Distinct regions of C-terminus of the high affinity neurotensin receptor mediate the functional coupling with pertussis toxin sensitive and insensitive G-proteins

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Abstract The functional coupling of C-terminally truncated mutants of the high affinity rat neurotensin (NT) receptor (NTS1) was characterized in transfected Chinese hamster ovary cells. On cells expressing NTRΔ372 (truncated NTS1 lacking the entire 52 amino acid C-terminus), NT failed to promote [^{35}S]guanosine 5′-[γ-^{35}S]triphosphate binding whereas a robust pertussis toxin (PTx) sensitive response was observed in cells expressing a partially truncated receptor (NTRΔ401 lacking the last 23 residues). Similar results were obtained when measuring the ability of NT to induce the production of arachidonic acid. Since neither deletions impaired the NT-induced phosphoinositide hydrolysis, these results indicate that the membrane proximal region of the C-terminus is specifically involved in the functional coupling of the receptor with PTx sensitive G-proteins. This region was also found to be involved in the control of receptor internalization. However, PTx failed to impair internalization, indicating that these two properties are not directly related.

Key words: Internalization; Arachidonic acid; Truncation; Guanosine-5′-*O*-(3-thio)triphosphate; Phospholipase

1. Introduction

In contrast to the simple, initially proposed, paradigm that a given G-protein coupled receptor (GPCR) interacts with a particular G-protein or to various G-proteins within one family, it is now well documented that simultaneous functional coupling with distinct unrelated G-proteins can be observed. Thus evidence for dual coupling to Gs and Gq type G-proteins [1–3] or to Gi and Gq type G-proteins [4–6] has been reported in a variety of cellular and tissular models. The importance of this genuine property of GPCRs was further revealed in recent studies showing that the coupling to multiple signaling pathways was dependent on the agonist used [7–11], showed distinct desensitization kinetics [12,13] and involved distinct molecular determinants of the receptor [14].

Most studies have highlighted the functional coupling of high affinity neurotensin (NT) receptor (NTS1) with phospholipase C (PLC), supporting evidence for the involvement of protein kinase C activation and inositol phosphate (InsP) dependent mobilization of intracellular calcium in the cellular responses to NT (for review, see [15]). However, we have previously shown that in transfected Chinese hamster ovary (CHO) cells, NT mediates a robust increase in guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding [16], an effect that is generally considered to reveal the functional coupling with Gi/o type G-proteins [17–19]. Accordingly, the response to NT is almost fully inhibited after pertussis toxin (PTx) treatment [20]. Based on these observations and from studies conducted with NTS1/Gα protein fusions expressed in *Escherichia coli* [21], we previously proposed a dual coupling of this receptor with both Gq and Gi/o type G-proteins. Whereas Gq protein mediates the well documented NT-induced InsP production and calcium mobilization, Gi/o proteins contribute to the NT-mediated activation of phospholipase A_2 (PLA_2) [20]. By evaluating the ability of C-terminally truncated NTS1 mutants in mediating [^{35}S]GTPγS binding, PLA_2 and PLC activation, we now provide evidence for the involvement of distinct regions of the receptor in the functional coupling with PTx sensitive (presumably of the Gi/o family) and insensitive (Gq) G-proteins.

2. Materials and methods

2.1. Materials

All culture media and materials were from Life Technologies. [^{35}S]GTPγS (specific activity 1000 Ci/mmol), [^{3}H]arachidonic acid (100 Ci/mmol) and [^{3}H]NT (101 Ci/mmol) were from NEN-Dupont, and *myo*-[2-^{3}H]inositol (70–120 Ci/mmol) was from Amersham. PTx, NT, 1,10-phenanthroline, dithiothreitol (DTT) and 5′-guanylylimidodiphosphate (Gpp(NH)p) were from Sigma.

