Low-affinity neurotensin receptor (NTS2) signaling: 
Internalization-dependent activation of ERK1/2

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Abbreviations: NT, neurotensin; NN, neuromedin N; Levo, levocabastine; CHO cells, Chinese hamster ovary cells; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1/2; EGFP, enhanced green fluorescent protein; PAO, phenylarsine oxide; MDC, monodansylcadaverine; PTX, Pertussis toxin.
Abstract

The role and signaling properties of the low-affinity neurotensin receptor (NTS2) are still a matter of controversy. In particular, it is unclear whether neurotensin acts as an agonist, inverse agonist or antagonist at this site. In view of the growing evidence for a role of NTS2 in antinociception, the elucidation of the pharmacological and coupling properties of this receptor is particularly critical. In the present study, we demonstrate that in CHO cells expressing the rat NTS2 receptor, neurotensin (NT), levocabastine, neuromedin N, and the high-affinity NT receptor antagonist SR48692 all bind to and activate the NTS2 receptor. This activation is followed by ligand-induced internalization of receptor-ligand complexes, as evidenced by confocal microscopy using a fluorescent NT analog. All compounds tested produced a rapid and sustained activation of extracellular signal-regulated kinases (ERK1/2), but were without specific effect on Ca\(^{2+}\) mobilization. The agonist-induced activation of ERK1/2 was completely abolished by preincubation of the cells with the endocytosis inhibitors, phenylarsine oxide and monodansylcadaverine, as well as overexpression of a dominant negative mutant of dynamin 1 (DynK44A), indicating that receptor internalization was required for ERK1/2 activation. NTS2-induced activation of ERK1/2 was not species-specific since the same agonistic effects of NT and analogs were observed in CHO cells transfected with the human NTS2 receptor. In conclusion, this study demonstrates that NTS2 is a \textit{bona fide} NT receptor and that activation of this receptor by NT or NT analogs results in an internalization-dependent activation of the ERK1/2 signaling cascade.
Introduction

Neurotensin (NT) is a tridecapeptide that exerts neuromodulatory functions in the central nervous system (CNS) and has endocrine/paracrine actions in the periphery (Vincent, 1995; Rostène and Alexander, 1997). NT has been shown to modulate dopaminergic transmission in the nigrostriatal and mesocorticolimbic pathways (Nemeroff, 1986; Kitabgi et al., 1989), thereby implicating this neuropeptide in the pathophysiology of several CNS disorders including Parkinson’s disease and schizophrenia (for reviews see Kitabgi et al., 1989; Binder et al., 2001; Kinkead and Nemeroff, 2002). In addition, NT injection in brain or ventricular system produces hypothermia (Martin et al., 1980), changes in blood pressure (Rioux et al., 1981), and non-opioid dependent analgesia (Kalivas et al., 1982).

NT mediates its central and peripheral effects through interaction with three receptor subtypes, referred to as NTS1, NTS2 and NTS3. NTS1 and NTS2 belong to the seven transmembrane domain/G protein-coupled receptor family (Tanaka et al., 1990; Vita et al., 1993; Chalon et al., 1996; Mazella et al., 1996; Vita et al., 1998), whereas NTS3 is a single transmembrane domain sorting receptor predominantly associated with vesicular organelles and the Golgi apparatus (Petersen et al., 1997; Mazella et al., 1998). Pharmacological and biochemical studies have indicated that the high-affinity (subnanomolar range) NT receptor NTS1 is coupled to cGMP, cAMP and inositol phosphate signaling cascades (for reviews see Hermans and Maloteaux, 1998; Vincent et al., 1999). Stimulation of NTS1 also induces the activation of extracellular signal-regulated kinases (ERK1/2) through coupling with both pertussis toxin-sensitive and insensitive G-proteins. This activation leads in turn to the expression of proliferative genes such as c-fos, Krox-24 and elk-1 (Poinot-Chazel et al., 1996;
Ehlers et al., 1998; Portier et al., 1998; Ehlers et al., 2000; Martin et al., 2002a). These effects are selectively blocked by the non-peptide NT antagonist SR48692, which displays a nanomolar affinity for NTS1 (Gully et al., 1993).

The low-affinity (nanomolar range) NT receptor, NTS2, differs from the NTS1 site not only by its tenfold lower affinity for NT, but also by its selective recognition of levocabastine, a non-peptide histamine H₁ receptor antagonist, which selectively inhibits NT binding to NTS2 without affecting its binding to NTS1 (Schotte et al., 1986; Kitabgi et al., 1987). NTS2 also displays a much lower affinity (IC₅₀ = 300 nM) than NTS1 (IC₅₀ = 5.6 nM) for the SR48692 compound (Gully et al., 1993). However, there is still considerable controversy concerning the pharmacological and signaling properties of NTS2. In particular, doubts have been cast regarding the agonistic properties of NT at this site, and hence about whether or not this protein may be regarded as a true NT receptor. Indeed, in CHO cells stably transfected with human NTS2, SR48692, but neither NT nor levocabastine, was found to activate classical second messenger systems, such as phosphoinositide hydrolysis, Ca²⁺ mobilization, or ERK1/2 phosphorylation (Vita et al., 1998). Furthermore, in transfected CHO and COS cells, this SR48692-induced activation of the human NTS2 was blocked by NT, suggesting that the endogenous peptide was acting as a competitive antagonist at these sites (Vita et al., 1998; Richard et al., 2001).

