Biochemical and Biophysical Research Communications 391 (2010) 1007–1013

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Intermolecular cross-talk between NTR1 and NTR2 neurotensin receptor promotes intracellular sequestration and functional inhibition of NTR1 receptors

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ARTICLE INFO

Article history:
Received 30 November 2009
Available online 5 December 2009

Keywords:
Neurotensin
G-protein-coupled receptor
Dimerization
Trafficking
Signal transduction

ABSTRACT

G-protein-coupled receptors (GPCR) are now regarded as being able to acquire heterodimer conformations affecting their pharmacology, signaling and trafficking. In co-immunoprecipitation studies using differentially epitope-tagged receptors, we herein provide direct evidence for heterodimerization of human neurotensin type 1 receptor (hNTR1) and type 2 receptor (hNTR2). Using chimeric constructs, we also identified the hNTR2 transmembrane 2 (TM2) to TM4 region as crucial for the formation of the dimerization interface. At the functional level, we demonstrated that the co-expression of hNTR2 suppressed hNTR1-mediated adenylate cyclase/cAMP and phospholipase C activation. Finally, confocal microscopy revealed that whereas tagged hNTR1 expressed alone were localized to the plasma membrane, co-expression of hNTR2 caused the retention of hNTR1 in sub-cellular compartments, indicating that heterodimerization with hNTR2 interferes with the proper recruitment of hNTR1 to the plasma membrane. Overall, this study proposes a novel function of NTR2 in the regulation of NTR1 activity.

Introduction

Neurotensin (NT), a brain-gut tridecapeptide, fulfils a dual function of neurotransmitter or neuromodulator in the central nervous system (CNS) and of local hormone at the periphery [1,2]. NT actions and related signal transduction depend on recognition of the peptide at the plasma membrane of target cells by three specific receptors referred to as NTR1, NTR2 and NTR3/sortilin. NTR1 and NTR2 belong to the family of G-protein-coupled receptors (GPCR) with seven transmembrane domains, whereas NTR3 is part of the Vps10p family of sorting receptors characterized by a single transmembrane domain [3–5].

NTR1 is known to link through $G_{i/o}$, G_q, and G_s protein, to a variety of signaling cascades, including formation of cAMP and cGMP, production of inositol phosphates through phospholipase C activation, activation/inhibition of mitogen-activated protein kinases (ERK1/2 and JNK) and serine/threonine protein kinase Akt [2]. In contrast to NTR1, the signaling properties of NTR2 are still controversial, exhibiting cell type- and species-dependent pharmacological properties [2,5]. For instance, NT analogs were reported to act as an agonist, inverse agonist, or competitive antagonist at NTR2 sites depending on the heterologous expression system (oocytes, CHO, COS-7, and HEK-293 cells) [6–11].

GPCRs have classically been assumed to exist and act as monomeric entities in neuronal and non-neuronal cells. However, a large number of data obtained using biochemical and functional approaches argue for the existence of these receptors as homo- or hetero-dimeric or even higher-structure oligomers [12]. Such intermolecular interactions are important for receptor function, including agonist binding, potency, efficacy, G-protein selectivity, trafficking to the plasma membrane, and agonist-promoted internalization [13]. The process of intramolecular cross-talk by which individual NT receptors interact to form heterodimeric or heteromultimeric complexes could be of particular importance in the NT family, since it is quite frequent that two NT receptors subtypes are co-expressed in NT-related brain regions, such as the dorsal raphe, substantia nigra and the ventral tegmental area [14] or in a given cell type [15–17]. In this context, the two structurally different receptors NTR1 and NTR3, co-express in several human cancer cell lines and interact physically to modulate both NT-induced phosphorylation of ERK1/2 and the phosphoinositide (PI)

Abbreviations: NTR, neurotensin receptor; NT, neurotensin; TM, transmembrane domain; IP, inositol phosphate; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; CRE, cAMP-response element; Luc, luciferase.

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doi:10.1016/j.bbrc.2009.12.007

turnover mediated by NTR1 [18]. Similarly, NTR2 was shown to exist as homodimers either *in vitro* or *in vivo* and to form heterodimeric complexes with the truncated splice variant vNTR2 isoform [15,16,19].

The aim of this study was therefore to investigate whether physical interactions occurring between human NTR1 and NTR2 influence NTR1 coupling to second messenger signaling cascades and to identify the NTR2 structural domains responsible for NTR1/NTR2 functional heterodimerization. Furthermore, alterations in trafficking of NTR1 by physical interaction with NTR2 were determined.

Materials and methods

Plasmids and construction of chimeras. Human NTR1 (hNTR1), rat NTR1 (rNTR1), and hNTR2 constructs are generous gifts from Dr. J. Mazella (CNRS, Valbonne, France). The cDNAs for bullfrog NTR1 (bfNTR1) and NTR4 (bfNTR4) were previously developed in our laboratory [20]. For domain swapping between hNTR2 and bfNTR4, individual cDNA fragments of interest were amplified using overlapping PCR, as previously described [21]. The plasmid encoding luciferase containing four copies of the cAMP-response element (CRE-luc) was obtained from Stratagene (La Jolla, CA). The plasmid for c-fos-luciferase (c-fos-luc) was generously provided by Dr. R. Prywes (Columbia University, NY). The pCMV-β-gal was purchased from Clontech (Mountain View, CA).

