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Low-Affinity Neurotensin Receptor (NTS2) Signaling: Internalization-Dependent Activation of Extracellular Signal-Regulated Kinases 1/2

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ABSTRACT

The role and signaling properties of the low-affinity neurotensin receptor (NTS2) are still controversial. 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 Chinese hamster ovary (CHO) cells expressing the rat NTS2 receptor, neurotensin (NT), levocabastine, neuromedin N, and the high-affinity NT receptor antagonist SR48692 [2-{[1-(-7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid] 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 1/2 (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, because 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 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.

Neurotensin (NT) is a tridecapeptide that exerts neuromodulatory functions in the central nervous system 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 central nervous system disorders, including Parkinson's disease and schizophrenia (for review, see Kitabgi et al., 1989; Binder et al., 2001; Kinkead and Nemeroff, 2002). In addition, NT injection in the 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

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ABBREVIATIONS: NT, neurotensin; ERK1/2, extracellular signal-regulated kinases 1/2; SR48692, 2-{[1-(-7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carbonyl]amino}adamantane-2-carboxylic acid; Levo, levocabastine; CHO, Chinese hamster ovary; COS, *Cercopithecus aethiops*; NN, neuromedin N; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; G-418, geneticin; MDC, monodansylcadaverine; PAO, phenylarsine oxide; PTX, pertussis toxin; PVDF, polyvinylidene difluoride; Fluo-NT, *N*α-BODIPY-neurotensin-(2-13); PCR, polymerase chain reaction; BSA, bovine serum albumin; bp, base pair(s); PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance; RT-PCR, reverse transcription-polymerase chain reaction; EGFP, enhanced green fluorescent protein.

transmembrane domain/G protein-coupled receptor family (Tanaka et al., 1990; Vita et al., 1993, 1998; Chalon et al., 1996; Mazella et al., 1996), 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 review, see Hermans and Maloteaux, 1998; Vincent et al., 1999). Stimulation of NTS1 also induces the activation of extracellular signal-regulated kinases 1/2 (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, 2000; Portier et al., 1998; Martin et al., 2002a). These effects are selectively blocked by the nonpeptide 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 10-fold lower affinity for NT, but also by its selective recognition of levocabastine, a nonpeptide histamine H_1 receptor antagonist that 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 (IC_{50} = 300 nM) than NTS1 (IC_{50} = 5.6 nM) for the SR48692 compound (Gully et al., 1993); however, the pharmacological and signaling properties of NTS2 are still extremely controversial. 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 classic second messenger systems, such as phosphoinositide hydrolysis, Ca^{2+} mobilization, or ERK1/2 phosphorylation (Vita et al., 1998). Furthermore, in transfected CHO and COS-7 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 laevis* oocytes expressing the mouse NTS2 receptor, NT, neuromedin N (NN), and levocabastine were all found to activate Ca^{2+}-dependent chloride currents (Mazella et al., 1996). In addition, 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 human embryonic kidney 293 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 caused by species differences between the two receptors or by variations in receptor coupling as a result of the cell type in which the receptor is expressed. In view of the growing evidence for a role of NTS2 (Dubuc et al., 1999a,b; 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 nonopioid analgesic drugs, the need for precise knowledge of the pharmacological and signaling properties of this receptor seems 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.

Materials and Methods

Materials. The chemicals used in the present study were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM) and F-12 medium, fetal bovine serum (FBS), glutamine, G-418, gentamicin, and LipofectAMINE were from Invitrogen (Carlsbad, CA); NT, monodansylcadaverine (MDC), phenylarsine oxide (PAO), sodium orthovanadate (Na_3VO_4), pertussis toxin (PTX), and staurosporine were from Sigma-Aldrich (St. Louis, MO); anti-phosphorylated ERK1/2 and anti-ERK1/2 antibodies were from New England Biolabs (Beverly, MA); horseradish peroxidase-conjugated anti-rabbit antibodies and the enhanced chemiluminescence detection system were from Amersham Biosciences Inc. (Piscataway, NJ); Complete protease inhibitor and polyvinylidene difluoride (PVDF) membranes were from Roche (Montreal, QC, Canada); NN was from Bachem California (Torrance, CA). Levocabastine and SR48692 were kindly provided by Janssen Pharmaceuticals (Antwerp, Belgium) and Sanofi Synthelabo (Toulouse, France), respectively. All other chemicals were of grade A purity.

