# Functional coupling with $G\alpha_q$ and $G\alpha_{i1}$ protein subunits promotes high-affinity agonist binding to the neurotensin receptor NTS-1 expressed in *Escherichia coli*

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**Abstract To analyze the coupling of Gα subunits to the rat neurotensin receptor NTS-1 (NTR), fusion proteins were expressed in *Escherichia coli* with various Gα subunits covalently linked to the receptor C-terminus. The presence of $G\alpha_q$ or $G\alpha_{i/q}$, in which the six C-terminal residues of $G\alpha_{i1}$ were replaced with those from $G\alpha_q$, increased the percentage of receptors in the agonist high-affinity state. This effect was less pronounced for wild-type $G\alpha_{i1}$ and not observed for $G\alpha_{i/s}$. Functional coupling of neurotensin receptor to Gα was demonstrated by neurotensin-induced [$^{35}$S]GTPγS binding for the $G\alpha_q$, $G\alpha_{i/q}$ and $G\alpha_{i1}$ subunits, but not for $G\alpha_{i/s}$. Our results extend previous findings of the dual coupling of NTR to pertussis toxin-sensitive and -insensitive G-proteins in Chinese hamster ovary cells with preference for the latter. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Neurotensin receptor; NTS-1; G-protein α subunit; Guanosine 5′-*O*-(3-thiotriphosphate); Seven-helix G-protein-coupled receptor–Gα fusion; *Escherichia coli*

## 1. Introduction

The rat high-affinity neurotensin receptor NTS-1 (NTR) [1] is an integral membrane protein and belongs to the large family of seven-helix G-protein-coupled receptors (GPCRs) (see [2]). Its natural ligand is neurotensin (NT), a 13 amino acid peptide [3], which is involved in intercellular communication within the central nervous system and peripheral organs. NTR and its N-terminally truncated form (T43NTR) have been expressed as maltose binding protein (MBP) fusions in *Escherichia coli* [4,5] and have been purified in the presence of detergent in functional form [5–7].

Binding studies using membrane-bound NTR expressed in mammalian cells or in insect cells have revealed high-affinity agonist binding sites which are converted into low-affinity binding sites in the presence of non-hydrolyzable GTP analogues [8–10]; this probably indicates the coupling of NTR to endogenous G-proteins. Furthermore, pertussis toxin (PTX)-sensitive and PTX-insensitive G-proteins, linked to the cAMP and inositol phosphate messenger systems, respectively, have been implicated in coupling [11–17]. However, the interpretation of such studies and the identification of the respective G-proteins coupled to NTR have often been complicated by the presence of multiple endogenous heterotrimeric G-proteins and differences in the cell lines used for investigation.

We have analyzed the coupling of Gα protein subunits to the bacterially expressed rat NTR using a GPCR–Gα fusion protein approach combined with [$^3$H]NT and GTPγS (guanosine 5′-*O*-(3-thiotriphosphate)) binding experiments. Functional expression of GPCRs in *E. coli* provides the opportunity to study receptor properties in a background free of heterotrimeric G-proteins and/or pharmacologically similar receptor subtypes [18–20]. We provide evidence for receptor interaction with both $G\alpha_q$- and $G\alpha_i$-type proteins covalently linked to the receptor C-terminus. Our results support previous data [21] of the dual coupling of NTR to PTX-sensitive and -insensitive G-proteins with predominant coupling to PTX-insensitive G-proteins.