Cell culture, transient transfection, and luciferase assay. HeLa and HEK-293 cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 U penicillin and 100 μg/ml streptomycin. Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, 1 day after cells were plated into 24-well plates. For each transfection, 100 ng CRE-luc or c-fos-luc and 50 ng of the internal control plasmid pCMV-β-galactosidase were used concomitantly with 100 ng of NTR plasmid constructs when not otherwise indicated. Thirty-six hours after transfection, cells were treated for 6 h with 100 nM of human NT synthesized by AnyGen (Gwangju, South Korea). Following treatment, cells were harvested and luciferase activity in cell extracts was determined using a luciferase assay system according to standard methods in a Lumat LB9501 (EB & G, Berthold, Germany). All assays were performed in triplicate and repeated three times; the data are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Newman–Keuls Post-test and $^{*}p < 0.05$ was considered statistically significant.

Inositol phosphate (IP) assay. HeLa cells were seeded into 12-well plates and transfected with plasmid encoding hNTR1 (500 ng) combined with either plasmid encoding hNTR2 (125–1000 ng) or empty vector. Twenty-four hours after transfection, cells were incubated for 24 h in Medium 199 (Life Technologies, Grand Island, NY) containing 1% FBS, 1 mM L-glutamine, and 1 μCi/well myo-[^{3}H]inositol (Amersham Pharmacia Biotech, Piscataway, NJ). Cells were washed with 0.5 ml buffer A (20 mM HEPES, 140 mM NaCl, 4 mM KCl, 8 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml fatty acid-free bovine serum albumin) and incubated in 0.5 ml buffer A containing 10 mM LiCl for 30 min at 37 °C. Cells were then incubated with 100 nM of NT for 30 min at 37 °C. Reaction was stopped by adding 0.5 ml of ice-cold 10 mM formic acid for 1 h at 4 °C. IPs were recovered using a Dowex ion exchange resin (BioRad, Hercules, CA). Total