Transfection of CHO Cells. CHO/K1 cells were cultured in DMEM/F-12 medium mixture (1:1) supplemented with 10% FBS and 50 mg/l gentamicin at 37°C in 75-cm^2 Falcon flasks in a humidified atmosphere of 95% air and 5% CO_2. For transfection, CHO/K1 cells were grown to subconfluence (70–80%) in 24-well Petri dishes and incubated for 4 h at 37°C in 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/F-12 medium, and the cells were transferred 36 h later to a 75-cm^2 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 *N*α-BODIPY-neurotensin-(2-13) (Fluo-NT) as described below. CHO/rNTS2 clone no. 16 and CHO/hNTS2 clone no. 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, Mississauga, ON, Canada) and submitted to reverse transcription (reverse transcription system kit; Promega, Madison, WI) 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 $MgCl_2$, 0.1% Triton X-100, 0.02% BSA, 200 μM dNTPs, and 0.5 units of *Taq* DNA polymerase) containing 100 ng of either one of the following three 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′-ACTTGT-ATTTCTCCCAGGCTG-3′) provided for the amplification of a fragment of rNTS2 receptor cDNA with a predicted size of 620 bp. 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 s, followed by 34 cycles at 95°C for 40 s, 54°C for 35 s, 72°C for 45 s, 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/F-12 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 $MgCl_2$, and 20 mM HEPES, 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}–10^{-5} M) of nonradioactive NT, levocabastine, NN, or SR48692. Cells were then washed twice with Earle's buffer and harvested in 1 ml of 0.1 M NaOH, and the radioactivity content was measured in a γ 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/acetoxymethyl ester (Molecular Probes, Eugene, OR) 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 $MgCl_2 \cdot 6H_2O$, and 10 mM HEPES, pH 7.35) at 37°C for 30 min to allow the acetoxymethyl ester form to be hydrolyzed. The coverslips were then mounted on the stage of a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, NY), 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 the 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 s using a CoolSnap$_{fx}$ charge-coupled device camera (Roper Scientific, Trenton, NJ) cooled at −35°C. Additional experiments were carried out using 0.01% DMSO in PBS-HEPES alone to test for possible nonspecific 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 Corporation, Downingtown, PA). Each Ca^{2+} curve in Fig 2 represents the average response of n cells.

Western Blotting Analyses of ERK1/2 Activity. CHO/rNTS2, CHO/hNTS2, and CHO/K1 cells were grown for 3 days in DMEM/F-12 medium containing 10% FBS, starved in serum-free DMEM for 1 h, and then stimulated for various time intervals (1–60 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) for 30 min or with PTX (G_i protein inhibitor) for 18 h (100 ng/ml) before stimulation with NT or NT analogs. The reaction was stopped by aspiration of the medium and the addition of ice-cold Hanks' 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*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 PVDF membranes as described previously (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 three washes with Tris-buffered saline/Tween 20. Detection of immunoreactive proteins was accomplished using horseradish peroxidase-conjugated anti-rabbit (1:2000) and an enhanced chemiluminescence 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 Scion Image (Scion Corporation, Frederick, MD). The statistical significance of the activation of ERK1/2 in stimulated versus nonstimulated cells was verified using ANOVA, and the p values were obtained from Dunnett's tables.

Binding of 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/F-12 medium containing 10% FBS and stimulated for 30 min at 37°C with 50 nM 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 (Polysciences, Warrington, PA) and examined using the Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) equipped with a Zeiss inverted microscope and a helium/neon laser (543 nm).

Immunofluorescence Studies. CHO/rNTS2 cells were grown on 12-mm glass coverslips for 3 days in DMEM/F-12 medium containing 10% FBS and then starved in serum-free DMEM for 1 h. Cells pretreated or not with PAO (10 μM, 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 the addition of ice-cold Hanks' 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 the Zeiss LSM510 confocal laser-scanning microscope equipped with a Zeiss inverted microscope, an argon laser (488 nm), and a helium/neon 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, Carleton University, Ontario, QC, Canada) and pEGFP-N1 (BD Biosciences, Mississauga, ON, 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 to 30% subconfluence 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 nontransfected CHO cells (CHO/K1) and on CHO cells transfected with 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 nontransfected 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 $[Ca^{2+}]_i$. As seen in Fig. 2, NT, Levo, and NN were ineffective at modifying $[Ca^{2+}]_i$, even when applied at concentrations as high as 1 μM (A, B, and C, respectively). By contrast, SR48692 (1 μM) markedly increased $[Ca^{2+}]_i$. This effect was caused by the drug itself, because it was not observed after the 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. 2, A, B, and C), suggesting that it was not mediated by NTS2. Congruent with this interpretation, SR48692 produced a similar increase in $[Ca^{2+}]_i$ in nontransfected CHO/K1 cells (Fig. 2D).