## 2. Materials and methods

### 2.1. Expression of NTR fusion proteins in E. coli and membrane preparation

The fusion protein MBP-T43NTR-TrxA-H10 consists of the *E. coli* MBP (Lys-1 to Thr-366), followed by glycine, serine, the N-terminally truncated NTR (T43NTR, Thr-43 to Tyr-424) [5], three alanine residues, the *E. coli* thioredoxin (TrxA, Ser-2 to Ala-109), glycine, threonine and a deca-histidine tail (H10) [6]. In MBP-T43NTR-Gq and MBP-T43NTR-Gi, Tyr-424 of NTR is followed by three alanine residues and the mouse $G\alpha_q$ protein (Thr-2 to Val-359) [22] or the rat $G\alpha_{i1}$ subunit (Gly-2 to Phe-354) [23], respectively. MBP-T43NTR-Gi/q and MBP-T43NTR-Gi/s contain chimeric $G\alpha_{i1}$ subunits in which the six C-terminal amino acid residues were replaced with the equivalent sequences of the mouse $G\alpha_q$ protein (Lys-Glu-Tyr-Asn-Leu-Val) or the rat $G\alpha_s$ subunit (Arg-Gln-Tyr-Glu-Leu-Leu) [23], respectively. Expression of membrane-inserted receptors in *E. coli* DH5α was performed at 20°C using the expression vector pRG/III-hs-MBP [5]. The preparation of crude membranes has been described previously [5].

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*Abbreviations:* CHO, Chinese hamster ovary; Gi/q, $G\alpha_{i1}$ subunit in which the six C-terminal amino acid residues were replaced with the six C-terminal residues of $G\alpha_q$; Gi/s, $G\alpha_{i1}$ subunit containing the six C-terminal amino acid residues of $G\alpha_s$; GPCR, seven-helix G-protein-coupled receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MBP, *Escherichia coli* maltose binding protein; NT, neurotensin; NTR, high-affinity neurotensin receptor NTS-1; PTX, pertussis toxin; TrxA, *Escherichia coli* thioredoxin

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Protein contents were determined by the method of Schaffner and Weissmann [24] using bovine serum albumin as the standard.

*2.2. Expression of NTR in Chinese hamster ovary (CHO) cells*

The characterization of CHO cells expressing the rat NTR in functional form (CHO-NTR) has previously been reported [25,26]. Binding experiments were performed on crude homogenates prepared as described [26].

*2.3. Ligand binding analyses*

Crude *E. coli* membranes (100 μl) were washed twice with 500 μl of ice-cold TE buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA) using a bench top microcentrifuge at $10\,500 \times g$ for 3 min at 4°C. Saturation binding analyses at equilibrium with the agonist [$^3$H]NT (New England Nuclear) on five different membrane preparations (MBP-T43NTR-TrxA-H10: 0.4–1.2 μg of membrane protein per assay tube, MBP-T43NTR-Gq: 0.3–0.6 μg, MBP-T43NTR-Gi: 0.2–0.3 μg, MBP-T43NTR-Gi/q: 0.2–0.4 μg, MBP-T43NTR-Gi/s: 0.2–0.3 μg) were performed in TEBB buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 40 μg of bacitracin per ml, 0.1% (w/v) bovine serum albumin) [27] in a final volume of 100 μl. When indicated, $MgCl_2$ was added to the assay buffer at a final concentration of 5 mM. Non-specific binding was determined in the presence of 2 μM unlabeled NT (Sigma). Samples were incubated for 30 min at room temperature. The assay was terminated by centrifugation ($10\,500 \times g$, 4 min) at room temperature. The membrane pellet was resuspended in 70 μl of 0.1 M formic acid after removal of the supernatant containing unbound [$^3$H]NT. Bound ligand was analyzed by liquid scintillation counting. All experiments were performed in duplicate.

Data from saturation assays were analyzed by non-linear least-squares curve-fitting using the program Ebda/Ligand [28,29]. Improvement of an individual fit assuming two states was considered significant if $P$ values of less than 0.05 were obtained in an $F$ test. $B_H$ values (percentage of receptors in the high-affinity state) and dissociation constants were compared by means of an unpaired $t$-test (two-tail) or one-way analysis of variance (Anova) (GraphPad Prism software). The threshold $P$ value was set to 0.05.