2.2. Expression of truncated mutants of the rat NTS1 in CHO cells

The molecular cloning of the cDNA sequence encoding the rat NTS1 in the expression vector pSVK3 (Pharmacia Biotech) has been previously described [22]. The cDNAs encoding the truncated receptors were obtained by polymerase chain reaction (PCR) per-

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Abbreviations: NT, neurotensin; NTS1, high affinity neurotensin receptor; InsP, inositol phosphate; CHO, Chinese hamster ovary; Gpp(NH)p, guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PTx, pertussis toxin; CHO-NTRwt, transfected CHO cells expressing the rat NTS1; NTRΔ372, truncated NTS1 lacking the last 52 amino acids; NTRΔ401, truncated NTS1 lacking the last 23 amino acids

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formed on the rat NTS1 full length cDNA. The 5′-oligonucleotide (5′-GAATTCGAATTCCCGGCACCCACCATGCACCTCAA-3′) corresponded to the 5′-end sequence of NTS1 cDNA and contained an *Eco*RI restriction site, the 3′-oligonucleotides (5′-TCTAGATCTA-GACCAGGTTGTAGAGGATGGGATT-3′ and 5′-GGGCTTCCT-CTAGAACGTTGGCCTCTTTCGG-3′) were complementary to nucleotides 1092–1126 and 1181–1212 of NTS1 cDNA, respectively, and contained an *Xba*I site and an in frame stop codon. The PCR products (corresponding to amino acids 1–372 and 1–401, respectively, i.e. lacking the last 52 and 23 amino acids, respectively) were cloned in pSVK3.

CHO cells were cultured in modified Eagle's medium (MEM) α medium supplemented with 10% fetal calf serum, 85 μg/ml streptomycin and 85 U/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2–95% air. Cells were co-transfected with the NTS1 constructs cloned in pSVK3 together with pSV2neo encoding the geneticin-resistant gene (9/1 ratio) by the calcium phosphate co-precipitation method. Transfected cells were selected in the presence of 600 μg/ml geneticin and resistant cell populations were submitted to limited dilutions in order to isolate cellular clones. Clones that displayed similar [^{3}H]NT specific binding were used in the present study. Transfected CHO cells were routinely cultured in the above mentioned medium supplemented with 150 μg/ml geneticin. When indicated, 100 ng/ml PTx was added in the culture medium 20 h before the experiment.

2.3. Binding of [^{35}S]GTPγS and [^{3}H]NT

The specific binding of [^{35}S]GTPγS (0.1 nM) and [^{3}H]NT (0.0625–2 nM) was measured on cell homogenates (25–50 μg protein/tube) in a buffer containing 50 mM Tris–HCl pH 7.4 containing 5 mM $MgCl_2$, 1 μM 1,10-phenanthroline and 0.1% bovine serum albumin (BSA), as previously described [20]. For [^{35}S]GTPγS binding studies, this buffer was also supplemented with 1 μM GDP, 150 mM NaCl and 1 mM DTT. The effect of guanylyl nucleotides on the affinity of [^{3}H]NT was measured by comparing saturation curves of [^{3}H]NT specific binding performed in the presence or in the absence of 100 μM Gpp(NH)p.

2.4. Measurement of [^{3}H]InsP and arachidonic acid release

The ability of NT to mediate PLC activation was evaluated by measuring the accumulation of [^{3}H]InsP in intact cells in the presence of 10 mM LiCl ion. Sample extraction and analysis of the [^{3}H]InsP fraction were performed as previously described [20]. NT-induced [^{3}H]arachidonic acid release was measured on cells grown in the presence of 0.5 pCi/well [^{3}H]arachidonic acid for 24 h as previously described [23].

2.5. Internalization of [^{3}H]NT

Receptor-mediated internalization of [^{3}H]NT was measured on intact cells by measuring the proportion of radioactivity that remained associated with the cells after acid-stripping of cell surface bound ligand [24–26]. Cells were grown to confluence in 24-well plates. Shortly, after washing with incubation buffer (Ham/F12 supplemented with 1 μM 1,10-phenanthroline and 0.1% BSA), cells were incubated in the same buffer for 15 min at 37°C. Thereafter, medium was replaced with fresh medium containing 1 nM [^{3}H]NT (with or without 1 μM unlabeled NT for determination of the non-specific binding). After incubation at 37°C for the period of time indicated, cells were placed on ice and washed rapidly once with ice cold neutral phosphate-buffered saline (PBS) (pH 7.4) and twice with either ice cold neutral PBS (for measurement of total and non-specific binding) or acidic PBS pH 2.5 (to measure internalized peptide). After a final wash with neutral PBS, cells were dissolved in 1% sodium deoxycholate (pH 11.3) and radioactivity was counted by liquid scintillation. Percentage of internalization was expressed as the ratio of acidic-resistant binding/specific binding.