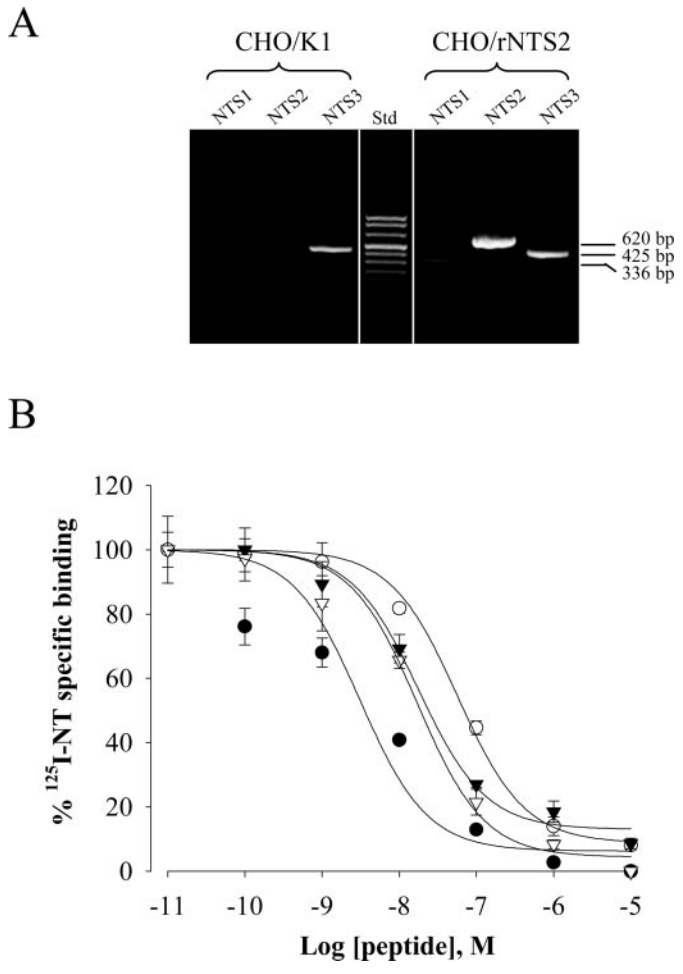

Fig. 1. Expression and binding properties of the rNTS2 receptor transfected in CHO cells. A, RT-PCR analysis of nontransfected CHO cells (CHO/K1) and CHO cells transfected with the rat NTS2 receptor cDNA (CHO/rNTS2). In CHO/K1 cells, a single band of the size expected for the NTS3 receptor fragment (425 bp) is visible. By contrast, in CHO/rNTS2 cells, a band 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.).

NTS2-Mediated ERK1/2 Phosphorylation. 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. 3, A and 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 mediated by NTS2, because it was not observed in nontransfected cells (Fig. 3, B and 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^{-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. 4, A and 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. 4, A and B), suggesting that the two drugs interacted with different sites. Yet the effects of SR48692, as well as those of levocabastine and NN, were mediated by NTS2, because none of these ligands was able to activate ERK1/2 in nontransfected, CHO/K1 cells (Fig. 4C). These effects were also independent from G_i, because 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. 5, A and C, stimulation of CHO/hNTS2 with 10^{-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^{-6} M levocabastine or 10^{-7} M SR48692 also induced a robust activation of ERK1/2 phosphorylation (Fig. 5, B and 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 Fluo-NT for 30 min at 37°C and examined by confocal microscopy. In CHO/rNTS2 cells, Fluo-NT pervaded the cyto-

plasm 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, because nontransfected CHO/K1 cells (not shown) and cells coincubated 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 nonstimulated cells, which were immunonegative (Figs. 6, D and G, and 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 preincubating 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 -nonexpressing cells. Stimulation of these dually transfected cells with 100 nM SR48692 increased phosphorylated ERK1/2 immunofluorescence in approximately 40% of the cells (Fig. 6, E and H), whereas 100% of the cells expressing only the rNTS2 receptor were activated after stimulation with SR48692 (Fig. 7B). This decrease was caused by the overexpression of the dynamin 1 dominant-negative mutant, because none of the cells confirmed to overexpress DynK44A, by virtue of their coexpression of EGFP, showed phosphorylated ERK1/2 immunofluorescence (Fig. 6, F and I, arrows).

Discussion

The present study demonstrates that neurotensin activates the mitogen-activated protein 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 that had reported antagonistic or inverse agonistic effects of these two drugs on the