*2.4. Binding of [$^{35}$S]GTPγS*

The binding experiments were performed at 30°C in a final volume of 1 ml in polypropylene tubes containing 20 μg (CHO-NTR homogenates) or 100 μg (*E. coli* membranes) of protein. The binding buffer consisted of 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 1 μM 1,10-phenanthroline, 0.1% bovine serum albumin, 1 μM GDP and 1 mM dithiothreitol. Binding reactions were initiated by the addition of [$^{35}$S]GTPγS (Amersham, specific activity 1000 Ci/mmol) at a final concentration of 0.1 nM. Non-specific binding was determined in the presence of 0.1 mM guanylylimidodiphosphate. After incubation for 30 min, the reactions were terminated by dilution with 3 ml of ice-cold washing buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl) and immediate filtration over GF/B glass fiber filters (Whatman) followed by two washes with washing buffer, using a 24-channel harvester (Semat, UK). The amount of [$^{35}$S]GTPγS trapped on the filter was determined by liquid scintillation counting. All binding data were analyzed by non-linear regression using the software GraphPad Prism.

## 3. Results

*3.1. Expression of MBP-T43NTR fusion proteins in E. coli*

The fusion proteins MBP-T43NTR-TrxA-H10, MBP-T43NTR-Gq, MBP-T43NTR-Gi, MBP-T43NTR-Gi/q and MBP-T43NTR-Gi/s (Fig. 1) were produced in membrane-inserted form in *E. coli*. Their presence was detected with anti-MBP antisera (New England Biolabs) as described [4] (data not shown). Their agonist binding properties are shown in Table 1.

*3.2. MBP-T43NTR-Gα fusion proteins expressed in E. coli bind agonist with high and low affinity*

[$^3$H]NT binding assays performed with the fusion proteins MBP-T43NTR-Gq, -Gi, -Gi/q and -Gi/s showed the presence of two independent populations of binding sites with high and low affinity for NT (Table 1, Fig. 2). This was also found for MBP-T43NTR-TrxA-H10 which lacks a C-terminal Gα moiety (R. Grisshammer, unpublished work). However, the percentage of receptors in the high-affinity state was significantly higher for the $G\alpha_q$ ($B_H = 46.9\%$) and $G\alpha_{i/q}$ ($B_H = 49.0\%$) proteins compared to that of MBP-T43NTR-TrxA-H10 ($B_H = 22.9\%$) ($t$-test: $P = 0.005$ and $P = 0.002$), indicating a possible stabilization of the receptor high-affinity state by the $G\alpha_q$ and $G\alpha_{i/q}$ moieties. In contrast, the percentage of MBP-T43NTR-Gi/s in the high-affinity state ($B_H = 28.2\%$) was not different from that observed for MBP-T43NTR-TrxA-H10 ($t$-test: $P = 0.53$). For MBP-T43NTR-Gi, an intermediate $B_H$ value of 34.6% was obtained. The difference between this value and that obtained for MBP-T43NTR-TrxA-H10 is not significant ($t$-test: $P = 0.08$), but is borderline significant when compared with the data obtained for MBP-T43NTR-Gq and MBP-T43NTR-Gi/q (Anova: $P = 0.047$).

*3.3. Agonist-induced [$^{35}$S]GTPγS binding to MBP-T43NTR-Gα fusion proteins expressed in E. coli*

As previously described for the CHO-NTR system, agonist-induced exchange of GDP for [$^{35}$S]GTPγS in the nucleotide binding pocket of Gα proteins requires the presence of NaCl [26]. The lower potency of NT found in these experiments is a consequence of the decreased affinity of NT for its receptor in the presence of sodium ions.

