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The signaling signature of the neurotensin type 1 receptor with endogenous ligands

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ABSTRACT

The human neurotensin 1 receptor (hNTS1) is a G protein-coupled receptor involved in many physiological functions, including analgesia, hypothermia, and hypotension. To gain a better understanding of which signaling pathways or combination of pathways are linked to NTS1 activation and function, we investigated the ability of activated hNTS1, which was stably expressed by CHO-K1 cells, to directly engage G proteins, activate second messenger cascades and recruit β-arrestins. Using BRET-based biosensors, we found that neurotensin (NT), NT(8-13) and neuromedin N (NN) activated the $G\alpha_q$-, $G\alpha_{i1}$-, $G\alpha_{oA}$-, and $G\alpha_{13}$-protein signaling pathways as well as the recruitment of β-arrestins 1 and 2. Using pharmacological inhibitors, we further demonstrated that all three ligands stimulated the production of inositol phosphate and modulation of cAMP accumulation along with ERK1/2 activation. Interestingly, despite the functional coupling to $G\alpha_{i1}$ and $G\alpha_{oA}$, NT was found to produce higher levels of cAMP in the presence of pertussis toxin, supporting that hNTS1 activation leads to cAMP accumulation in a $G\alpha_s$-dependent manner. Additionally, we demonstrated that the full activation of ERK1/2 required signaling through both a PTX-sensitive $G_{i/o}$-c-Src signaling pathway and PLCβ-DAG-PKC-Raf-1-dependent pathway downstream of G_q. Finally, the whole-cell integrated signatures monitored by the cell-based surface plasmon resonance and changes in the electrical impedance of a confluent cell monolayer led to identical phenotypic responses between the three ligands. The characterization of the hNTS1-mediated cellular signaling network will be helpful to accelerate the validation of potential NTS1 biased ligands with an improved therapeutic/adverse effect profile.

1. Introduction

G protein-coupled receptors (GPCRs) constitute the largest superfamily of cell-surface proteins involved in signal transduction (Bockaert, 1999). A large and diverse group of ligands activates GPCRs, which transduce intracellular signals by coupling to G proteins or other proteins such as arrestins (Marinissen and Gutkind, 2001). Today, GPCRs are the targets of up to 40% of the total drug market and hence continue to be of great interest for the development of therapeutic agents.

Given that a GPCR can couple to different signaling pathways and that the latter can be selectively engaged or blocked led to the emergence of a novel paradigm called bias signaling (Kenakin, 2013). However, to fully exploit bias signaling, a clear profile of the signaling pathways activated by a GPCR is crucial. Here, we focused on deciphering the signaling signature of human NTS1 following stimulation by endogenous ligands. The neurotensin receptor family is composed of three subtypes, NTS1, NTS2, and NTS3 (Vincent et al., 1999). Among these subtypes, NTS1 (Tanaka et al., 1990; Vita et al., 1993) and NTS2 (Chalon et al., 1996; Mazella et al., 1996; Vita et al., 1998) belong to the rhodopsin-like family of GPCRs, whereas NTS3 belongs to the sortilin receptor family (Mazella and Vincent, 2006; Mazella et al., 1998). Since its discovery, NTS1 has been detected in peripheral tissues such as the vascular endothelium and gastrointestinal tract (Azriel and Burcher, 2001; Coppola et al., 2008) as well as in the central nervous system (CNS) (Roussy et al., 2008), suggesting that

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it is involved in a broad variety of physiological functions (Feng et al., 2015; Osadchii, 2015). Indeed, NTS1 mediates blood pressure lowering (Rioux et al., 1982), ileum contraction or relaxation (Carraway and Mitra, 1994), analgesia (Roussy et al., 2008), and hypothermia (Feifel et al., 2010).