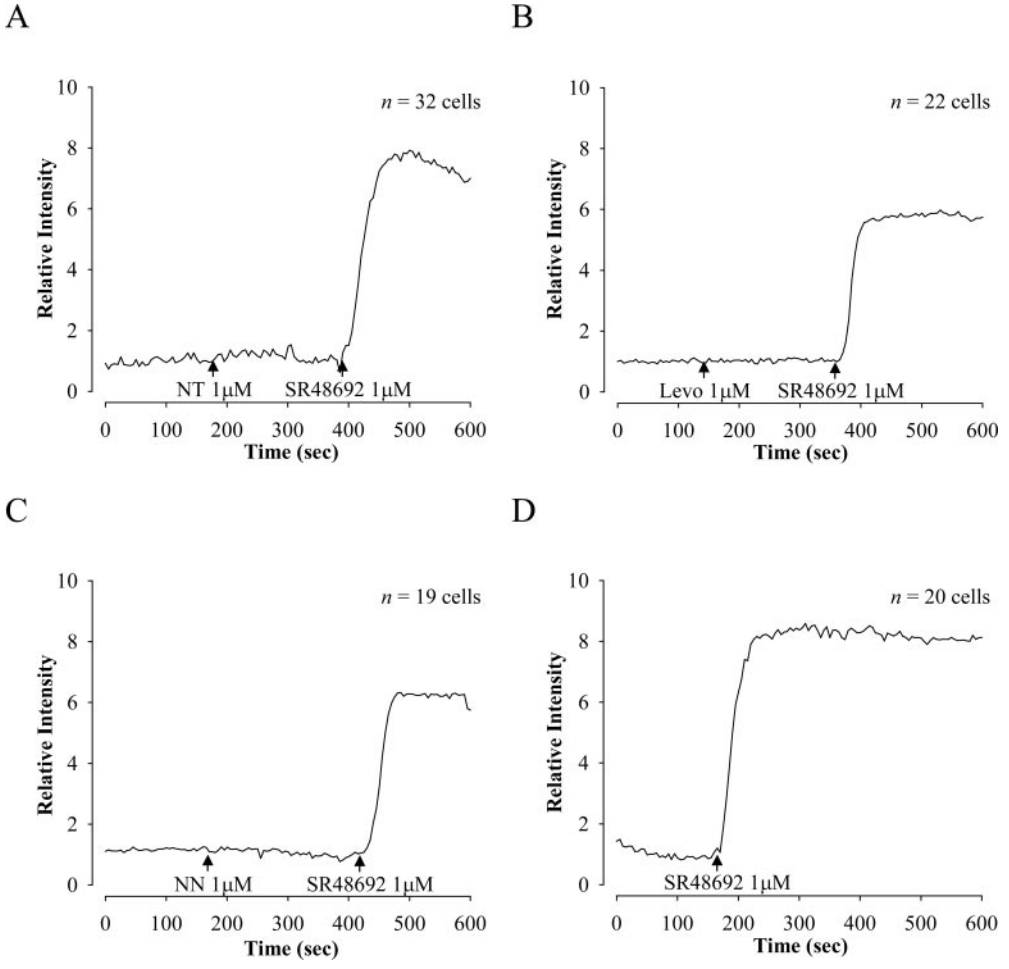

Fig. 2. Intracellular Ca^{2+} mobilization in Fluo-4-loaded CHO cells. A–C, application of 1 μM SR48692 to CHO/rNTS2 cells induces an increase, followed by a plateau, in intracellular Ca^{2+}. By contrast, no increase in intracellular Ca^{2+} is observed after 1 μM NT, 1 μM Levo, or 1 μM NN, nor are any of these drugs able to prevent the Ca^{2+} mobilization effect of SR48692. D, same type of SR48692-induced response observed in CHO/K1 cells (nontransfected cells). The curves represent the means of *n* responding cells and five experiments.

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 caused by species differences or by 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. ^{125}I-NT was accordingly 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, levocabastine, nor 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 nontransfected cells, in the present study, the effects of SR48692 were not blocked by NT, NN, or levocabastine and were equally strong in nontransfected cells, suggesting that they were not mediated by NTS2. 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 whether NT activated ERK1/2 in transfected CHO/rNTS2 cells as in rat cerebellar granule cells (Sarret et al., 2002). 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 mediated by NTS2, because it could not be elicited in nontransfected 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 caused by artifactitious coupling of the receptor subsequent to its aberrant expression in CHO cells.

Levels of ERK1/2 activation comparable with 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 with those of NT is congruent with results in *X. laevis* oocytes, which showed that NT, NN, and levocabastine all stimulated to the same extent an NTS2-mediated Ca^{2+}-activated inward Cl^- 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 related 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