NTR has been reported to couple to both PTX-sensitive and PTX-insensitive G-proteins [21]. This prompted us to investigate NT-induced [$^{35}$S]GTPγS binding to fusion proteins with either $G\alpha_{i1}$ or $G\alpha_q$ covalently linked to the receptor C-

Table 1
Saturation binding analyses with [$^3$H]NT of MBP-T43NTR fusion proteins using washed membrane preparations from *E. coli*

| Fusion protein | Additions to TEBB assay buffer | p$K_{DH}$ | p$K_{DL}$ | $B_{max}$ (pmol/mg) | $B_H$ (%) |
|---|---|---|---|---|---|
| MBP-T43NTR-TrxA-H10 ($n = 6$) | none | 9.80 ± 0.09 | 8.67 ± 0.11 | 23.7 ± 2.3 | 22.9 ± 4.4 |
| MBP-T43NTR-Gq ($n = 5$) | none | 10.15 ± 0.08* | 8.86 ± 0.11 | 12.4 ± 0.5 | 39.6 ± 3.4 |
| MBP-T43NTR-Gq ($n = 5$) | 5 mM $MgCl_2$ | 10.05 ± 0.06 | 8.97 ± 0.11 | 10.7 ± 0.6 | 46.9 ± 4.9 |
| MBP-T43NTR-Gi ($n = 4$) | 5 mM $MgCl_2$ | 9.91 ± 0.09 | 8.95 ± 0.07 | 35.0 ± 2.5 | 34.6 ± 2.4 |
| MBP-T43NTR-Gi/q ($n = 4$) | 5 mM $MgCl_2$ | 10.08 ± 0.05 | 8.82 ± 0.12 | 12.9 ± 1.5 | 49.0 ± 1.7 |
| MBP-T43NTR-Gi/s ($n = 4$) | 5 mM $MgCl_2$ | 9.81 ± 0.12 | 8.89 ± 0.11 | 30.1 ± 1.1 | 28.2 ± 7.3 |
| MPB (negative control) ($n = 1$) | none | ND | ND | ND | ND |

Ligand binding assays were performed in TEBB buffer. $MgCl_2$ was added when indicated. Data are shown ± S.E.M. $B_{max}$ values are given for the combined high- and low-affinity states. The differences between the mean p$K_{DL}$ values (low-affinity state) are not statistically significant (Anova: $P = 0.37$); similarly, the differences between the p$K_{DH}$ values (high-affinity state) are not statistically significant (Anova: $P = 0.11$) except that marked by *. $B_H$: average percentage of receptors in the high-affinity state; negative control: crude membranes prepared from *E. coli* DH5α containing the parental expression plasmid pRG/III-hs-MBP; ND: no specific [$^3$H]NT binding detected.

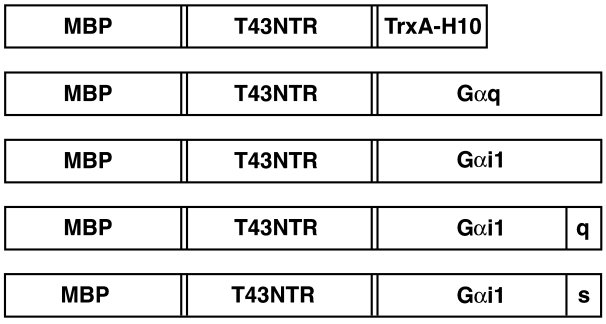


Fig. 1. Schematic representation of the MBP-T43NTR fusion proteins analyzed in this study. The boxes shown are not drawn to scale. The abbreviations for the modules of the receptor fusion proteins are as follows. MBP, mature *E. coli* MBP; T43NTR, truncated rat NTR; TrxA-H10, *E. coli* thioredoxin with a deca-histidine tail; Gq, mouse $G\alpha_q$ protein; Gi, rat $G\alpha_{i1}$ subunit; Gi/q, $G\alpha_{i1}$ subunit in which the six C-terminal amino acid residues were replaced with the six C-terminal residues of $G\alpha_q$; Gi/s, $G\alpha_{i1}$ subunit containing the six C-terminal amino acid residues of $G\alpha_s$.