By contrast, in Xenopus oocytes expressing the mouse NTS2 receptor, NT, NN, and levocabastine were all found to activate Ca²⁺-dependent chloride currents (Mazella et al., 1996). Additionally, application of NT or levocabastine on rat cerebellar granule cells, which endogenously express the NTS2 but not the NTS1 receptor, induced a sustained activation of the ERK1/2 signaling cascade (Sarret et al., 2002). Congruent with an agonist role of NT at this site, rodent NTS2 receptors were found to efficiently internalize via clathrin-coated pits upon NT
binding, both in stably transfected HEK293 cells (Botto et al., 1998) and in rat cerebellar granule cell cultures (Sarret et al., 2002).

It is unclear whether the reported agonistic/antagonistic effects of NT on the human versus rodent NTS2 receptor are due to species differences between the two receptors or to variations in receptor coupling due to the cell type in which the receptor is expressed. In view of the growing evidence for a role of NTS2 (Dubuc et al., 1999a; Dubuc et al., 1999b; Remaury et al., 2002; Yamauchi et al., 2003), in addition to that of NTS1 (Tyler et al., 1999; Pettibone et al., 2002), in antinociception and therefore of the possibility that NTS2 might represent a new target for the development of non-opioid analgesic drugs, the need for precise knowledge of the pharmacological and signaling properties of this receptor appears particularly critical. Thus, the aim of the present study was to characterize the pharmacological and signaling properties of the rat NTS2 receptor expressed in stably transfected CHO cells and to compare these properties with those of the human NTS2 receptor expressed in the same cell line as well as with our own earlier data on the properties of the rat NTS2 receptor endogenously expressed in rat cerebellar granule cells.
Material and methods

Material

The chemicals used in the present study were obtained from the following sources: Dulbecco's Modified Eagle's Medium (DMEM) and F12-medium, fetal bovine serum (FBS), glutamine, G-418, gentamicin, and lipofectAMINE from Gibco (Burlington, Ontario, Canada); neurotensin (NT), monodansylcadaverine (MDC), phenylarsine oxide (PAO), sodium orthovanadate (Na3VO4), Pertussis toxin (PTX) and staurosporine from Sigma (St Louis, MI, USA); anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies from New England Biolabs (Beverly, MA, USA); horseradish peroxidase-conjugated anti-rabbit antibodies and enhanced chemiluminescence (ECL) detection system from Amersham Pharmacia Biotech Inc. (Baie d'Urfé, Quebec, Canada); Complete™ protease inhibitor and polyvinylidene difluoride (PVDF) membranes from Roche Laboratories (Montreal, Quebec, Canada). Neuromedin N (NN) was from Bachem (Torrance, CA, USA); levocabastine was kindly provided by Janssen Research (Beerse, Belgium) and SR48692 by Sanofi Synthelabo (Toulouse, France). All other chemicals were of grade A purity.

Transfection of CHO cells

CHO/K1 cells were cultured in DMEM/F12 medium mixture (1:1) supplemented with 10% FBS and 50 mg/l gentamicin at 37°C in 75 cm² Falcon flasks in a humidified atmosphere of 95% air and 5% CO2. For transfection, CHO/K1 cells were grown to subconfluency (70-80%) in 24-well Petri dishes and incubated during 4 h at 37°C in the transfection medium (mixture of pTARGET-rNTS2 (1 µg/ml) (Sarret et al., 2003) or pTARGET-hNTS2 and 40 µg/ml
LipofectAMINE in serum-free DMEM). Transfection medium was then replaced with DMEM/F12 medium and the cells were transferred 36 h later to a 75 cm² flask containing fresh medium supplemented with G-418 at a concentration of 800 µg/ml. After 2 weeks of selection with G-418, a total of 23 and 12 individual clones were isolated for CHO/rNTS2 and CHO/hNTS2, respectively. Each clone was separately grown and tested for its capacity to internalize Fluo-NT as described below. CHO/rNTS2 clone #16 and CHO/hNTS2 clone #1 were used for all experiments.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNAs (2 µg) were extracted from CHO/rNTS2 and CHO/K1 cells using Qiagen RNeasy Mini Spin columns (Qiagen Inc., Mississauga, Ontario, Canada) and submitted to reverse transcription (reverse transcription system kit; Promega) for 1 h at 42°C. First strand cDNAs were then subjected to 35 cycles of PCR in a final reaction volume of 50 µl of the reaction buffer (50 mM KCl, 10 mM Tris, pH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.02% BSA, 200 µM dNTPs, 0.5 unit of Taq DNA polymerase) containing 100 ng of either one of the following 3 pairs of sense and antisense primers as described previously (Sarret et al., 2002). The first pair (5’-ACACCCATTGTGGACACAGCC-3’ and 5’-TTCATCCGAGATATAGCAGAA-3’) provided for the amplification of a fragment of rNTS1 receptor cDNA with a predicted size of 335 bp. The second pair (5’-GAATGTGCTGGTGTCCTTCGC-3’ and 5’-ACTTGTATTCTCCAGGCTG-3’) provided for the amplification of a fragment of rNTS2 receptor cDNA with a predicted size of 620 bp and the third pair (5’-TCCCGAGAACTCTGGAAAGGT-3’ and 5’-CACAGAGGCGAAGAGGAAACG-3’) provided for the amplification of a fragment of rNTS3 receptor cDNA with a predicted size of 426 bp. Amplification was carried out with the
first cycle at 95°C for 3 min, 54°C for 2 min, 72°C for 45 sec, followed by 34 cycles at 95°C for 40 sec, 54°C for 35 sec, 72°C for 45 sec, and a final extension step at 72°C for 5 min. PCR products were then analyzed on a 1.5% agarose gel.