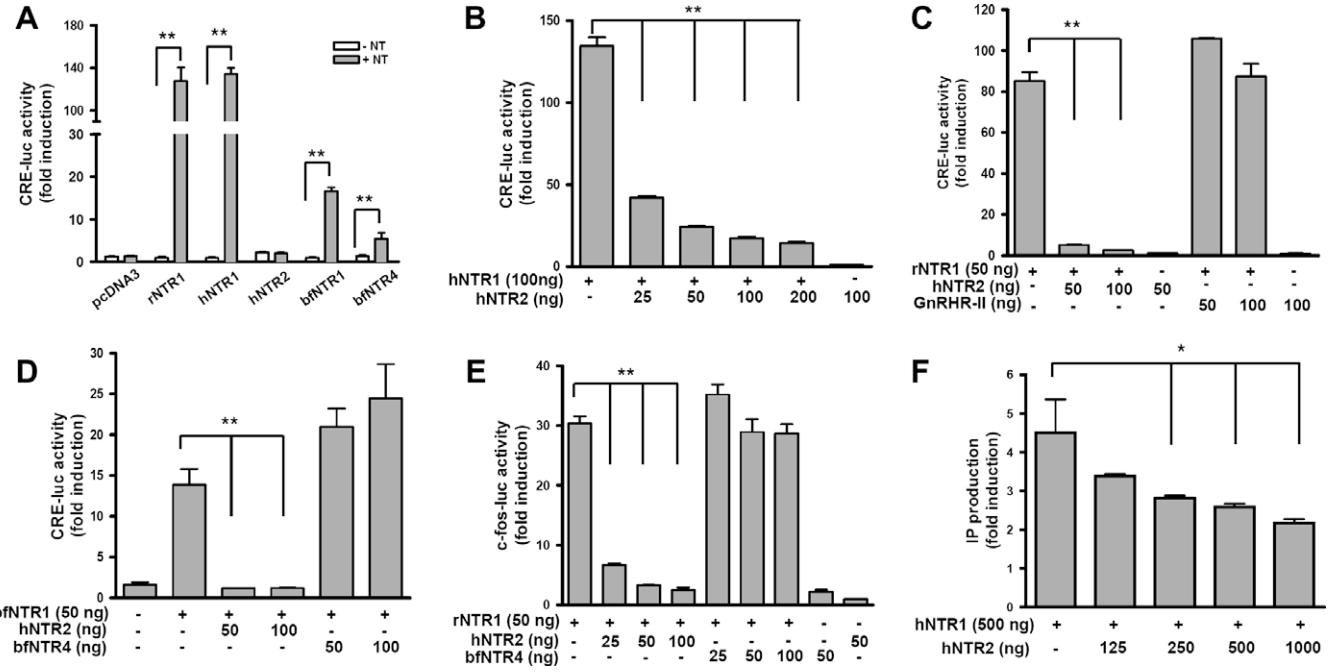

Fig. 1. Inhibition of NTR1-dependent signaling pathways by NTR2. Modulation of PKA, PKC pathways and IP pools by NT receptors were examined on transiently transfected HeLa cells using the CRE-luciferase (A–D), c-fos luciferase (E) and IP pool determination (F) assays, respectively. (A) HeLa cells were transfected with plasmids containing CRE-luc (100 ng) and β-gal (50 ng) together with 100 ng of the plasmids containing rNTR1, hNTR1, bfNTR1, hNTR2 or bfNTR4. Cells were treated 24 h after transfection, with 100 nM NT for 6 h. Bioluminescence of cell lysates are expressed as mean fold induction over β-galactosidase values. NTR1 and bfNTR4 receptors activate the PKA pathway under NT stimulation ($^{**}p < 0.01$). (B) hNTR2 expression inhibits hNTR1 PKA activation in a dose-dependent manner ($^{**}p < 0.01$). (C) The cross-family receptor control Monkey GnRH receptor type II does not stimulate PKA pathway by itself and is not affecting rNTR1 signaling unlike hNTR2. (D) hNTR2 demonstrates a cross-specie inhibition over bfNTR1 ($^{**}p < 0.01$) but not bfNTR4. (E) HeLa cells were transfected with 100 ng of c-fos-luc plasmid, cells were serum-starved for 20 h 1 day after transfection, followed by a 6 h treatment of 100 nM NT. Luciferase activity for experimental groups was normalized as a fold induction over the value obtained in the absence of NT. hNTR2 incurs a cross-specie inhibition of rNTR1 ($^{**}p < 0.01$) but not bfNTR4. (F) HeLa cells were transfected with hNTR1 in combination with various concentrations of hNTR2. Inositol phosphate (IP) production was determined 30 min after treatment of cells with 100 nM NT by scintillation counting. Data are determined as fold increase of IP production over the values obtained in the absence of NT. hNTR2 inhibits hNTR1 IP production ($^{*}p < 0.05$). All results shown are expressed as mean ± SEM of experiments performed in triplicate.

IPs were eluted with 1 M ammonium formate containing 0.1 M formic acid and radioactivity counted using a liquid scintillation counter (TRI-CARB 2900 TR, Packard Bioscience, Boston, MA).

Co-immunoprecipitation of hNTR1 and hNTR2. Twenty-four hours after transfection with either Flag-hNTR1 (1 μg), HA-hNTR2 (1 μg) or both receptors, cells were washed with cold PBS and lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate) including protease inhibitor cocktail (Roche, Mannheim, Germany). Soluble proteins were collected by centrifugation at 17,000*g* for 10 min at 4 °C and supernatants were incubated with mouse anti-Flag conjugated to 12 μl agarose beads (M2, Sigma, Saint Louis, MO) for 2 h at 4 °C. The agarose beads were washed four times with RIPA buffer and receptor immunoprecipitated proteins were separated 8% or 15% SDS–PAGE gel. Immunoreactive proteins were then revealed by incubation with the HA-conjugated mouse monoclonal antibody (1:5000 dilution) (Sigma).

Immunocytochemistry. HeLa cells were plated on poly-L-lysine-coated glass chambers 1 day before transient transfection with plasmids expressing HA-tagged hNTR1 (100 ng) with and without hNTR2-GFP (100 ng) or bf/hTM2-4-GFP (100 ng). HeLa cells were washed with cold PBS, fixed in 4% formaldehyde for 10 min at RT, washed with PBS and permeabilized PBST-1 (0.2% Triton X-100) for 10 min, and blocked with PBST-2 (10% FBS, 0.1% Triton X-100) for 30 min at RT. Then, cells were first incubated, for 1.5 h at RT, with mouse anti-HA antibody (1:1000 dilution) followed with the secondary antibody Cy3-conjugated goat anti-mouse IgG (1:1000 dilution) (ZyMED, San Francisco, CA) for 1 h at RT. Nuclei were stained with Hoechst 33342 (1:1000 dilution, Molecular Probes). HA-tagged receptors were visualized by confocal microscopy performed with a LSM510 (Carl Zeiss, Oberkochen, Germany).

Results

hNTR2 exerts an inhibitory effect on NTR1-mediated signaling

With regards to G-protein coupling, NTR1 is a G_q- and G_s-preferring receptor [2]. In this study, we first examined whether NTR1 receptor stimulation resulted in adenylate cyclase/PKA and phospholipase C/PKC activation, by using CRE- and c-fos-luciferase (luc) assays, respectively [20]. In HeLa cells transiently expressing either hNTR1 or rNTR1, stimulation with NT for up to 6 h induced a pronounced increase in CRE-luc activity (Fig. 1A). Activation of the recently cloned bfNTR1 and bfNTR4 by human NT also stimulated significantly CRE-luc activity, although to a lesser extent than mammalian NTR1s (Fig. 1A). By contrast, NT failed to modify levels of CRE-luc activity in HeLa cells expressing hNTR2, indicating that NT binding to hNTR2 was unable to excite this class of second messengers.