3. Results

Truncated forms of NTS1 lacking either the last 23 (clone NTRΔ401) or 52 (clone NTRΔ372) residues were expressed in transfected CHO cells (Fig. 1). The mutated DNA sequences were obtained by PCR with specific oligonucleotides designed in order to introduce termination codons within the cDNA sequence encoding the intracellular C-terminus of the receptor, yielding truncated receptors. Clones expressing similar levels of the wild-type or the mutated receptors were selected and further characterized. As shown in Table 1, the affinity of [^{3}H]NT measured on homogenates from cells expressing truncated receptor was not significantly different from that measured on transfected CHO cells expressing the rat NTS1 (CHO-NTRwt cells). As commonly observed with most GPCRs, the affinity of NT for its receptor is dependent on the functional coupling with G-protein, and disruption of this coupling with guanylyl nucleotides decreases the binding affinity. Addition of Gpp(NH)p significantly decreased the affinity of [^{3}H]NT for the wild-type receptor expressed in CHO cells, whereas such effect was not observed after truncation of the entire C-terminus (Table 1). In contrast, the binding of [^{3}H]NT to CHO-NTRΔ401 was decreased in the presence of Gpp(NH)p in a similar fashion to what is observed with the wild-type receptor. Taken together, these results indicate that the membrane proximal region of NTS1 is required in order to pharmacologically reveal disruption of G-protein coupling with guanylyl nucleotides.

In both CHO-NTRΔ372 and CHO-NTRΔ401 cells, NT-induced phosphoinositide hydrolysis with a similar potency to that observed in CHO-NTRwt (pEC_{50} values were 9.10 ± 0.14, 8.99 ± 0.13 and 9.43 ± 0.13 for CHO-NTRwt, CHO-NTRΔ372 and CHO-NTRΔ401, respectively) (Fig. 2A). The maximal response was found to be significantly higher (Student *t*-test) in cells expressing the truncated receptors as compared to those expressing the wild-type receptor (32% higher for CHO-NTRΔ372, $P < 0.05$ and 126% higher for CHO-NTRΔ401, $P < 0.001$).

Using optimized experimental conditions, we previously showed that in homogenates of transfected CHO cells expressing NTS1, NT induces a robust increase in [^{35}S]GTPγS binding [16]. We recently showed that this response mainly reflects the functional activation of PTx-sensitive G-proteins, presumably Gi [20,21]. The ability of NT to promote [^{35}S]GTPγS

Table 1
Characterization of [^{3}H]NT specific binding on membranes of transfected CHO cells expressing NTRwt, NTRΔ372 or NTRΔ401

Clone	K_D (nM)	B_{Max} (pmol/mg protein)	*n*
CHO-NTRwt	0.24 ± 0.05	0.98 ± 0.06	5
CHO-NTRwt+Gpp(NH)p 100 μM	0.91 ± 0.06***	0.82 ± 0.16	5
CHO-NTRΔ372	0.20 ± 0.02	1.60 ± 0.42	4
CHO-NTRΔ372+Gpp(NH)p 100 μM	$0.32 \pm 0.04^{n.s.}$	1.73 ± 0.53	4
CHO-NTRΔ401	0.23 ± 0.04	1.49 ± 0.16	4
CHO-NTRΔ401+Gpp(NH)p 100 μM	1.26 ± 0.17***	1.05 ± 0.14	4

The specific binding of [^{3}H]NT (0.06–2 nM) was measured on cell homogenates in the presence or in the absence of 100 μM Gpp(NH)p. Data indicated are K_D and B_{max} values determined by non-linear analysis of the saturation curves and are mean ± S.E.M. from *n* different experiments performed in duplicate. The influence of Gpp(NH)p on the K_D of [^{3}H]NT was statistically analyzed by one way ANOVA followed by Newman–Keuls test (***$P < 0.001$; n.s., non-significant).