**Binding of $^{125}$I-NT to CHO/rNTS2 cells**

For binding experiments, cells were grown on 24-well plates and incubated at 37°C in DMEM/F12 medium 48 h before the assay. Cells were equilibrated for 10 min at 37°C in Earle’s buffer (130 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgCl$_2$, HEPES 20 mM, pH 7.4) supplemented with 0.2% BSA and 0.1% glucose. Cells were then incubated with 2.5 nM $^{125}$I-NT (100 Ci/mmol) for 30 min at 37°C in 250 µl of Earle’s buffer containing 0.8 mM ortho-phenanthroline in the presence of increasing concentrations (from $10^{-11}$ to $10^{-5}$ M) of nonradioactive NT, levocabastine, NN, or SR48692. Cells were then washed twice with Earle’s buffer, harvested in 1 ml of 0.1 M NaOH, and the radioactivity content was measured in a gamma counter. IC$_{50}$ values were determined from competition curves as the concentration of unlabeled ligand necessary to inhibit 50% of $^{125}$I-NT specific binding.

**Intracellular calcium measurements**

For intracellular calcium ([Ca$^{2+}$]$_i$) measurements, the CHO/rNTS2 and CHO/K1 cells were cultured on 22-mm glass coverslips and incubated in serum-free DMEM supplemented with 4 µM fluo-4/acetoxyethyl ester (Molecular Probes) at 37°C for 30 min. Cells were then washed three times with 0.5% BSA and further incubated in PBS-HEPES (140 mM NaCl, 5.4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$·6H$_2$O, 10 mM HEPES, pH 7.35) at 37°C for 30 min in order to allow the acetoxyethyl ester form to be hydrolyzed. The coverslips were then mounted on the stage of a
Nikon Eclipse TE300 inverted microscope and the cells were maintained at 37°C throughout the experiments with a heating Peltier element.

NT, NN, levocabastine, or SR48692, diluted in fresh PBS-HEPES containing, for solubilization of the latter two drugs, 0.01% dimethylsulfoxide (DMSO), were added to the cells at a final concentration of 1 µM and images of fluorescence were acquired every 5 seconds using a CoolSnap fₓ CCD camera (Roper Scientific, Tucson, AZ) cooled at –35°C. Additional experiments were carried out using 0.01% DMSO in PBS-HEPES alone, to test for possible non specific effects of the solubilizing agent. Band pass filters were used for excitation and emission (450-490 and 520-560 nm, respectively). Average fluorescence intensity for each cell was measured using Metafluor software package (Universal Imaging Corp, West Chester, PA). Each Ca²⁺ curve represents the average response of n cells as indicated on the figure.

Western blotting analyses of ERK1/2 activity

CHO/rNTS2, CHO/hNTS2, and CHO/K1 cells were grown for 3 days in DMEM/F12 medium containing 10% FBS, starved in serum-free DMEM during 1 h, and then stimulated for various time intervals (from 1 to 60 min) with NT (100 nM), levocabastine (100 nM or 1 µM), NN (100 nM) or SR48692 (100 nM) at 37°C in serum-free medium. In some experiments, cells were preincubated with PAO or MDC (two endocytosis inhibitors) during 30 min or with PTX (Gi-protein inhibitor) for 18 h (100 ng/ml) prior to stimulation with NT or NT analogs. The reaction was stopped by aspiration of the medium and the addition of ice-cold Hank’s balanced salt solution containing 0.1 µM staurosporine and 1 mM sodium orthovanadate. Cells were then left for 30 min at 4°C and lysed in 50 mM HEPES, pH 7.8, containing 1% Triton X-100, 0.1 µM staurosporine, 1
mM sodium orthovanadate, and Complete™ protease inhibitor. The cell lysates were centrifuged at 8000 x g for 15 min at 4°C, and the supernatants were stored at −20°C until use.

For each lysate, equal amounts of proteins (25 µg) were separated on 10% SDS-polyacrylamide gels and electrotransferred on polyvinilidene difluoride (PVDF) membranes as previously described (Gendron et al., 2003). PVDF membranes containing proteins were incubated for 2 h at room temperature with anti-phosphorylated ERK1/2 (1:1000) or anti-ERK1/2 (1:1000) rabbit antibodies, followed by 3 washes with Tris-buffered saline/Tween 20. Detection of immunoreactive proteins was accomplished using horseradish peroxidase-conjugated anti-rabbit (1:2000) and an ECL detection system.

To quantify the effect of NT and NT analogs on ERK1/2 phosphorylation, the ratios of phosphorylated ERK1/2 over total ERK1/2 levels were determined by densitometry, using NIH Scion Image Imaging Software. The statistical significance of the activation of ERK1/2 in stimulated versus non-stimulated cells was verified using ANOVA test, and the p values were obtained from Dunnett’s tables.

**Binding of Nα-Bodipy-Neurotensin-(2-13) (Fluo-NT) to CHO/rNTS2 cells**

CHO/rNTS2 cells were grown for 2 days on 12-mm poly-L-lysine-coated glass coverslips in DMEM/F12 medium containing 10% FBS and stimulated for 30 min at 37°C with 50 nM Nα-Bodipy-Neurotensin-(2-13) (Fluo-NT) in serum-free DMEM containing 0.8 mM ortho-phenanthroline, alone or in the presence of levocabastine (10 µM) or phenylarsine oxide (endocytosis inhibitor) (10 µM). At the end of the incubation, cells were washed twice with ice-cold PBS, air-dried, mounted on glass slides with Aquamount, and examined using a Zeiss confocal...
laser-scanning microscope LSM510 (Carl Zeiss Canada Ltd., Toronto, ON) equipped with a Zeiss inverted microscope and a He/Ne laser (543 nm).