To determine whether co-expression of hNTR2 affects NT signaling through NTR1, CRE-luc assays were carried out in cells expressing NTR1 alone or together with hNTR2. The CRE-luc activity mediated by hNTR1 decreased in the presence of hNTR2 in a dose-dependent manner (Fig. 1B and Supplemental Fig. 1). rNTR1 stimulation of CRE-luc reporter was also reversed by co-expression of hNTR2 (Fig. 1C). Similar CRE-luc inhibition was observed in cells expressing bfNTR1 together with hNTR2 (Fig. 1D), suggesting cross-species functional interaction between NTR1 and NTR2 receptors. This effect also appeared to be NTR2-specific since co-expression of NTR1 with other GPCRs, such as the type II gonadotropin-releasing hormone receptor (GnRHR-II) [21] did not inhibit rNTR1 activity (Fig. 1C). Likewise, bfNTR4 failed to decrease bfNTR1-induced CRE-luc activity (Fig. 1D).

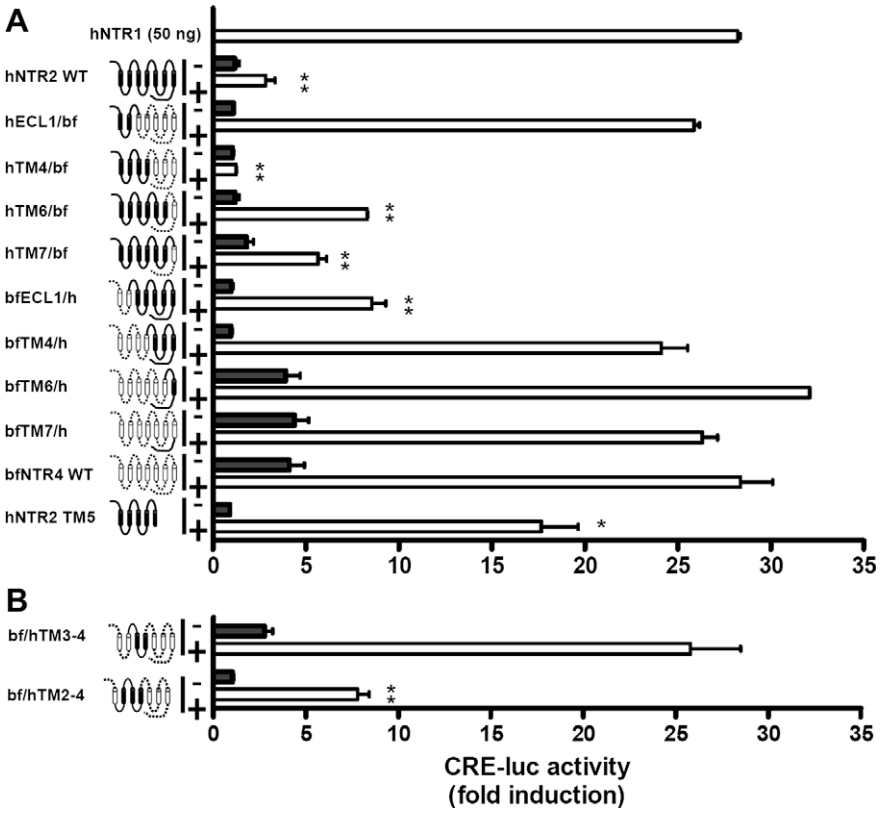

Fig. 2. Mapping of the functional interaction between NTR1 and NTR2. The region responsible for the inhibitory effects of NTR2 on NTR1 was determined using the CRE-luc reporter assay (A,B). Chimeric constructs built with hNTR2 and bfNTR4 regions are described as schematic representations in black and white, respectively. HeLa cells were transfected with plasmids containing hNTR1 (50 ng), CRE-luc (100 ng) and β-gal (50 ng) in the absence or presence of chimeric constructs. An additional hNTR2 construct truncated at TM5 was also transfected to evaluate its influence on NTR1 signaling. CRE-luc activity is represented as a fold induction over the value obtained in the absence of NT treatment. Results shown are the mean ± SEM of triplicate observations. $^{*}p < 0.05$, $^{**}p < 0.01$.

We then examined the consequences of hNTR2 co-expression on NTR1-induced phospholipase C/PKC signaling by measuring c-fos-luc activity and IP production. Increasing concentrations of hNTR2, but not bfNTR4, inhibited rNTR1-induced phospholipase C/PKC activation (Fig. 1E). Similarly, in HeLa cells expressing both receptors, hNTR1-mediated IP accumulation was dose-dependently reduced by the presence of hNTR2 (Fig. 1F), demonstrating that hNTR2 negatively regulates NTR1-triggered intracellular signaling cascades.