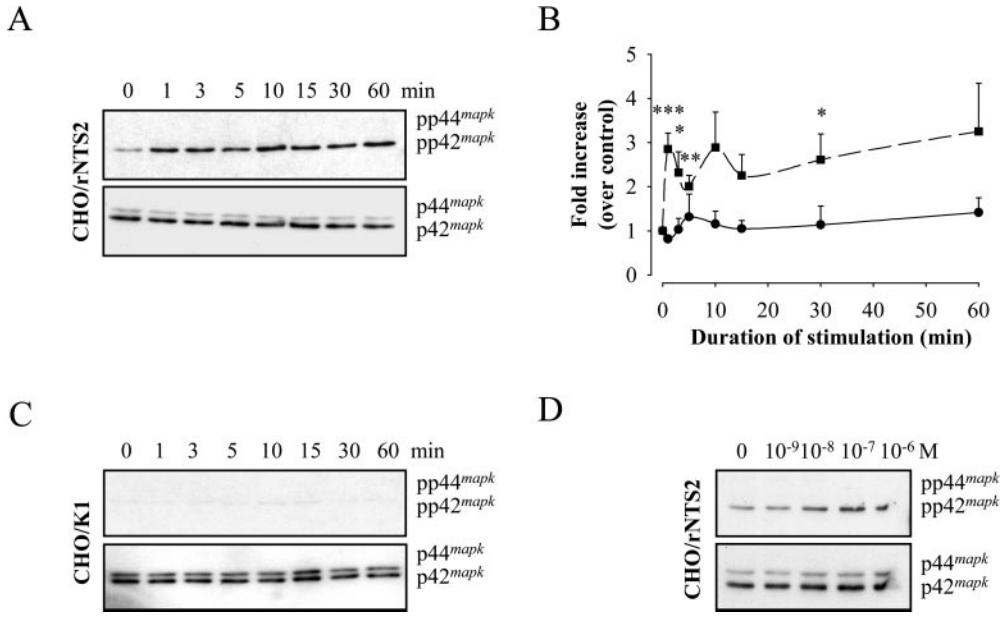

Fig. 3. Neurotensin activation of ERK1/2 in rNTS2-transfected CHO cells. CHO/rNTS2 (A, B, and D) and CHO/K1 (C) cells were stimulated with various concentrations of NT (D) for 0 to 60 min (A–C), and ERK1/2 phosphorylation levels were determined as described under *Materials and Methods*. A, C, and D, top, phosphorylated ERK1/2; bottom, 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 ± S.E.M. (●, CHO/K1, $n = 3$; ■, CHO/rNTS2, $n = 4$) (ANOVA and Dunnett's). *, $p < 0.05$; **, $p < 0.02$; and ***, $p < 0.01$ compared with control, untreated cells. C, stimulation of wild-type, nontransfected CHO cells with 100 nM NT has no effect on ERK1/2 phosphorylation. D, dose-dependent activation of ERK1/2 after application of 10^{-9} to 10^{-6} M NT for 5 min (representative of two independent experiments).

plasmid as in cells transfected with the rNTS2, suggesting that the differences between the present and earlier results are not the result of differences between rat and human NTS2 but rather of 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^{2+} mobilization, these effects were mediated by NTS2, because they were not observed in nontransfected 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 (present study; 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; Barroso et al., 2000).

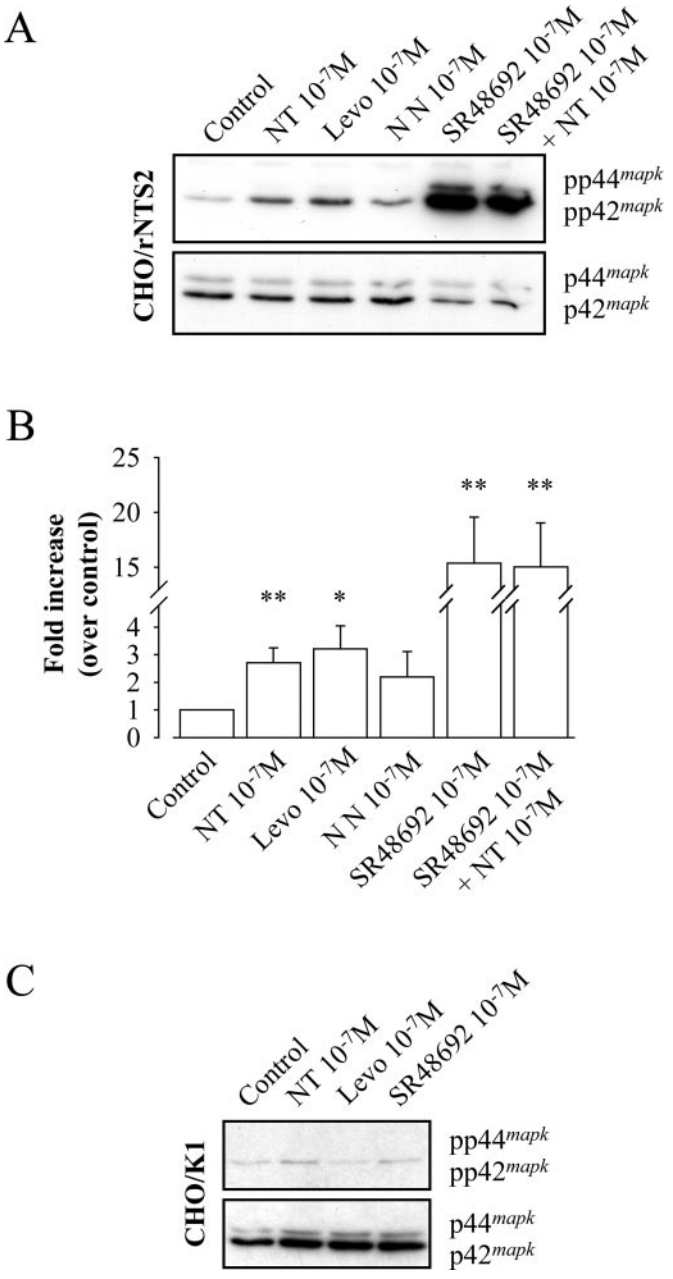

Fig. 4. Effect of neurotensin, levocabastine, neuromedin N, and SR48692 on ERK1/2 phosphorylation in CHO/rNTS2. CHO/rNTS2 (A and B) and CHO/K1 (C) cells were treated or not for 5 min with a battery of NTS2 agonists and harvested for the determination of ERK1/2 phosphorylation levels as described under *Materials and Methods*. NT (100 nM), Levo (100 nM), 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 ± S.E.M. ($n = 3$) (ANOVA and Dunnett's). *, $p < 0.1$ and **, $p < 0.05$ compared with control, untreated cells.