**Immunofluorescence studies**

CHO/rNTS2 cells were grown on 12-mm glass coverslips for 3 days in DMEM/F12 medium containing 10% FBS and then starved in serum-free DMEM during 1 h. Cells, pretreated or not with PAO (10µM, 10 min at 37°C), were then treated or not with SR48692 (100 nM) for 5 min at 37°C in serum-free DMEM. The reaction was stopped by aspiration of the medium and addition of ice-cold Hank’s balanced salt solution containing 0.1 µM staurosporine and 1 mM sodium orthovanadate. After 10 min of incubation on ice, cells were fixed for 20 min with methanol at –20°C and rehydrated with Hanks’ balanced salt solution for 30 min at room temperature. Phosphorylated ERK1/2 were labeled overnight at 4°C using anti-phosphorylated ERK1/2 rabbit antibodies (1:100) and revealed using goat anti-rabbit Alexa488- or Alexa594-conjugated secondary antibodies (Molecular Probes; diluted 1:500 in Hanks’ balanced salt solution) for 60 min at room temperature. After washing, coverslips were mounted on glass slides using Aquamount and examined using a Zeiss confocal laser-scanning microscope LSM510 equipped with a Zeiss inverted microscope, an Argon laser (488 nm) and a He/Ne laser (543 nm). Images were all taken using the same acquisition settings.

To determine whether ligand-induced receptor internalization was necessary for NTS2-induced ERK1/2 phosphorylation, the above immunofluorescence assay was repeated on CHO/rNTS2 cells preincubated for 10 min with PAO (10 µM) as well as on CHO/rNTS2 cells cotransfected with pcDNA1-DynK44A (kindly provided by Dr Stephen S. Ferguson) and pEGFP-N1 (BD Biosciences, Mississauga, Ontario, Canada). For this purpose, pcDNA1-
DynK44A (1 µg/ml) and pEGFP-N1 (0.1 µg/ml) plasmids were mixed with 40 µg/ml LipofectAMINE and the mixture was kept at room temperature for 30 min before being added to the culture medium. CHO/rNTS2 cells grown to 25-30% subconfluency on 12-mm poly-L-lysine-coated glass coverslips were then transfected for 4 h at 37°C with this DNA-lipid complex. At the end of the incubation, transfection medium was replaced with fresh medium and cells were processed 36 h later for immunolabeling of the phosphorylated-ERK1/2 as described above.
Results

Expression and binding properties of rNTS2 in transfected CHO cells

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of rat NTS1, NTS2 and NTS3 expression was performed on non-transfected CHO cells (CHO/K1) and on CHO cells transfected with the rat NTS2 receptor cDNA (CHO/rNTS2). As shown in Fig. 1A, a 620 bp band corresponding to the size of the NTS2 receptor fragment was observed in CHO/rNTS2 cells, but not in CHO/K1 cells. In contrast, a 425 bp product corresponding to the NTS3 receptor was detected in both transfected and non-transfected cells. The PCR product for the NTS1 receptor (expected at 336 bp) was observed in neither of these cell lines but was present in CHO/rNTS1 cells used as positive controls (not shown). As illustrated in Fig. 1B, NT, levocabastine, NN, and SR48692 all inhibited competitively specific $^{125}$I-NT binding with IC$_{50}$ values of 3.5, 74, 21 and 31 nM, respectively.

Lack of NTS2-induced intracellular Ca$^{2+}$ mobilization

The capacity of NT, levocabastine, NN, and SR48692 to induce Ca$^{2+}$ mobilization was tested in CHO/rNTS2 cells, using Fluo-4 as a fluorescent marker of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$). As seen in Fig. 2, NT, levocabastine (Levo) and NN were ineffective at modifying [Ca$^{2+}$]$_i$ even when applied at concentrations as high as 1 $\mu$M (Panels A, B and C, respectively). By contrast, SR48692 (1 $\mu$M) markedly increased [Ca$^{2+}$]$_i$. This effect was due to the drug itself, as it was not observed following application of the DMSO-containing vehicle alone. The SR48692-induced increase in [Ca$^{2+}$]$_i$ was neither prevented nor modified by concomitant (not shown) or prior stimulation with NT, levocabastine or NN (Fig. 2A, B and C),
suggesting that it was not NTS2-mediated. Congruent with this interpretation, SR48692 produced a similar increase in [Ca\textsuperscript{2+}]\textsubscript{i} in non-transfected CHO/K1 cells (Fig. 2D).

**NTS2-mediated ERK1/2 phosphorylation**

In order to determine whether heterologously-expressed rNTS2 were functionally coupled to the ERK1/2 pathway, CHO/rNTS2 cells were stimulated with 100 nM NT for various periods of time. Western blot analysis of phosphorylated ERK1/2 revealed that NT rapidly enhanced the level of phosphorylation of ERK1/2 (Fig. 3A, B). This increase in ERK1/2 phosphorylation was already apparent after 1 min of stimulation, peaked at 10 min (2.89 ± 0.80 fold increase over control) and was sustained for at least 1 h. This effect was NTS2-mediated as it was not observed in non-transfected cells (Fig. 3B, C). As shown in Fig. 3D, the NT-induced ERK1/2 activation measured after 5 min of stimulation was dose-dependent and readily detectable at concentrations as low as 10\textsuperscript{-8}M (i.e. within the range of the k_d value of NT for the rNTS2 receptor).

We then tested whether other documented NTS2 ligands similarly affected ERK1/2 phosphorylation. As shown in Fig. 4A, B, levocabastine (100 nM) and NN (100 nM) both activated ERK1/2 to the same extent as NT (3.2 ± 0.8 and 2.2 ± 0.9 fold increase over control, respectively). Furthermore, the NTS1 antagonist SR48692, which binds the NTS2 receptor with less affinity than NT (Fig. 1B), induced a phosphorylation of ERK1/2 that was considerably more robust than that produced by either of the other NTS2 agonists tested (15.4 ± 4.2 fold increase over control). This effect of SR48692 was unaffected by the addition of 100 nM NT in the incubation medium (Fig. 4A, B), suggesting that the two drugs interacted with different sites. Yet the effects of SR48692, as well as those of levocabastine and NN, were NTS2-mediated
since none of these ligands were able to activate ERK1/2 in non-transfected, CHO/K1 cells (Fig. 4C). These effects were also independent from G\textsubscript{i} since they were unaffected by an overnight preincubation of CHO/rNTS2 cells with PTX (not shown).