NTS1 is endogenously activated by neurotensin (NT) as well as by neuromedin N (NN), both of which are derived from the same pro-NT/NN precursor (Rostène and Alexander, 1997; Rovere et al., 1996). Structure-activity studies of NT have revealed that the minimal bioactive fragment corresponds to the C-terminal hexapeptide of NT, NT(8-13) (St-Pierre et al., 1981).

To date, depending on the cell type, NTS1 has been linked, through $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_s$ coupling, to a variety of intracellular signaling cascades including cyclic AMP (cAMP), inositol phosphate (IP), and arachidonic acid accumulation as well as the activation/inhibition of mitogen-activated protein kinases (ERK1/2 and JNK) and serine/threonine protein kinase Akt (Müller et al., 2011). Interestingly, NTS1-G protein interactions involve different receptor domains; interaction with $G\alpha_{q/11}$ requires the intact third intracellular loop of the receptor, and coupling to $G\alpha_s$ and $G\alpha_{i/o}$ involves the receptor's C-terminal domain (Gailly et al., 2000; Grisshammer and Hermans, 2001; Najimi et al., 2002; Skrzydelski et al., 2003). NT binding to NTS1 also induces the internalization of the receptor–ligand complex via a clathrin-dependent endocytic process requiring the recruitment of dynamin, intersectin, and β-arrestins to the GRK2/GRK5 phosphorylated receptor (Inagaki et al., 2015; Oakley et al., 2001, 2000; Savdie et al., 2006; Vandenbulcke et al., 2000; Zhang, 1999). Like many GPCRs, NTS1 internalization has been shown to lead to G protein-independent signaling (Souazé et al., 1997; Toy-Miou-Leong et al., 2004). Although the coupling to diverse G proteins has been demonstrated through the modulation of second messenger cascades, there is still no direct evidence of the interaction between NTS1 and G proteins in a unique cell type.

In the present study, we used bioluminescence resonance energy transfer (BRET) biosensors and label-free whole-cell assays to study the ability of NT, NT(8-13) and NN to activate signaling pathways following their binding to hNTS1 expressed in CHO-K1 cells.

2. Materials and methods

2.1. Materials

Neurotensin 1-13 (NT), IBMX (3-isobutyl-1-methylxantine), SR48692, and forskolin were obtained from R & D Systems, Inc. (Minneapolis, MN, USA). NT(8-13) was synthesized at the peptide synthesis facility of the Université de Sherbrooke. NN was purchased from GenScript USA Inc. (Piscataway, NJ, USA). Coelenterazine 400 A (DeepBlueC) was purchased from Gold Biotechnology Inc. (St. Louis, MO, USA). The peptide sequences of NT, NT(8-13) and NN are shown in Table 1. Ham's F12, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Penicillin-streptomycin-glutamine and fetal bovine serum (FBS) were obtained from Wisent (St. Bruno, QC, Canada), Opti-MEM was acquired from Invitrogen (Burlington, ON, Canada). Lance Ultra cAMP and pERK1/2 assay kits were purchased from Perkin Elmer (Montréal, QC, Canada). A Cisbio IP1 assay kit was purchased through Cedarlane (Burlington, ON, Canada). All inhibitors used in this study were from Santa-Cruz Biotechnologies (Dallas, TX, USA), with the exception of UBOQIC, which was purchased from Bonn University; Pertussis Toxin (PTX) which was purchased from List Biological Laboratories (Campbell, CA, USA); Dynasore, which was obtained from Tocris (Minneapolis, MN, USA); and Y27632, which was obtained from Cell Signaling Technology (Danvers, MA, USA).

Table 1
Peptide sequence of NT, NT(8-13) and NN.