terminus. Experiments conducted on *E. coli* membranes containing MBP-T43NTR-Gi revealed a highly significant ($P < 0.001$ as compared to basal nucleotide binding, *t*-test) response to NT (Fig. 3A). Concentration–response curves (Fig. 3B) gave an $pEC_{50}$ value of $7.88 \pm 0.25$ which is identical to the potency of NT measured in [$^{35}$S]GTPγS assays using CHO-NTR cell homogenates [26]. Experiments with MBP-T43NTR-Gq did not reveal an increase in [$^{35}$S]GTPγS binding after the addition of NT using the assay conditions described in Section 2.4. However, an indication for the functional coupling of T43NTR with $G\alpha_q$ came from [$^{35}$S]GTPγS binding in the presence of $(NH_4)_2SO_4$ at a concentration of 0.5 M [30]. After addition of NT, a 24% increase in [$^{35}$S]GTPγS specific binding to MBP-T43NTR-Gq was observed (one experiment in quadruplicate, data not shown). To further show receptor interaction with $G\alpha_q$-type proteins, T43NTR was fused to a chimeric $G\alpha_{i1}$ subunit, in which the six C-terminal amino acid residues were replaced with the equivalent sequences of the $G\alpha_q$ protein (MBP-T43NTR-Gi/q). This approach accounts for the importance of the Gα C-terminus for receptor specificity, and benefits from the high intrinsic guanine nucleotide exchange activity of the $G\alpha_i$ subunit. As shown in Fig. 3A, highly significant NT-induced [$^{35}$S]GTPγS binding was observed with the MBP-T43NTR-Gi/q construct. The agonist-induced increase in [$^{35}$S]GTPγS specific binding above basal was significantly higher ($P < 0.005$, *t*-test) for the chimeric -Gi/q fusion ($269 \pm 41\%$) compared to that for the wild-type -Gi construct ($77 \pm 2.5\%$) (Fig. 3B). The $pEC_{50}$ value of $7.95 \pm 0.16$ for NT-induced [$^{35}$S]GTPγS binding was similar to that obtained with MBP-T43NTR-Gi (Fig. 3B).

To demonstrate that coupling of T43NTR was specific for $G\alpha_q$ and $G\alpha_{i1}$, but not the effect of simple physical proximity of the receptor and the Gα subunit in the fusion constructs, MBP-T43NTR-Gi/s, containing the chimeric $G\alpha_{i1}$ subunit with the six C-terminal amino acid residues of $G\alpha_s$, was subject to [$^{35}$S]GTPγS binding assays. Although no functional response to NT was measured (Fig. 3A), a substantial basal binding of [$^{35}$S]GTPγS to MBP-T43NTR-Gi/s was detected similar to that obtained with MBP-T43NTR-Gi and MBP-T43NTR-Gi/q, indicating that the $G\alpha_{i/s}$ subunit was able to bind nucleotide under our experimental conditions.

The NT-induced [$^{35}$S]GTPγS binding to MBP-T43NTR-Gi and MBP-T43NTR-Gi/q was found to be completely inhibited by the competitive antagonist SR48692 [31] (used at a concentration of 1 μM) (Fig. 3C). Such inhibition was also obtained with CHO-NTR membranes. Under our experimental conditions, the compound SR48692 did not reduce the basal binding of [$^{35}$S]GTPγS to *E. coli* model membranes or to CHO-NTR membranes.

## 4. Discussion

We have analyzed the coupling of NTR to Gα proteins by agonist and GTPγS binding assays using GPCR–Gα fusion constructs expressed in *E. coli*. Our experimental results suggest the interaction of NTR with $G\alpha_q$ and $G\alpha_{i1}$, but not with $G\alpha_s$.