To determine whether the NTS2-mediated effects of NT and NT analogs on ERK1/2 activation were species-specific, Western blot analyses of ERK1/2 phosphorylation were repeated on CHO cells transfected with cDNA encoding the human NTS2 receptor (CHO/hNTS2). As shown in Fig. 5A, C, stimulation of CHO/hNTS2 with 10\textsuperscript{-7} M NT for 3 to 60 min resulted in a significant increase in ERK1/2 phosphorylation. As in CHO/rNTS2 cells, this effect peaked at 10 min but was somewhat less sustained in that phosphorylation levels returned to baseline by 1 h (Fig. 5A). Stimulation of CHO/hNTS2 cells for 5 min with 10\textsuperscript{-6} M levocabastine or 10\textsuperscript{-7} M SR48692 also induced a robust activation of ERK1/2 phosphorylation (Fig. 5B, C). As in CHO/rNTS2 cells, the effects of SR48692 were significantly greater than those of NT (3.07 ± 0.57 versus 1.79 ± 0.31 fold increase over control; Fig. 5C).

**Internalization-dependence of the NTS2-induced ERK1/2 activation**

To determine whether heterologously-expressed NTS2 internalized upon agonist binding, CHO/rNTS2 and CHO/K1 cells were incubated with 50 nM N\textalpha-Bodipy-neurotensin-(2-13) (Fluo-NT) for 30 min at 37°C and examined by confocal microscopy. In CHO/rNTS2 cells, Fluo-NT pervaded the cytoplasm in the form of small, endosome-like fluorescent clusters (Fig. 6A). By contrast, Fluo-NT labeling was confined to the cell surface after pretreatment with the endocytosis inhibitor, PAO (10 µM, 10 min) (Fig. 6B). Fluo-NT internalization was NTS2-specific, since non-transfected CHO/K1 cells (not shown) and cells co-incubated with Fluo-NT and an excess of levocabastine (Fig. 6C) were entirely fluorescent-negative.
To determine whether ligand-induced NTS2 receptor internalization was necessary for ERK1/2 activation, CHO/rNTS2 cells were pretreated or not with PAO (10 µM) or MDC (400 µM) and stimulated for 5 min with 100 nM NT, levocabastine, or SR48692. PAO and MDC both completely inhibited the effect of stimulation by either ligand on ERK1/2 phosphorylation as measured by Western blotting (Fig. 7A).

The effect of stimulation with SR48692 on ERK1/2 activation was also verified by immunofluorescence in CHO/rNTS2 cells, using antibodies against phosphorylated-ERK1/2. In contradistinction with non-stimulated cells, which were immunonegative (Fig. 6D, G and Fig. 7B), cells stimulated for 5 min with SR48692 (100 nM) exhibited intense phosphorylated-ERK1/2 immunoreactivity, mainly within their nucleus (Fig. 7B, arrowheads). This induction of phosphorylated ERK1/2 immunoreactivity was totally prevented by pre-incubating the cells with PAO (Fig. 7B).

To further confirm that the NTS2-induced ERK1/2 activation was dependent on ligand-induced internalization, CHO/rNTS2 cells were transiently transfected with a dominant-negative mutant of dynamin 1, DynK44A, together with the fluorescent protein EGFP (with a ratio of 10:1), to distinguish DynK44A-expressing from non-expressing cells. Stimulation of these dually transfected cells with 100 nM SR48692 increased phosphorylated-ERK1/2 immunofluorescence in approximately 40% of the cells (Fig. 6E, H), whereas 100% of the cells expressing only the rNTS2 receptor were activated after stimulation with SR48692 (Fig. 7B). This decrease was due to the overexpression of the dynamin 1 dominant negative mutant since none of the cells confirmed to overexpress DynK44A, by virtue of their co-expression of EGFP, showed phosphorylated ERK1/2 immunofluorescence (Fig. 6F, I, arrows).
Discussion

The present study demonstrates that neurotensin activates the MAP kinase cascade through its interaction with either rat or human NTS2 receptors in transfected CHO cells. It also indicates that ligand-induced internalization of this receptor is required for NTS2-mediated signaling.

We previously demonstrated that stimulation of rat cerebellar granule cells, which endogenously express the NTS2 receptor, with either NT or levocabastine resulted in ERK1/2 activation (Sarret et al., 2002). These results differed from those obtained by other groups, which had reported antagonistic or inverse agonistic effects of these two drugs on the human NTS2 receptor heterologously expressed in COS (Richard et al., 2001) and CHO cells (Vita et al., 1998) respectively. A first objective of the present study was therefore to determine whether these discrepancies were due to species differences or to endogenous versus heterologous expression of the NTS2 receptor.

For this purpose, we first established a stable cell line of CHO cells expressing the rat NTS2 receptor (CHO/rNTS2 cells). RT-PCR analysis confirmed that these cells did express the NTS2 receptor, to the exclusion of the NTS1. Accordingly, \(^{125}\text{I}\)-NT was found to bind to these cells with a pharmacology characteristic of that of NTS2, both in terms of affinity for NT and of relative affinity for the NT analogs levocabastine, NN and SR48692 (Chalon et al., 1996; Mazella et al., 1996; Botto et al., 1998; Vita et al., 1998; Sarret et al., 2002).