The region between transmembrane domains 2 (TM2) and TM4 of hNTR2 is required for the inhibition of hNTR1 coupling

To identify the region of hNTR2 involved in NTR1 signaling inhibition, we created chimeras of hNTR2 and bfNTR4, initially sharing 45% identity [20]. Since the presence of bfNTR4 did not modify the signal transduction mediated by NTR1, large segments from the extracellular loop 1 (ECL1) to N-terminus were alternatively swapped with a corresponding region of hNTR2, and *vice versa* (Fig. 2). Each chimeric construct was expressed alone or in combination with hNTR1 in HeLa cells to record NT-induced CRE-luc activity. The relative expression level of every construct was verified in FACS scan and compared to hNTR1. There was no significant difference in expression that could account for a bias of inhibition (data not shown). First of all, substituting the N-terminal domain (hECL1/bf) and the core domain of bfNTR4 (bfTM4/h, bfTM6/h, bfTM7/h) with the corresponding hNTR2 receptor segments did not impair hNTR1 signaling (Fig. 2A). Conversely, substitution of regions of the C-terminal tail up to the second extracellular loop (chimeras hTM7/bf, hTM6/bf, hTM4/bf), or substitution of the N-terminal to TM2, inhibited NTR1 signal transduction similarly as did the WT hNTR2. A truncated mutant of hNTR2 lacking the third intracellular loop to the C-terminal tail (hNTR2 TM5) inhibited hNTR1 activity, but to a lesser extent than the wild-type receptor, suggesting that the distal region of hNTR2 is partially involved in hNTR1 modulation. To further delineate the interaction domain, we constructed additional chimeric receptors (Fig. 2B). The chimeric receptor presenting the human NTR2 TM2-4 (bf/hTM2-4) had