There is good evidence that activation of NTR leads to hydrolysis of inositol phospholipid [32–34] by activation of phospholipase C through both PTX-sensitive [11] and PTX-insensitive G-proteins [14,16], presumably belonging to the $G_{i/o}$ and $G_{q/11}$ families, respectively. Furthermore, both positive and negative modulation of adenylyl cyclase activity in response to NT have been reported in mammalian cells expressing NTR [13,17]. To investigate the interaction of rat NTR with G-proteins, we used a GPCR–Gα fusion protein approach [20,35–41] because initial reconstitution experiments with partially purified $G\alpha_q\beta\gamma$ (expressed in insect cells) and *E. coli* membrane-inserted MBP-T43NTR-TrxA-H10 revealed that the detergents used for the purification of $G\alpha_q\beta\gamma$ by immobilized metal affinity chromatography [42] interfered with NT binding to T43NTR (data not shown).

[$^3$H]NT binding to MBP-T43NTR-Gq revealed a significantly higher proportion of receptors in the high-affinity state compared to that of MBP-T43NTR-TrxA-H10 (Table 1). This may indicate the stabilization of the receptor high-affinity state by the interaction of T43NTR with $G\alpha_q$. The incomplete conversion of MBP-T43NTR-Gq into its high-affinity conformation is likely due to inherent properties of this type of fusion construct; similar results have been found and dis-

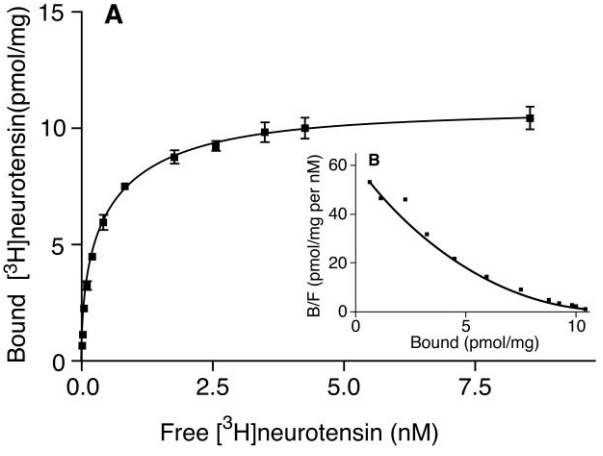


Fig. 2. Binding of [$^3$H]NT to the membrane-inserted fusion protein MBP-T43NTR-Gq. (A) Saturation binding at equilibrium. Washed *E. coli* membranes were incubated for 30 min in TEBB buffer supplemented with $MgCl_2$, with increasing concentrations of radiolabeled agonist. Separation of bound from free ligand was achieved by centrifugation. The results shown are from a typical experiment performed in duplicate. In many cases, the error bars are smaller than the symbols. (B) Scatchard transformation of the data.