We then tested the effects of NT and of various NT analogs on the mobilization of [Ca\(^{2+}\)]\(_i\) in these transfected cells. As previously reported for cortical cerebellar neurons endogenously expressing the rat NTS2 receptor (Sarret et al., 2002), or for transfected CHO cells expressing
the human NTS2 receptor (Vita et al., 1998), neither NT nor levocabastine or NN affected Ca\(^{2+}\) mobilization in CHO/rNTS2 cells. By contrast, incubation with the NTS1 antagonist, SR48692 caused a marked elevation of intracellular calcium in the same cells. This increase conformed to earlier reports of SR48692-induced Ca\(^{2+}\) mobilization in CHO cells transfected with either human (Vita et al., 1998) or rat (Yamada et al., 1998) NTS2 receptors. However, whereas in these previous studies the effects of SR48692 were antagonized by concomitant administration of an excess of NT, NN or levocabastine and could not be elicited in non-transfected cells, in the present study, the effects of SR48692 were not blocked by NT, NN or levocabastine and were equally strong in non-transfected cells, suggesting that they were not NTS2-mediated. Likewise, in rat cerebellar granule cells, SR48692 induced a robust [Ca\(^{2+}\)]\(_i\) increase that was unaffected by concomitant application of NT or levocabastine and was therefore interpreted as being NTS2-independent (Sarret et al., 2002).

We then sought to determine if as in rat cerebellar granule cells (Sarret et al., 2002), NT activated extracellular signal-regulated kinases (ERK1/2) in transfected CHO/rNTS2 cells. Application of 100 nM NT to CHO/rNTS2 cells induced a robust, dose-dependent increase in ERK1/2 phosphorylation. This activation was rapid and sustained over 60 min. It also was NTS2-mediated, since it could not be elicited in non-transfected cells. The similarity of these findings with those obtained in neurons in culture (Sarret et al., 2002) suggests that the observed activation is physiological and not due to artifactitious coupling of the receptor subsequent to its aberrant expression in CHO cells.

Levels of ERK1/2 activation comparable to those obtained after stimulation with NT were achieved by incubating CHO/rNTS2 cells with either NN or levocabastine. That these two drugs would display effects comparable to those of NT is congruent with results in *Xenopus*.
oocytes, which showed that NT, NN, and levocabastine all stimulated to the same extent an
NTS2-mediated Ca\textsuperscript{2+}-activated inward Cl\textsuperscript{-} current (Mazella et al., 1996; Botto et al., 1997;
Dubuc et al., 1999b). However, the present results differ from those of Vita et al. (1998), who
found no effect of NT, NN or levocabastine on ERK1/2 activation in CHO cells transfected with
the human NTS2 receptor. To determine whether this discrepancy was due to species
differences, we repeated the experiments in CHO cells transfected with hNTS2 in lieu of rNTS2.
Our results showed the same NTS2-mediated activation of ERK1/2 phosphorylation in cells
transfected with the human plasmid as in cells transfected with the rNTS2, suggesting that the
differences between the present and earlier results are not due to differences between rat and
human NTS2, but rather to variations in the sensitivity of the methods employed for the detection
of ERK1/2 phosphorylation.

Stimulation with the NTS1 antagonist, SR48692 also resulted in a marked increase in
ERK1/2 activation in both CHO/rNTS2 and CHO/hNTS2 cells. Unlike the effects of SR48692
on Ca\textsuperscript{2+} mobilization, these effects were NTS2-mediated since they were not observed in non-
transfected CHO cells. Previous studies have reported on the agonistic properties of SR48692 on
both rodent (Botto et al., 1997; Yamada et al., 1998) and human (Vita et al., 1998) NTS2.
Surprising here was the fact that although SR48692 displayed a much lower affinity than NT,
NN or levocabastine for the NTS2 receptor (this study and Gully et al., 1993; Mazella et al.,
1996; Botto et al., 1998; Vita et al., 1998; Yamada et al., 1998; Nouel et al., 1999; Richard et
al., 2001; Sarret et al., 2002), it induced ERK1/2 phosphorylation much more efficiently (~7-fold
more efficient than NT in cells transfected with the rat receptor). To determine whether this
discrepancy could be explained by the binding of SR48692 to a site distinct from the target of
NT or its analogs, we repeated the SR48692 stimulation experiments in the presence of 100 nM
NT. Despite its higher affinity for the receptor, NT had no competitive inhibiting effect on the SR48692-induced ERK1/2 activation, suggesting that the two drugs interact with different binding pockets, as they do on the NTS1 receptor (Labbé-Jullié et al., 1995; see also Barroso et al., 2000).

Immunofluorescent studies confirmed that stimulation of CHO/rNTS2 cells with SR48692 produced a robust increase in phosphorylated ERK1/2 levels. Furthermore, they demonstrated that this increase mainly occurred in the nucleus, suggesting that some of the targets of activated ERK1/2 may be transcription factors such as Elk-1, Ets, Stat1/3, or cMyc/N-Myc and, by extension, that activation of the NTS2 receptor results in the modulation of gene expression.

It was recently shown that in COS-7 cells transfected with the human NTS2 receptor, the receptor was constitutively active and that NT and levocabastine behave as neutral antagonist and inverse agonist, respectively, on the production of inositol phosphate (Richards et al. 2001). The present NT- or levocabastine-induced effects on ERK1/2 phosphorylation are unlikely to be likewise due to neutral antagonistic or inverse agonistic properties of the drugs since there was no evidence of constitutive NTS2 receptor activity in our system. Indeed, no difference was observed between the basal phosphorylation level of ERK1/2 in CHO/rNTS2 and in non-transfected CHO cells. Furthermore, had NT or levocabastine acted as inverse agonists, they should not, as they did, have increased phosphorylation of ERK1/2 to levels higher than those measured in non-transfected cells.