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 c-Myc/N-Myc and, by extension, that the 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 a neutral antagonist and inverse agonist, respectively, on the production of inositol phosphate (Richard et al., 2001). By contrast, the present NT- or levocabastine-induced effects on ERK1/2 phosphorylation are unlikely to be caused by neutral antagonistic or inverse agonistic properties of the drugs, because 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 nontransfected 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 nontransfected 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 mediated by clathrin, as documented for most G protein-coupled receptors (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 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 mitogen-activated protein kinase pathway is predicated on the internalization of receptor-ligand complexes via a dynamin-dependent and G_i 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 the 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 nonopioid 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 interaction with NTS2 as well as with NTS1 receptors (Dubuc et al., 1999a,b; 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 seems to be exerted to the exclusion of other signaling systems. Thus, stimulation of NTS2 does not seem to induce Ca^{2+} mobilization (present study; Sarret et al., 2002) or cAMP or cGMP production (Chalon et al., 1996; Botto et al., 1998). Further studies will obviously be

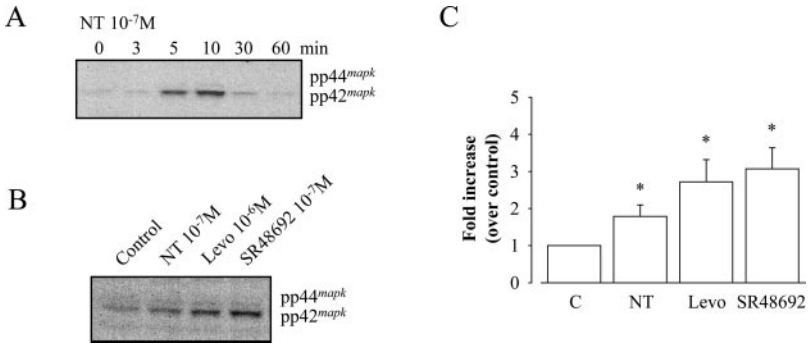

Fig. 5. Effect of neurotensin, levocabastine, and SR48692 on ERK1/2 phosphorylation in CHO/hNTS2 cells. CHO/hNTS2 cells were treated or not for 0 to 60 min with 100 nM NT (A) or for 5 min with a battery of NTS2 agonists (B) and harvested for the determination of ERK1/2 phosphorylation levels as described under *Materials and Methods*. C, densitometric measurements of ERK1/2 activation expressed as -fold increase over control ± S.E.M. (n = 5–7) (ANOVA and Dunnett's). *, $p < 0.001$ compared with control, untreated cells. NT (100 nM), Levo (1 μM), and SR48692 (100 nM) all induce ERK1/2 phosphorylation in CHO/hNTS2 cells.