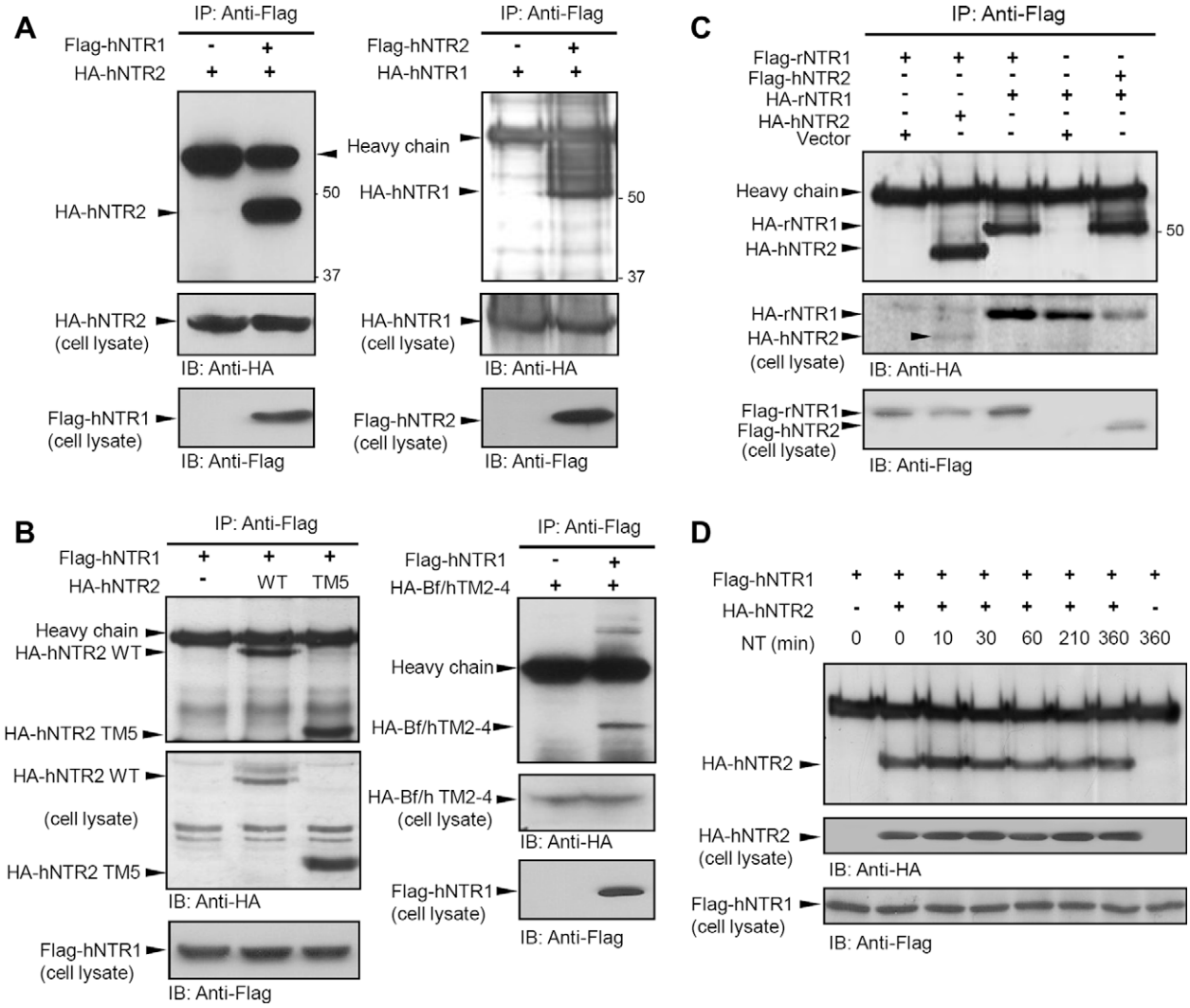

Fig. 3. Physical interactions between hNTR1 and hNTR2. Anti-Flag-conjugated agarose beads immunoprecipitations (IP) of NT receptors were performed 24 h after HeLa cells transfection with Flag- and HA-epitope tagged NTR1 and NTR2 receptors. Immunoblots were performed on polyacrylamide gel with an anti-HA antibody revelation. (A) In lane 2 of left panel, a band at 46 kDa corresponding to the awaited HA-hNTR2 protein is revealed by the co-immunoprecipitation of Flag-hNTR1. A similar IP is observed when using HA-hNTR1 over Flag-hNTR2 exposing the expected band at 50 kDa (lane 2 of right panel). (B) hNTR1 preserves the interaction with hNTR2 truncated construct TM5 (lane 3 of left panel 30 kDa) and a chimeric construct bf/hTM2-4 (lane 2 of right panel) as unveiled by the Flag-hNTR1 IP. (C) Cross-species interaction is revealed between rat NTR1 and human NTR2 (lane 2 and 5) corresponding to 50 and 46 kDa, respectively. Lane 3 discloses a homodimer of rat NTR1 immunoprecipitated by Flag-rNTR1 over a HA-rNTR1 revelation. Western blots for cell lysate are seen in the bottom of IP data. (D) Incubation with 100 nM of the selective agonist NT does not alter immunoprecipitation of hNTR1/hNTR2 dimer as shown by the unaffected staining of HA-hNTR2 over time.

the ability to inhibit hNTR1 function. However, when limiting the mutant to human TM3-4 (bf/hTM3-4), NTR1 signaling was not impaired. Thus, these corroborant results strongly suggest that the regions comprised between TM2 and TM4 are required to the inhibitory actions of hNTR2 on hNTR1.

Physical interaction between hNTR1 and hNTR2 receptors

To determine whether the inhibitory activity of hNTR2 may be mediated by a direct interaction with hNTR1, Flag- and HA-tagged hNTR1 and hNTR2 were expressed either individually or co-expressed in HeLa cells. When membrane extracts from these cells were immunoprecipitated using the anti-Flag antibody, the anti-HA antibody specifically detected a band at approximately 46 kDa, consistent with the molecular weight of the monomeric form of the NTR2 ([16]; Fig. 3A, left panel). Conversely, cell lysates prepared from HeLa cells expressing Flag-tagged hNTR2 and HA-tagged hNTR1 were immunoprecipitated with the anti-Flag antibody and then immunoblotted with the anti-HA antibody (Fig. 3A, right panel). Thus, these data strongly suggest that hNTR1 and hNTR2 form heterodimers in HeLa cells.

We then tested the ability of HA-hNTR2 TM5 truncation mutant and HA-bf/hTM2-4 chimeric receptor to interact physically with Flag-hNTR1. As shown in Fig. 3B, co-immunoprecipitation followed by Western blotting analysis revealed that both hNTR2 TM5 and bf/hTM2-4 chimeric receptor were co-immunoprecipitated with

