A NTR1 (B), wild-type NTR2 (C), or A79D NTR2 (D) in absence (closed symbols) or presence (open symbols) of 15 mM NaCl (A), 145 mM NaCl (B), 225 mM NaCl (C), or 50 mM NaCl (D). Data are from a representative experiment performed two to five times for each receptor.

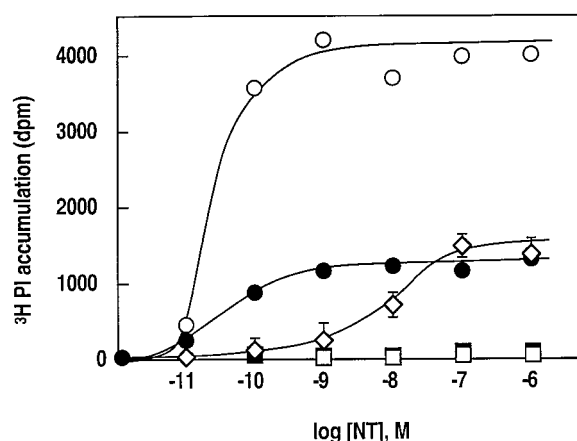


charged residue is also necessary for an efficient coupling of the NTR1 to phospholipase C.

The absence of efficient coupling to phospholipase C for the NTR2 stably expressed in eukaryotic cell system (Botto et al., 1998) was initially correlated with its insensitivity to physiological concentrations of  $\text{Na}^+$  ions ( $\text{IC}_{50} = 250 \text{ mM}$ ) (Mazella et al., 1996). We hypothesized that this property was the consequence of the absence of an highly conserved Asp residue in the second TM spanning domain of the NTR2, the corresponding position being occupied by an alanine residue. The substitution of this alanine by an Asp residue (A79D-NTR2) effectively reconstituted a relatively good sensitivity of the type 2 NT receptor to  $\text{Na}^+$  ions ( $\text{IC}_{50} = 55 \text{ mM}$ ), and we verified that this sensitivity was due to a decrease of the



**Fig. 5.** Effect of  $\text{GTP}\gamma\text{S}$  on specific  $^{125}\text{I}$ -labeled  $\text{Tyr}_3\text{-NT}$  binding to wild-type and mutated NTR1 and NTR2. Specific binding of  $^{125}\text{I}$ -labeled  $\text{Tyr}_3\text{-NT}$  ( $0.4 \text{ nM}$ ) to homogenates prepared from COS-7 cells transfected with wild-type (●) or D113A (○) NTR1 and with wild-type (■) or A79D (□) NTR2 was measured in presence of increasing concentrations of  $\text{GTP}\gamma\text{S}$ . Results are expressed as percentage of specific binding measured in absence of  $\text{GTP}\gamma\text{S}$ . Each point represents mean of duplicate determinations from four different experiments.



**Fig. 6.** Effect of NT on PI production in cells transfected with wild-type and mutated NT receptors. COS-7 cells were transfected with wild-type NTR1 (●, ○), D113A NTR1 (◇), wild-type NTR2 (■), or A79D NTR2 (□). Transfected cells were incubated for 15 min at  $37^\circ\text{C}$  with increasing concentrations of NT, and PI production was measured as detailed in *Materials and Methods*. Results are expressed as amount of  $^3\text{H}$ PI accumulation for each receptor. Values are means  $\pm$  S.E.M. of three independent experiments for D113A NTR1 (◇). For wild-type NTR1, average of triplicate determinations are shown for expression of NTR1 at  $1000 \text{ fmol/mg}$  (○) or at  $200 \text{ fmol/mg}$  (●).

affinity for NT in the presence of  $\text{Na}^+$  ions. However, the A79D mutant NTR2 remained uncoupled to phospholipase C when expressed into COS-7 cells. The lack of coupling for both the wild-type NTR2 and the A79D-NTR2 mutant was in agreement with their insensitivity to  $\text{GTP}\gamma\text{S}$  (Fig. 5) and their inability to increase the PI turnover in response to NT (Fig. 6). These data indicate that the ability of NTR2 to couple to G proteins does not depend solely upon the presence of an Asp residue in its second TM domain.