Compound	Sequence
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH
Neurotensin (8-13)	H_2N- Arg-Arg-Pro-Tyr-Ile-Leu-COOH
Neuromedin N	H_2N- Lys-Ile-Pro-Tyr-Ile-Leu-COOH

2.2. Plasmids and constructs

The cDNAs encoding the human neurotensin receptor type 1 and the $G_{\beta 1}$ subunit were obtained from the Missouri S & T cDNA Resource Center (Rolla, MO, USA). The fusion vector pIREShygro3-GFP10 and RlucII-β-arrestin 1 or 2 plasmids were kindly provided by Dr. Michel Bouvier (Dept. of Biochemistry and IRIC, Université de Montréal, Montréal, QC, Canada). Using an InFusion advantage PCR cloning kit (Clontech Laboratories, Mountain View, CA, USA), the hNTS1 construct was inserted into the pIREShygro3-GFP10 vector as previously described (Demeule et al., 2014). The plasmids encoding $G\alpha_q$-RlucII (Breton et al., 2010), $G\alpha_{oA}$-RlucII (Richard-Lalonde et al., 2013), $G\alpha_{13}$-RlucII (Demeule et al., 2014), $G\alpha_{i1}$-RlucII, GFP10-$G\gamma_1$, and GFP10-$G\gamma_2$ (Galés et al., 2006) were kindly provided by Dr. Michel Bouvier. All constructs were verified by DNA sequencing.

2.3. Cell culture and transfections

CHO-K1 cells stably expressing hNTS1 (CHO-hNTS1 cells) were purchased from Perkin Elmer (Montréal, QC, Canada) and cultured in Ham's F12 containing 20 mM HEPES, 10% FBS, 0.4 mg/ml G418, and penicillin (100 U/ml)-streptomycin (100 µg/ml)-glutamine (2 mM) under 5% CO_2 at 37 °C in a humidified atmosphere. The CHO-K1 cells were cultured in the same conditions as above but without G418. For the transient expression of recombinant proteins, T75 flasks were seeded with 3×10^6 cells, and 24 h later, the cells were transfected using PEI (Ehrhardt et al., 2006).

2.4. $BRET^2$ assay

To monitor direct G protein activation, we used the following biosensor couples: $G\alpha_q$-RlucII, GFP10-$G\gamma_1$, and $G\beta_1$ (Breton et al., 2010); $G\alpha_{oA}$-RlucII, GFP10-$G\gamma_1$, and $G\beta_1$ (Richard-Lalonde et al., 2013); $G\alpha_{13}$-RlucII, GFP10-$G\gamma_1$, and $G\beta_1$; or $G\alpha_{i1}$-RlucII, GFP10-$G\gamma_2$, and $G\beta_1$ (Galés et al., 2006). G protein biosensors were transfected into CHO-hNTS1 cells. At 24 h post-transfection, the cells were detached with trypsin-EDTA and plated (50,000 cells/well) in white opaque 96-well plates (BD Falcon, Corning, NY, USA). At 48 h post-transfection, the cells were washed once with PBS, and 90 µL of HBSS containing 20 mM HEPES was then added. Ligands were added at increasing concentrations for 20 min followed by coelenterazine-400A (5 µM). The $BRET^2$ measurements were collected in the 400–450 nm window (RlucII) and in the 500–550 nm window (GFP10) using the $BRET^2$ filter set on a GENios Pro plate reader (Tecan, Durham, NC, USA). The $BRET^2$ ratio was determined as the light emitted by the acceptor GFP10 over the light emitted by the donor RlucII. The monitoring of β-arrestin recruitment was done by the transient transfection of CHO-K1 cells with plasmids containing cDNAs encoding hNTS1-GFP10 and RlucII-β-arrestin 1 or 2. The same protocol as the one used for G protein activation was then used.

2.5. IP-One and Lance Ultra cAMP assays

The IP-One assay was performed according to the manufacturer's recommendations. Briefly, 15,000 CHO-hNTS1 cells per well (384-well shallow well plate) were treated with increasing concentrations of NT, NT (8-13), or NN for 30 min. IP1-d2 and anti-IP1-Cryptate were added for at least 1 h. The plates were read on a GENios Pro plate reader with HTRF filters (excitation at 320 nm and emission at 620 and 665 nm).