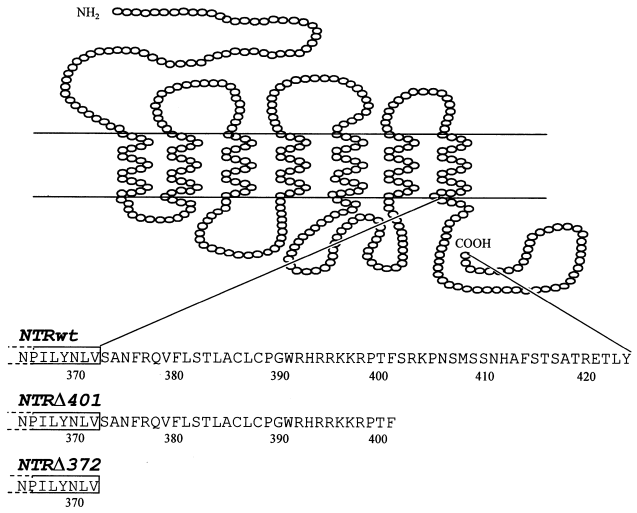

Fig. 1. Proposed structure of NTS1 and detail of the amino acid sequence of the C-terminus in the wild-type receptor (NTRwt) and the truncated receptors examined in the present study.

binding was used to examine the functional coupling of truncated NTS1s with Gi/o-type G-proteins when expressed in CHO cells. As shown in Fig. 2B, NT-induced nucleotide binding was almost not detected in CHO-NTRΔ372 cells, whereas in CHO-NTRΔ401 cells, the response to NT was identical to that obtained in CHO-NTRwt cells (non-significant difference between the potency and efficacy of NT, see legend of Fig. 2B). As previously reported for cells expressing the wild-type receptor, NT-induced [^{35}S]GTPγS binding was almost undetectable on PTx-pretreated CHO-NTRΔ401 cells.

In a variety of models, including transfected CHO cells, NT was shown to activate PLA_2, causing the release of arachidonic acid [20]. Although calcium mobilization appears to enhance this response, we showed that NT-mediated arachidonic acid release mainly results from the coupling with PTx-sensitive G-proteins. In cells expressing the partially truncated receptor (CHO-NTRΔ401), NT induced a robust increase in arachidonic acid release that was indistinguishable from that measured in CHO-NTRwt cells and completely suppressed after PTx treatment. In contrast, no such functional response was detected in CHO-NTRΔ372 cells (Fig. 2C). Together, these results indicate that the membrane proximal region of the NTS1 C-terminus plays a critical role for both NT-induced [^{35}S]GTPγS binding and NT-induced arachidonic acid release whereas the conservation of the more distal region is not absolutely required.

Incubation of transfected CHO cells expressing NTS1 with NT is followed by the internalization of the peptide through receptor-mediated internalization. CHO-NTRwt cells rapidly internalize [^{3}H]NT (up to 90% internalization after 30 min, see Fig. 3A), whereas a dramatic decrease in the rate of internalization is observed after truncation of the entire C-terminus of the receptor (CHO-NTRΔ372, less than 40% internalization after 30 min). In contrast, although the rate of internalization was lower than in CHO-NTRwt cells, a robust [^{3}H]NT internalization (up to ~80% after 30 min) was measured in cells expressing NTRΔ401. These results indicate that within the C-terminus of NTS1, the membrane proximal region is in-