The NTR1 belongs to the family of G protein-coupled receptors in which the Asp residue (D113), located in the second TM domain, is strictly conserved (Probst et al., 1992). The affinity of the NTR1 for NT was therefore classically regulated by  $\text{Na}^+$  ions ( $\text{IC}_{50} = 17.7 \text{ mM}$ ) and by  $\text{GTP}\gamma\text{S}$  ( $\text{IC}_{50} = 3 \text{ nM}$ ). The NTR1 expressed in COS-7 cells responded to NT by a dose-dependent accumulation of IPs with an  $\text{EC}_{50}$  of about  $0.1 \text{ nM}$  and this whatever the amount of expressed receptors (Fig. 6). The substitution of Asp113 by alanine did not modify the affinity of the mutated receptor for NT but reduced its sensitivity for  $\text{Na}^+$  ions ( $\text{IC}_{50} = 144 \text{ mM}$ ). Consequently, the affinity of D113A-NTR1 for NT became insensitive to  $\text{Na}^+$  ions concentrations, which are effective on the wild-type NTR1 (i.e.,  $17 \text{ mM}$ ). It is of interest to note that both mutant receptor (D113A-NTR1 and A79D-NTR2) are expressed at a lower level than their respective wild types (Fig. 4). This indicates that single mutations in the second spanning domain are sufficient to decrease receptor expression. Surprisingly,  $\text{GTP}\gamma\text{S}$  inhibited the binding of NT on homogenates from cells transfected with this mutant NTR1 receptor with an efficiency ( $\text{IC}_{50} = 3 \text{ nM}$ ) similar to that measured on the wild-type receptor (Fig. 5). This led us to believe that the NTR1 belonged to the category of receptors such as the *sst2A* somatostatin receptor (Kong et al., 1993a), in which the mutation of the Asp residue present in TM II did not alter receptor-G protein association. However, the potency of NT to stimulate PI accumulation on COS-7 cells transfected with the D113A-NTR1 mutant was actually dramatically reduced ( $\text{EC}_{50} = 10.3 \text{ nM}$ ) as compared with the wild-type ( $\text{EC}_{50} = 0.09 \text{ nM}$ ) (Fig. 6), indicating that the association with G protein was effectively affected by the loss of the negatively charged amino acid residue. These data show that conclusions driven from the modulatory effects of GTP analogs on the affinity of a ligand for its receptor should be interpreted with caution when experimental results are obtained in acellular systems. Although observation of a decrease in affinity probably reflects the existence of an interaction between the receptor and a G protein, the nonphysiological character of these experiments precludes their interpretation on a quantitative basis. Thus, the 100-fold decrease in potency of NT on the PI response resulting from the D113A mutation of the NTR1 (Fig. 6) could not be detected by measuring the  $\text{GTP}\gamma\text{S}$ -induced changes in NT binding properties of both receptors (Fig. 5). Another possible explanation is that the discrepancy between  $\text{GTP}\gamma\text{S}$  effect and coupling efficiency could reflect a distinction between determinants for the binding of the receptor to G proteins and determinants for the activation of G proteins by the receptor. Thus, Asp113 in the NTR1 would be necessary for G protein activation but not for G protein binding.

In conclusion, we showed that the presence of an Asp residue in the second TM domain of the two cloned NTRs is

a necessary condition to observe sodium modulation of the NTR affinity. This same Asp residue is also involved in the coupling efficiency of the type 1 NT receptor to G proteins. However, mutation of the Asp residue does not abolish G protein coupling. The mode of interaction of the NTR1 with G proteins is therefore more similar to that of the sst2 receptor than to that of the  $\alpha$ -2 and  $\beta$  adrenergic receptors.

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