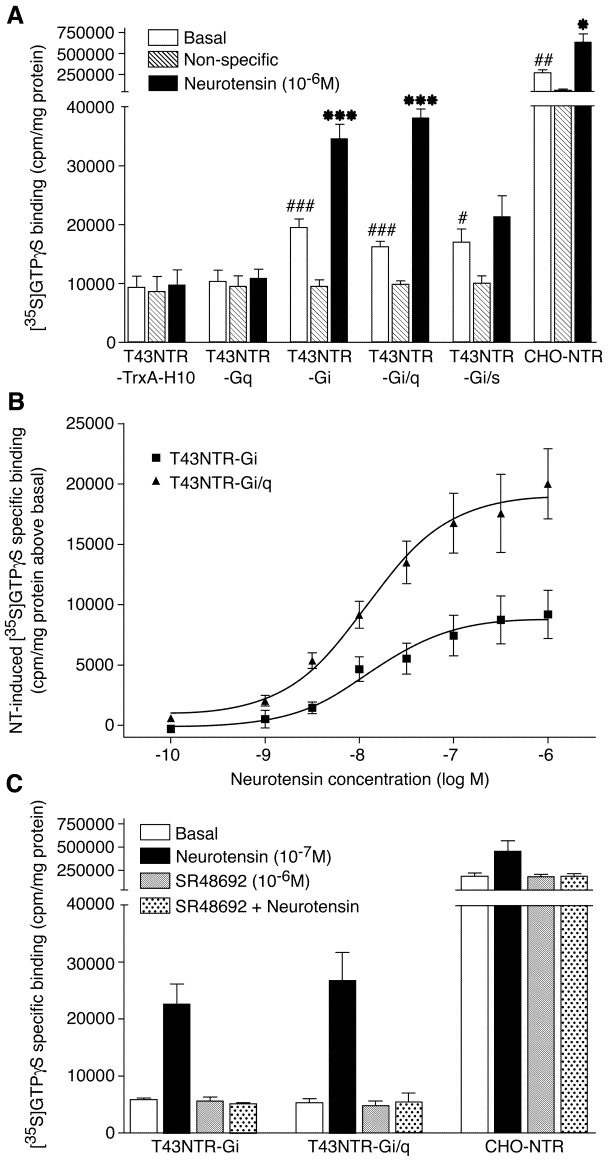


Fig. 3. GTPγS binding to *E. coli* membranes containing MBP-T43NTR fusion proteins. (A) NT-induced [$^{35}$S]GTPγS binding to *E. coli* membrane-inserted MBP-T43NTR-Gα fusion proteins or to homogenates of transfected CHO-NTR cells expressing the rat NTR. Data shown are average ± S.E.M. from 3–10 different experiments performed at least in duplicate. The amplitudes of the responses (percent increase in [$^{35}$S]GTPγS binding above basal) measured at a NT concentration of $10^{-6}$ M are 23 ± 33% for MBP-T43NTR-Gq ($n = 3$), 183 ± 34% for MBP-T43NTR-Gi ($n = 10$), 388 ± 52% for MBP-T43NTR-Gi/q ($n = 10$), 59 ± 21% for MBP-T43NTR-Gi/s ($n = 4$) and 170 ± 19% for CHO-NTR ($n = 4$). Statistical analysis by *t*-test: #, ## and ###, basal binding is significantly different from non-specific binding with a $P$ value $< 0.05$, $< 0.01$ and $< 0.001$, respectively; * and ***, NT-induced binding is significantly different from basal binding with $P$ values $< 0.05$ and $< 0.001$, respectively. (B) Concentration–response curves for NT-induced [$^{35}$S]GTPγS binding to MBP-T43NTR-Gi (squares) or MBP-T43NTR-Gi/q (triangles). Data shown are average ± S.E.M. from five (-Gi) and six (-poGi/q) different experiments performed at least in duplicate. $pEC_{50}$ values of 7.88 ± 0.25 and 7.95 ± 0.16, and average values for basal specific binding of 11 675 ± 4357 cpm/mg protein and 7096 ± 653 cpm/mg protein were obtained for the -Gi and the -Gi/q fusion proteins, respectively. The amplitudes of the maximal responses calculated from these experiments (percent increase in [$^{35}$S]GTPγS specific binding above basal) are 77 ± 2.5% for MBP-T43NTR-Gi and 269 ± 41% for MBP-T43NTR-Gi/q. (C) Inhibition by SR48692 of NT-induced [$^{35}$S]GTPγS binding to MBP-T43NTR-Gi and -Gi/q fusion proteins or to homogenates of transfected CHO-NTR cells. Data shown are average ± S.E.M. from three different experiments performed in quadruplicate.
←