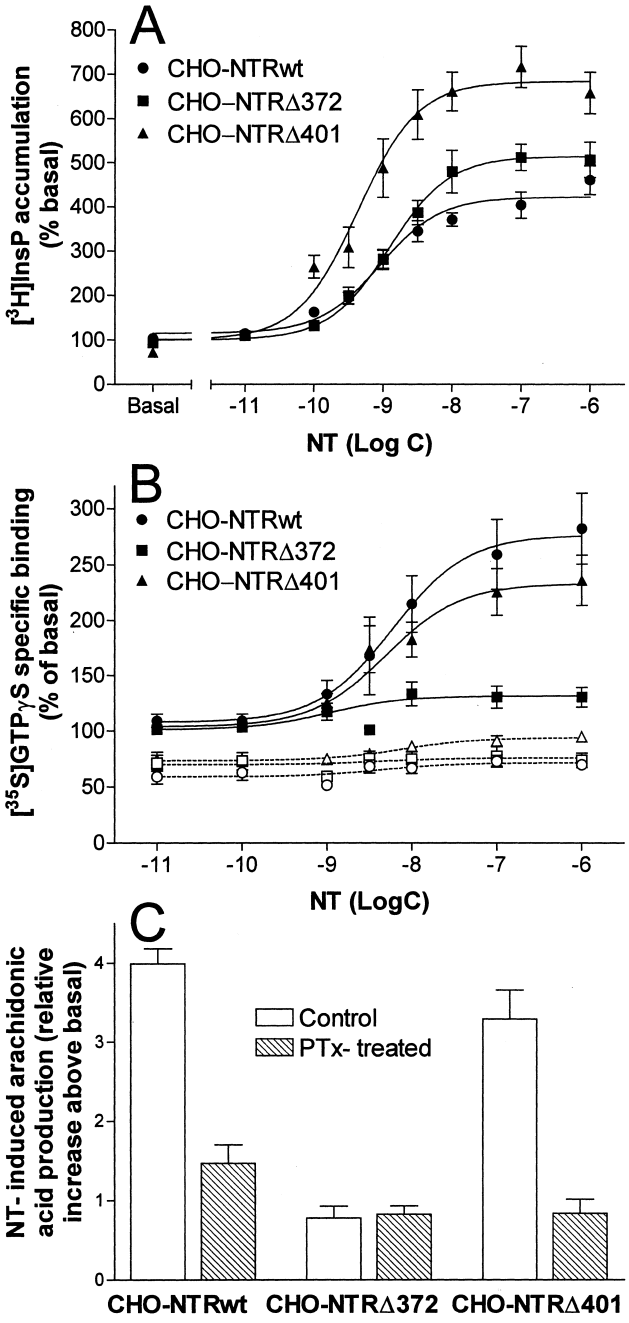

Fig. 2. A: NT-induced [^{3}H]InsP accumulation in CHO-NTRwt, CHO-NTRΔ372 and CHO-NTRΔ401 cells. Data shown are mean ± S.E.M. from at least five different experiments performed in triplicate. B: NT-induced [^{35}S]GTPγS binding on homogenates from CHO-NTRwt, CHO-NTRΔ372 and CHO-NTRΔ401 cells. The potency (pEC_{50}) of NT in CHO-NTRwt and CHO-NTRΔ401 was determined by non-linear analysis of sigmoidal concentration response curves and revealed no significant difference (8.20 ± 0.11 and 8.20 ± 0.07, respectively). Data shown with open symbols and dotted line indicate the responses measured on cells previously treated in with PTx (100 ng/ml, 20 h). Data are mean ± S.E.M. from at least six (no PTx) or two (with PTx) different experiments performed in duplicate. C: NT (10 nM)-induced arachidonic acid production. Data shown are mean ± S.E.M. from four to 22 different determinations.

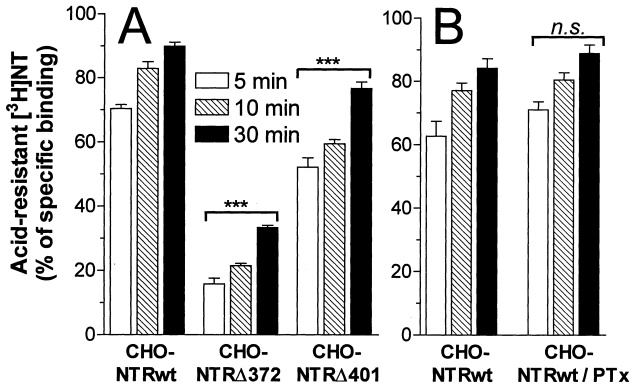

Fig. 3. A: Receptor-mediated internalization of [^{3}H]NT in intact CHO-NTRwt, CHO-NTRΔ372 and CHO-NTRΔ401 cells. After incubation with [^{3}H]NT (1 nM) for the periods of time indicated, cells were washed three times with either neutral PBS or acidic PBS. Percentage of internalization was expressed as the fraction of acidic-resistant specific binding. Internalization data obtained in cells expressing the truncated receptors were compared with the corresponding values obtained from CHO-NTRwt cells by one way ANOVA followed by Newman–Keuls test (***$P<0.001$). B: Effect of PTx pretreatment (100 ng/ml, 20 h) on [^{3}H]NT internalization in intact CHO-NTRwt cells (n.s., non-significant difference between corresponding values measured on untreated cells).

volved in the control of receptor-mediated peptide internalization. In order to evaluate the influence of NTS1 coupling with PTx sensitive G-proteins, internalization of [^{3}H]NT was measured in CHO-NTRwt cells previously treated with PTx. As shown on Fig. 3B, PTx pretreatment had no significant effect on the ability of the cells to internalize [^{3}H]NT.