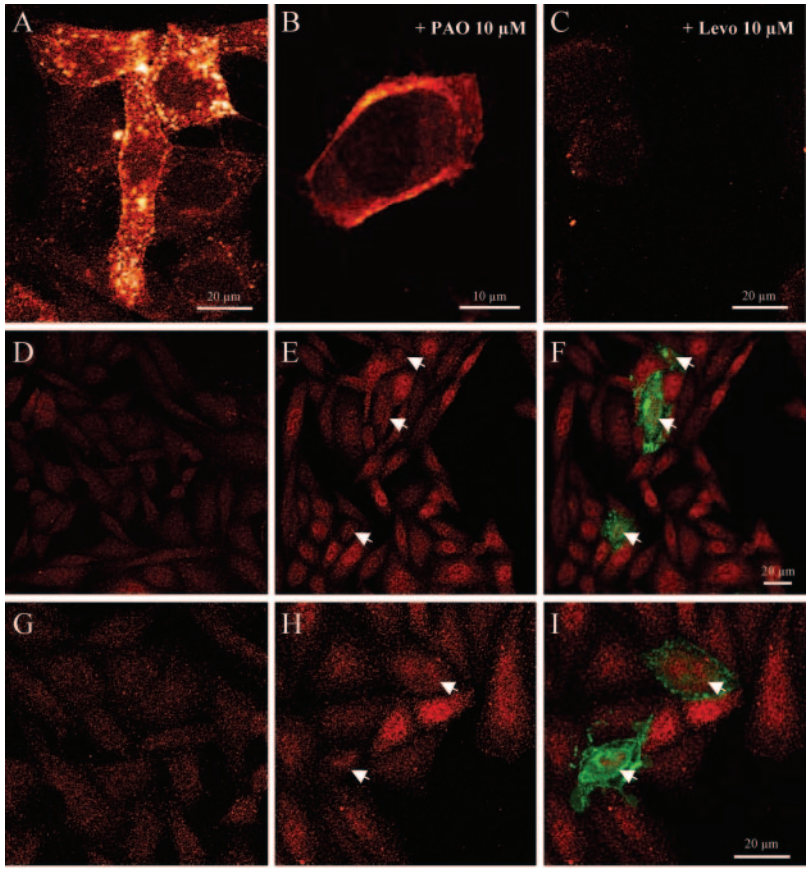

Fig. 6. Role of NTS2 receptor internalization in ligand-induced ERK1/2 activation. A–C, CHO/rNTS2 cells incubated with 50 nM Fluo-NT for 30 min at 37°C and examined by confocal microscopy. A, punctate Fluo-NT labeling is evident throughout the cytoplasm of CHO/rNTS2 cells; B, Fluo-NT labeling is confined to the periphery of the cells in CHO/rNTS2 cells preincubated with the endocytosis inhibitor PAO (10 μM); C, Fluo-NT labeling is specific and receptor-mediated, because the labeling is completely abolished by an excess of levocabastine (10 μM). Images were acquired using the same parameters and represent three different experiments. D–I, CHO/rNTS2 cells transfected with a 10:1 ratio of dynamin 1 (DynK44A) and pEGFP and processed for immunofluorescence detection of phosphorylated ERK1/2. Because of the transfection ratio, most of the EGFP-positive cells (green) can be assumed to express DynK44A. D and G, basal level of phosphorylated ERK1/2 immunoreactivity in nontreated cells. E–H, phosphorylated ERK1/2-immunoreactive signal is evident within the nucleus of a subpopulation of NTS2-expressing cells after 5-min exposure to 100 nM SR48692. F and 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 three different experiments).

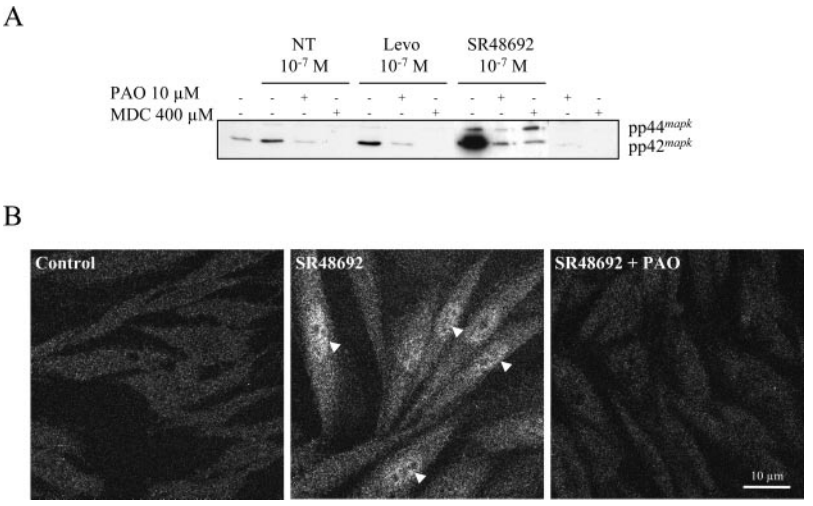

Fig. 7. ERK1/2 activation requires internalization of the NTS2 receptor. A (Western Blot analysis), 30-min preincubation with PAO (10 μM) or MDC (400 μM), two endocytosis inhibitors, prevents NT-, Levo-, and SR48692-induced ERK1/2 phosphorylation (5 min of stimulation) in CHO/rNTS2 cells. Immunoblots represent three different experiments. B (immunofluorescence labeling), 30-min preincubation with PAO (10 μM) prevents immunofluorescence labeling of phosphorylated ERK1/2 in rNTS2-transfected CHO cells treated (100 nM SR48692) for 5 min. 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 represent two different experiments.

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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References

Barroso S, Richard F, Nicolas-Ethève D, Reversat JL, Bernassau JM, Kitabgi P, and Labbé-Jullié C (2000) Identification of residues involved in neurotensin binding and modeling of the agonist binding site in neurotensin receptor 1. *J Biol Chem* **275:**328–336.

Binder EB, Kinkead B, Owens MJ, and Nemeroff CB (2001) Neurotensin and dopamine interactions. *Pharmacol Rev* **53:**453–486.

Botto JM, Chabry J, Sarret P, Vincent JP, and Mazella J (1998) Stable expression of the mouse levocabastine-sensitive neurotensin receptor in HEK 293 cell line: binding properties, photoaffinity labeling and internalization mechanism. *Biochem Biophys Res Commun* **243:**585–590.

Botto JM, Guillemare E, Vincent JP, and Mazella J (1997) Effects of SR 48692 on neurotensin-induced calcium-activated chloride currents in the *X. laevis* oocyte expression system: agonist-like activity on the levocabastine-sensitive neurotensin receptor and absence of antagonist effect on the levocabastine insensitive neurotensin receptor. *Neurosci Lett* **223:**193–196.