As previously demonstrated for mouse and human NTS2 receptors in transfected cells (Botto et al., 1998; Martin et al., 2002b), and for rat NTS2 receptors in cerebellar granule cells (Sarret et al., 2002), stimulation of rat NTS2 receptors heterologously expressed in CHO cells
resulted in a ligand-induced internalization of receptor-ligand complexes. This effect was inhibited by the endocytosis inhibitor phenylarsine oxide, suggesting that it was clathrin-mediated, as documented for most GPCRs (Kranenburg et al., 1999; Pierce et al., 2000; Miller and Lefkowitz, 2001; Claing et al., 2002). That a fluorescent analog of NT was able to induce NTS2 internalization further argues in favor of its playing an agonistic role at the NTS2 receptor.

It is now well documented that seven transmembrane domain/G-protein-coupled receptors may activate ERK1/2 via G protein-independent mechanisms, involving interaction of the receptor with endocytic proteins such as dynamin (Kranenburg et al., 1999; Pierce et al., 2000) and β-arrestins (Miller and Lefkowitz, 2001; Claing et al., 2002). In the present study, we found that blocking receptor internalization with phenylarsine oxide or monodansylcadaverine completely impaired the ability of NT, as well as of all other NTS2 agonists tested, to activate ERK1/2 in CHO/rNTS2 cells. Furthermore, overexpression of DynK44A, a dominant negative mutant form of dynamin 1, was found to selectively inhibit SR48692-induced ERK1/2 activation in cells dually expressing the NTS2 and the dominant negative mutant. These results strongly suggest that the NTS2-mediated activation of the MAP kinase pathway is predicated on the internalization of receptor-ligand complexes via a dynamin-dependent, and Gi protein-independent, mechanism.

In conclusion, the present results reveal that NT, as well as many of the known NTS2 receptor ligands act as agonists at this site, at least as pertains to promotion of ERK1/2 phosphorylation. This finding is important in that it lends further support to the premise that NT is an endogenous ligand at this receptor. It also suggests that NTS2-acting NT analogs may constitute a promising new class of non-opioid analgesic drugs provided that these drugs do not, as does SR48692, exert other actions (e.g. NTS1 antagonism and NTS2-independent induction of
Ca^{2+} mobilization). Indeed, recent studies have demonstrated that NT, but not SR48692 (Dubuc
et al., 1994), induces antinociceptive effects in the mouse, through its interaction with NTS2 as
well as with NTS1 receptors (Dubuc et al., 1999a; Dubuc et al., 1999b; Tyler et al., 1999;
Pettibone et al, 2002; Yamauchi et al., 2003). An intriguing observation is that the sustained,
NTS2-mediated activation of ERK1/2 documented here appears to be exerted to the exclusion of
other signaling systems. Thus, stimulation of NTS2 does not appear to induce Ca^{2+} mobilization
(this study and Sarret et al., 2002), nor was it shown to induce cAMP or cGMP production
(Chalon et al., 1996; Botto et al., 1998). Further studies will obviously be needed to determine
how diverse NTS2-mediated signals truly are and whether some account, in contrast to those
reported here, for short-term NT signaling.
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Figure legends

Figure 1. Expression and binding properties of the rNTS2 receptor transfected in CHO cells

A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis of non-transfected CHO cells (CHO/K1) and of CHO cells transfected with the rat NTS2 receptor cDNA (CHO/rNTS2). In CHO/K1 cells, a single band, of the size expected for the NTS3 receptor fragment (425 bp), is visible. By contrast, in CHO/rNTS2 cells, a band of the size of the rNTS2 receptor fragment (620 bp) is amplified in addition to rNTS3. Neither cell type expressed the rNTS1 receptor (expected size of the rNTS1 receptor fragment is 336 bp).

B: Competition inhibition of $^{125}$I-NT (2 nM) binding to CHO/rNTS2 cells by neurotensin (●), levocabastine (○), neuromedin N (▽), or SR48692 (▼). Each point is the mean of two separate experiments performed in triplicate (mean ± S.D.).

Figure 2. Intracellular Ca$^{2+}$ mobilization in fluo-4-loaded CHO cells

A, B and C: Application of 1 µM SR48692 to CHO/rNTS2 cells induces an increase, followed by a plateau, in intracellular Ca$^{2+}$. By contrast, no increase in intracellular Ca$^{2+}$ is observed following 1 µM neurotensin (NT), 1 µM levocabastine (Levo) nor 1 µM neuromedin N (NN). Neither are any of these drugs able to prevent the Ca$^{2+}$ mobilization effect of SR48692. D: The same type of SR48692-induced response is observed in CHO/K1 cells (non-transfected cells). The curves represent the means of n responding cells and are representative of 5 experiments.
Figure 3. Neurotensin activation of ERK1/2 in rNTS2-transfected CHO cells

CHO/rNTS2 (A, B, D) and CHO/K1 (C) cells were stimulated with various concentration of NT (D) for 0-60 min (A, B, C) and ERK1/2 phosphorylation levels were determined as described in Material and Methods. Upper panels of A, C and D, phosphorylated ERK1/2; lower panels, total ERK1/2. A: Stimulation of CHO/rNTS2 cells with 100 nM NT induces a rapid and sustained activation of ERK1/2. B: Densitometric measurements of ERK1/2 activation (phosphorylated ERK1/2 over total ERK1/2) expressed as fold increase over control ± SEM (○, CHO/K1, n = 3 and ■, CHO/rNTS2, n = 4). (ANOVA, Dunnett’s *, p<0.05; **, p<0.02; ***, p<0.01 as compared with control, untreated cells). C: Stimulation of wild-type, non-transfected CHO cells, with 100 nM NT has no effect on ERK1/2 phosphorylation. D: Dose-dependent activation of ERK1/2 following application of 10−9-10−6 M NT during 5 min (representative of 2 independent experiments).