cussed for β-adrenergic receptor $G\alpha_s$ and A1 adenosine receptor $G\alpha_i$ fusion proteins [35,38,40,43]. To test functional coupling of T43NTR with $G\alpha_q$, [$^{35}$S]GTPγS binding experiments were performed using MBP-T43NTR-Gq. Guanine nucleotide exchange experiments have been found to be much more difficult to perform for $G\alpha_q$ due to lower intrinsic guanine nucleotide exchange kinetics than for $G\alpha_i$ proteins [30,37,38,44,45]. However, we observed NT-induced nucleotide exchange in the presence of $(NH_4)_2SO_4$, which has been reported to accelerate the dissociation of bound GDP from $G\alpha_q$ [30]. To further show that coupling of T43NTR was specific for $G\alpha_q$, T43NTR was fused to a chimeric $G\alpha_{i1}$ subunit, in which the six C-terminal amino acid residues were replaced with the equivalent sequences of the $G\alpha_q$ protein (MBP-T43NTR-Gi/q). This approach relies on the notion that the extreme C-terminus of a Gα subunit encodes much (although not all) of its specificity for the interaction with a receptor [46–49]. In addition, this approach benefits from the high intrinsic guanine nucleotide exchange activity of the $G\alpha_i$ subunit. Indeed, significant NT-induced [$^{35}$S]GTPγS binding was observed with the MBP-T43NTR-Gi/q construct (Fig. 3). Furthermore, [$^3$H]NT saturation binding revealed a similar proportion of the fusion being in the high-affinity state as was observed for MBP-T43NTR-Gq (Table 1). To show that the coupling specificity to T43NTR was contributed by the $G\alpha_q$ C-terminus, but not the result of simple physical proximity of receptor and the $G\alpha_{i/q}$ subunit, the fusion construct MBP-T43NTR-Gi/s, containing the chimeric $G\alpha_{i1}$ subunit with the six C-terminal amino acid residues of $G\alpha_s$, was subject to [$^{35}$S]GTPγS and [$^3$H]NT binding assays. No NT-induced nucleotide exchange was observed (Fig. 3A), nor did the presence of the $G\alpha_{i/s}$ moiety lead to an increase in the percentage of receptors in the high-affinity state compared to that of MBP-T43NTR-TrxA-H10 (Table 1). Taken together, these data suggest interaction of T43NTR with $G\alpha_q$.

Experimental evidence indicates that NTR couples to PTX-sensitive G-proteins when expressed in CHO cells [21]. Therefore, the fusion protein MBP-T43NTR-Gi was subject to [$^3$H]NT and [$^{35}$S]GTPγS binding experiments to investigate a possible interaction of T43NTR with the $G\alpha_{i1}$ subunit. Agonist binding assays gave an average $B_H$ value of 34.6% (Table 1). This was lower than that obtained for MBP-T43NTR-Gq. However, [$^{35}$S]GTPγS experiments showed a robust response after addition of NT (Fig. 3) supporting evidence for functional coupling of T43NTR with $G\alpha_{i1}$.

Most of the effects of NT in tissue and eukaryotic cell models have been attributed to the activation of phospholipase C suggesting the involvement of $G_{q/11}$-type proteins, but fewer reports have noted the coupling of NTR to other G-proteins (for review see [33]). Indeed, our data show the preferential interaction of T43NTR with $G\alpha_q$ compared to that with $G\alpha_{i1}$. The percentage of receptors in the high-affinity state is higher for MBP-T43NTR-Gq and MBP-T43NTR-Gi/q than that for MBP-T43NTR-Gi (Table 1). In addition, the specific NT-induced increase in [$^{35}$S]GTPγS binding is up to 9-fold higher for MBP-T43NTR-Gi/q compared to that of

MBP-T43NTR-Gi when calculated in molar terms of the receptor fusion proteins, given their different expression levels determined by agonist binding (Table 1, Fig. 3B). These results may reflect the predominant functional coupling with $G_{q/11}$-type proteins as was found in CHO-NTR cells [21].

In this report we have addressed the selectivity of the interaction of NTR with Gα protein subunits. We find that bacterially expressed MBP-T43NTR fusion proteins show functional coupling to $G\alpha_q$ and $G\alpha_{i1}$ subunits linked to the receptor C-terminus. The results support our previous findings of the dual coupling of NTR to PTX-sensitive and -insensitive G-proteins in CHO cells with preference for $G_{q/11}$-type proteins.

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