4. Discussion

On the basis of the numerous studies aiming at depicting the molecular basis of receptor/G-protein coupling, it becomes more and more evident that no consensus model will ever be proposed for defining the structural features that determine the possible coupling of hundreds of receptors with dozens of G-proteins (for review, see [27]). Moreover, functional coupling is probably coordinated by several different regions of the receptor. In the case of the rat NTS1, previous studies have shown that partial deletions within its third intracellular loop completely prevented the coupling with PLC [28]. The specific involvement of this region of the receptor in the coupling with Gq type G-protein is confirmed by showing that partial or total deletion of the C-terminus did not impair NT-mediate InsPs accumulation. Intriguingly this response was higher with C-terminally truncated receptor, in accordance with previous studies showing that many GPCR more efficiently activate PLC after deletion of their C-terminus which is proposed to contain inhibitory sequences [29–31].

Both NT-induced [^{35}S]GTPγS binding, and arachidonic acid production were almost undetectable in cells expressing the receptor lacking its entire intracellular C-terminus. In contrast, cells expressing a partially truncated receptor responded to NT with the same efficacy and potency as compared to cell expressing the wild-type receptor. Furthermore, in cells expressing the NTRwt or NTRΔ401, [^{35}S]GTPγS binding and arachidonic acid production were prevented after treatment of the cells with PTx. Together, these data indicate that the membrane proximal region of the NTS1 intracellular C-terminus (residues 373–401) is involved in its functional coupling with PTx sensitive G-proteins.

Functional interaction of a receptor with G-proteins is also frequently examined by testing the effect of non-hydrolysable guanylyl nucleotides on the agonist binding. These nucleotides disrupt the ternary complex (agonist/receptor/G protein) and thereby decrease the affinity of agonists. According to other studies conducted on various tissular and cellular models, the affinity of NT for NTS1s is significantly decreased in the presence of Gpp(NH)p [20,32–34]. The binding of NT was modulated by Gpp(NH)p on cells expressing the partially truncated NTS1 while no similar effect was observed after deletion of the entire intracellular C-terminus, indicating that the membrane proximal region of the intracellular C-terminus plays a crucial role in determining the sensitivity of agonist binding to G-protein coupling. It is noteworthy that [^{3}H]NT binding experiments conducted on CHO-NTRΔ372 cell homogenates both in the presence or in the absence of guanylyl nucleotide revealed 'high affinity state' binding. This suggests that the lack of effect of Gpp(NH)p is not indicative of a disrupted G-protein coupling, but rather indicates that removal of the entire C-terminus constrains the receptor in the high-affinity conformation, irrespective of G-protein coupling.

As many GPCRs, NTS1 is subject to agonist-induced regulation through rapid signaling desensitization, receptor internalization and down regulation (for review, see [15]). In many models, including transfected CHO cells, NT was shown to be rapidly internalized through receptor-mediated endocytosis. Although we previously showed that deletion of the entire intracellular C-terminus of NTS1 dramatically impaired NT internalization [25]. The present data indicate that a partial truncation modestly affected the rate and amplitude of NT internalization, revealing that the membrane proximal region of the C-terminus was playing a critical role in mediating receptor internalization. Although this coincides with the region of the receptor involved in Gi/o coupling, pretreatment of CHO-NTRwt with PTx did not alter the internalization process, indicating that activation of this subset of G-proteins and downstream functional responses, including PLA_2 activation, does not control receptor internalization. Therefore, although the membrane proximal region of the NTS1 C-terminus is clearly involved in the coupling with PTx-sensitive G-proteins and in the control of receptor internalization, these two processes appear not to be mechanistically related. Previous studies have indicated that the NT-mediated activation of PLC was neither required [28] nor sufficient [25] to observe receptor internalization.

In conclusion, the present study indicates that the dual coupling of NTS1 with PTx sensitive (presumably of the Gi/o family) and insensitive (Gq/11) G-proteins requires distinct molecular determinants of the receptor. This confirms our previous findings suggesting that the multiple responses triggered by NT are the consequence of independent signaling cascades. It also shows that some regions involved in G-protein coupling are also involved in the control of the receptor regulation triggered by agonist stimulation. These observations suggest the possibility of developing agonists able to induce different receptor conformations mediating distinct responses. It also raises the question whether these multiple signaling pathways could be differentially regulated during agonist stimulation.

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