Figure 4. Effect of neurotensin, levocabastine, neuromedin N and SR48692 on ERK1/2 phosphorylation in CHO/rNTS2

CHO/rNTS2 (A, B) and CHO/K1 (C) cells were treated or not for 5 min with a battery of NTS2 agonists and harvested for determination of ERK1/2 phosphorylation levels as described in Material and Methods. Neurotensin (NT, 100 nM), levocabastine (Levo, 100 nM), neuromedin N (NN, 100 nM) and SR48692 (100 nM) all induce ERK1/2 phosphorylation in CHO/rNTS2 cells (A), but not in CHO/K1 cells (C). Note that ERK1/2 phosphorylation levels are markedly higher in cells stimulated with SR48692 than with other drugs, an effect that is not modified by coincubation with NT (100 nM). B: Densitometric measurements of ERK1/2 activation
(phosphorylated ERK1/2 over total ERK1/2) expressed as fold increase over control ± SEM (n = 3). (ANOVA, Dunnett’s *, p<0.1; **, p<0.05 as compared with control, untreated cells).

Figure 5. Effect of neurotensin, levocabastine and SR48692 on ERK1/2 phosphorylation in CHO/hNTS2

CHO/hNTS2 cells were treated or not for 0-60 min with 100 nM NT (A) or for 5 min with a battery of NTS2 agonists (B) and harvested for determination of ERK1/2 phosphorylation levels as described in Material and Methods. C: Densitometric measurements of ERK1/2 activation expressed as fold increase over control ± SEM (n = 5-7). (ANOVA, Dunnett’s *, p<0.001 as compared with control, untreated cells). Neurotensin (NT, 100 nM), levocabastine (Levo, 1 µM) and SR48692 (100 nM) all induce ERK1/2 phosphorylation in CHO/hNTS2 cells.

Figure 6. Role of NTS2 receptor internalization in ligand-induced ERK1/2 activation

A-C: CHO/rNTS2 cells were incubated with 50 nM Nα-Bodipy-Neurotensin (2-13) (Fluo-NT) for 30 min at 37°C and examined by confocal microscopy. A: Punctate Fluo-NT labeling is evident throughout the cytoplasm of CHO/rNTS2 cells. B: In CHO/rNTS2 cells preincubated with the endocytosis inhibitor phenylarsine oxide (PAO, 10 µM), Fluo-NT labeling is confined to the periphery of the cells. C: Fluo-NT labeling is specific and receptor-mediated since the labeling is completely abolished by an excess of levocabastine (10 µM). Images acquired using the same parameters and representative of 3 different experiments. D-I: CHO/rNTS2 cells transfected with a 10:1 ratio of Dynamin 1 K44A and pEGFP and processed for immunofluorescence detection of phosphorylated ERK1/2. Because of the transfection ratio, most of the EGFP positive cells (in green) can be assumed to express dynamin 1 K44A. D, G:
Basal level of phosphorylated ERK1/2 immunoreactivity in non-treated cells. **E, H:** Following 5 min exposure to 100 nM SR48692, phosphorylated ERK1/2 immunoreactive signal is evident within the nucleus of a subpopulation of NTS2-expressing cells. **F, I:** In merged images of phosphorylated ERK1/2- and EGFP-labeled fields, all EGFP-positive (e.g. DynK44A-expressing) cells (white arrows), are phosphorylated ERK1/2-immunonegative, indicating that internalization blockade prevents ERK1/2 activation. Representative of 3 different experiments.

**Figure 7. ERK1/2 activation requires internalization of the NTS2 receptor**

**A:** *Western Blot analysis.* In CHO/rNTS2 cells, 30 min preincubation with phenylarsine oxide (PAO, 10 µM) or monodansylcadaverine (MDC, 400 µM), two endocytosis inhibitors, prevents neurotensin- (NT), levocabastine- (Levo) and SR48692-induced ERK1/2 phosphorylation (5 min of stimulation). Immunoblots are representative of 3 different experiments.

**B:** *Immunofluorescence labeling.* Immunofluorescence labeling of phosphorylated ERK1/2 in rNTS2-transfected CHO cells treated (100 nM SR48692) or not (control) during 5 min is prevented by a 30 min preincubation with phenylarsine oxide (PAO, 10 µM). Note that ERK1/2 phosphorylation is evident in all cells and that phosphorylated ERK1/2 preferentially accumulates into the nucleus (arrowheads). All images were acquired using the same parameters and are representative of 2 different experiments.
Figure 1

A

[Image of gel electrophoresis showing bands at 620, 425, and 336 bp for CHO/K1 and CHO/rNTS2]

B

[Graph showing % 125I-NT specific binding vs. Log [peptide], M]

CHO/K1 CHO/rNTS2

Std

% 125I-NT specific binding

Log [peptide], M

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Figure 3

A

B

C

D

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Figure 4

A

B

C
Figure 5

A. NT 10^{-7} M

B. Control, NT 10^{-7} M, Levo 10^{-6} M, SR48692 10^{-7} M

C. Fold increase (over control)
Figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>NT 10^{-7} M</th>
<th>Levo 10^{-7} M</th>
<th>SR48692 10^{-7} M</th>
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<tr>
<td>PAO 10 \mu M</td>
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<td>MDC 400 \mu M</td>
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B

- Control
- SR48692
- SR48692 + PAO

pp44^{mapk} pp42^{mapk}