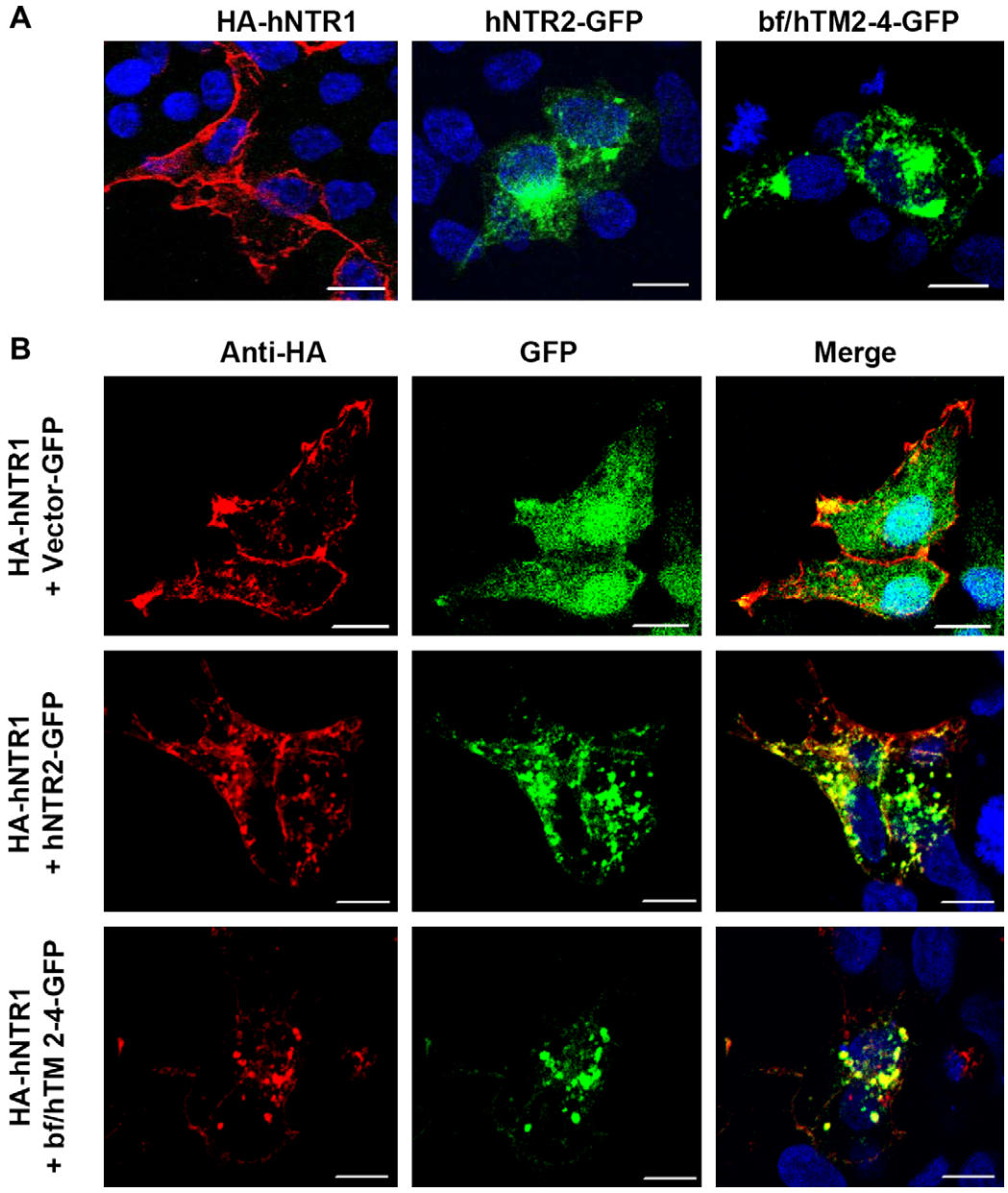

Fig. 4. Co-expression of hNTR2 impairs hNTR1 cell surface targeting. Twenty-four hours after transfection, HeLa cells were stained with mouse anti-HA and anti-Cy3-conjugated antibodies and visualized by confocal microscopy. Anti-HA and -GFP are visualized in the red and green filter channels, respectively. GFP and empty vector are both used as background controls. (A) Confocal images of HA-hNTR1 (red), hNTR2-GFP (green) and bf/hTM2-4-GFP (green) fluorescence in HeLa cells. HA-hNTR1 was primarily present at the plasma membrane in HeLa cells while both hNTR2-GFP and bf/h TM 2-4-GFP are in the cytoplasm. (B) Sub-cellular localization of HA-hNTR1 when co-expressed with hNTR2-GFP or bf/hTM2-4-GFP. hNTR1 are mostly distributed at the plasma membrane (top, Vector-GFP is used as background controls). When hNTR2 or bf/hTM2-4 is co-expressed, the hNTR1 staining overlaps with the labeling of hNTR2 (middle) and bf/hTM2-4 (bottom), forming cytoplasmic punctate. Scale bars, 10 μm.

hNTR1 (Fig. 3B). Further, HA-hNTR2 immunoprecipitated with Flag-rNTR1 (Fig. 3C, lane 2) and HA-rNTR1 also co-immunoprecipitated with Flag-hNTR2 (Fig. 3C, lane 5), implying that hNTR2 interacts physically with rNTR1. Additionally, we demonstrated that HA-rNTR1 co-immunoprecipitated with Flag-rNTR1 (Fig. 3C, lane 3), revealing that NTR1 was not only able to form heterodimers with NTR2, but also constituted homodimers with itself. Finally, we examined whether hNTR1–hNTR2 heterodimerization was affected by ligand stimulation. Thus, HeLa cells were treated for different period of times with 100 nM of NT and subjected to co-immunoprecipitation assays. As observed in Fig. 3D, similar amounts of HA-hNTR2 were detected, regardless of the duration of NT treatment, indicating that hNTR1–hNTR2 heterodimers pre-existed in cells prior to NT stimulation.

Cytoplasmic trafficking of the hNTR1–hNTR2 heterodimer

We next examined the effect of hNTR1–hNTR2 heterodimerization on receptor cell surface density and trafficking using HeLa cells expressing either hNTR1 or hNTR2 alone or co-expressing both receptors. The sub-cellular distribution of receptor proteins was analyzed by confocal microscopy. In cells expressing one of these receptors alone, HA-hNTR1 receptors were predominantly confined to the plasma membrane (Fig. 4A, left panel), whereas hNTR2-GFP and bf/hTM2-4 chimeric receptors were accumulated in the cytoplasm (Fig. 4A, middle and right panel). However, in co-expressing cells, HA-hNTR1 co-localized extensively with hNTR2-GFP (Fig. 4B, middle) and bf/hTM2-4-GFP (Fig. 4B, bottom) within the same intracellular compartment. Cell surface expression of hNTR1 in the presence or absence of hNTR2 was further examined using flow cytometry analysis. Cells expressing HA-hNTR1 together with hNTR2-GFP or bf/hTM2-4-GFP were stained with anti-HA- and Cy3-labled antibody in non-permeable condition, showing that cell expression level of HA-hNTR1 was 24.5 ± 3.8% in hNTR2-GFP-cotransfected cells and 24.4 ± 4.1% in bf/hTM2-4-GFP-cotransfected cells compared with that of HA-hNTR1 cells (Supplemental Fig. 2). Altogether, these results suggest that hNTR2 receptors regulate the targeting of hNTR1 to the plasma membrane, which may also account for the observed differences in the ability to activate second messenger signaling cascades.

Discussion

The present study demonstrates that co-expression of hNTR2 is able to impede NTR1-mediated adenylyl cyclase/cAMP and phospholipase C activation. The lack of the effects observed after co-transfection of NTR1 with closely related GPCRs, GnRHR-II and bfNTR4 reinforces the specificity of this functional interaction. This inhibitory effect of hNTR2 is likely due to heterodimerization of hNTR1 and hNTR2, which, in turn, retains a large portion of hNTR1 in the cytoplasm.

hNTR1 and hNTR2 form stable heterodimers independent of ligand stimulation. GPCRs dimerize through various mechanisms. Dimer or oligomer formation occurs through (1) covalent bonds (i.e., disulfide bonds) formed between extracellular domains, (2) coiled-coil interaction involving the C-terminal tail and (3) interactions between transmembrane helices [12,13]. In the case of hNTR1/hNTR2 heterodimerization, we point here to hydrophobic interactions within TM domains. Using a chimeric receptor approach, we demonstrated that the segment TM2 to TM4 of hNTR2 (including the second intracellular loop) is required for the functional inhibition of hNTR1. Additionally, we also observed that the hNTR2 TM5 truncation mutant partly inhibits hNTR1 activity, which would further indicate that the sequence corresponding to TM2–TM4 participates in the dimerization interface. Since the hNTR2 TM5 truncation mutant shares sequence similarity with the native vNTR2, we could also speculate that the endogenously expressed splice variant isoform may heterodimerize with NTR1 and interfere with its physiological function.

The physical interaction of hNTR2 with hNTR1 alters the cytoplasmic trafficking of hNTR1 to the plasma membrane. Confocal microscopy revealed that whereas HA- or GFP-tagged hNTR1 expressed alone were localized to the plasma membrane, co-expression of hNTR2 caused the retention of hNTR1 in a sub-cellular compartment. Considering the presence of endogenous NTR2 within the TGN/Golgi complex in cerebellar granule cells as well as in spinal cord neurons [9,22], it is possible that hNTR1–hNTR2 heterodimers are sequestered in the TGN. Alternatively, hNTR1–hNTR2 heterodimers can be constitutively internalized without stimulation by ligand.

In conclusion, our results provide direct evidence for a role of hNTR1/hNTR2 heterodimers in NTR1 function in a heterologous expression system, but also raise the question of the physiological relevance of this physical interaction. Indeed, the formation of NTR1/NTR2 heterodimers represents a versatile mechanism whereby the cell generates a new "receptor" with specific properties distinct from those of their monomers/homodimers, therefore granting additional functional resources to the cell. Thus, the NTR1/NTR2 heterodimer may have functional implication *in vivo* as the two receptors are co-expressed and linked in pain-processing pathways [23]. Further studies are needed to substantiate the pathophysiological consequences of NTR1/NTR2 heterodimerization *in vivo* and to determine whether it will be possible to exploit the phenomenon of NT receptor oligomerization to develop new drugs.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

This work was supported by Grants from the Korea Research Foundation (KRF-2006-005-J03001), the Ministry of Sciences and Technology (R01-2004-000-10163-0), and the Canadian Institutes of Health Research (CIHR, MOP-74618) awarded, respectively, to J.Y.S., H.B.K. and P.S. P.S. is a CIHR new investigator and member of the FRSQ-funded Centre de Recherche Clinique Étienne Lebel.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.007.

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