Chalon P, Vita N, Kaghad M, Guillemot M, Bonnin J, Delpech B, Le Fur G, Ferrara P, and Caput D (1996) Molecular cloning of a levocabastine-sensitive neurotensin binding site. *FEBS Lett* **386:**91–94.

Claing A, Laporte SA, Caron MG, and Lefkowitz RJ (2002) Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol* **66:**61–79.

Dubuc I, Costentin J, Terranova JP, Barnouin MC, Soubrié P, Le Fur G, Rostène W, and Kitabgi P (1994) The nonpeptide neurotensin antagonist, SR 48692, used as a tool to reveal putative neurotensin receptor subtypes. *Br J Pharmacol* **112:**352–354.

Dubuc I, Remande S, and Costentin J (1999a) The partial agonist properties of levocabastine in neurotensin-induced analgesia. *Eur J Pharmacol* **381:**9–12.

Dubuc I, Sarret P, Labbé-Jullié C, Botto JM, Honoré E, Bourdel E, Martinez J, Costentin J, Vincent JP, Kitabgi P, et al. (1999b) Identification of the receptor subtype involved in the analgesic effect of neurotensin. *J Neurosci* **19:**503–510.

Ehlers RA, Bonnor RM, Wang X, Hellmich MR, and Evers BM (1998) Signal transduction mechanisms in neurotensin-mediated cellular regulation. *Surgery* **124:**239–246; discussion 246–247.

Ehlers RA, Zhang Y, Hellmich MR, and Evers BM (2000) Neurotensin-mediated activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, MIA PaCa-2. *Biochem Biophys Res Commun* **269:**704–708.

Gendron L, Oligny JF, Payet MD, and Gallo-Payet N (2003) Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells. *J Biol Chem* **278:**3606–3614.

Gully D, Canton M, Boigegrain R, Jeanjean F, Molimard JC, Poncelet M, Gueudet C, Heaulme M, Leyris R, Brouard A, et al. (1993) Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proc Natl Acad Sci USA* **90:**65–69.

Hermans E and Maloteaux JM (1998) Mechanisms of regulation of neurotensin receptors. *Pharmacol Ther* **79:**89–104.

Kalivas PW, Gau BA, Nemeroff CB, and Prange AJ Jr (1982) Antinociception after microinjection of neurotensin into the central amygdaloid nucleus of the rat. *Brain Res* **243:**279–286.

Kinkead B and Nemeroff CB (2002) Neurotensin: an endogenous antipsychotic? *Curr Opin Pharmacol* **2:**99–103.

Kitabgi P, Hervé D, Studler JM, Tramu G, Rostène W, and Tassin JP (1989) Neurotensin/dopamine interactions. *Encephale* **15:**91–94.

Kitabgi P, Rostène W, Dussaillant M, Schotte A, Laduron PM, and Vincent JP (1987) Two populations of neurotensin binding sites in murine brain: discrimination by the antihistamine levocabastine reveals markedly different radioautographic distribution. *Eur J Pharmacol* **140:**285–293.

Kranenburg O, Verlaan I, and Moolenaar WH (1999) Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. *J Biol Chem* **274:**35301–35304.

Labbé-Jullié C, Botto JM, Mas MV, Chabry J, Mazella J, Vincent JP, Gully D, Maffrand JP, and Kitabgi P (1995) [^{3}H]-SR48692, the first nonpeptide neurotensin antagonist radioligand: characterization of binding properties and evidence for distinct agonist and antagonist binding domains on the rat neurotensin receptor. *Mol Pharmacol* **47:**1050–1056.

Martin GE, Bacino CB, and Papp NL (1980) Hypothermia elicited by the intracerebral microinjection of neurotensin. *Peptides* **1:**333–339.

Martin S, Navarro V, Vincent JP, and Mazella J (2002a) Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line. *Gastroenterology* **123:**1135–1143.

Martin S, Vincent JP, and Mazella J (2002b) Recycling ability of the mouse and the human neurotensin type 2 receptors depends on a single tyrosine residue. *J Cell Sci* **115:**165–173.

Mazella J, Botto JM, Guillemare E, Coppola T, Sarret P, and Vincent JP (1996) Structure, functional expression and cerebral localization of the levocabastine-sensitive neurotensin/neuromedin N receptor from mouse brain. *J Neurosci* **16:**5613–5620.

Mazella J, Zsürger N, Navarro V, Chabry J, Kaghad M, Caput D, Ferrara P, Vita N, Gully D, Maffrand JP, et al. (1998) The 100-kDa neurotensin receptor is gp95/sortilin, a non-G-protein-coupled receptor. *J Biol Chem* **273:**26273–26276.

Miller WE and Lefkowitz RJ (2001) Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr Opin Cell Biol* **13:**139–145.

Nemeroff CB (1986) The interaction of neurotensin with dopaminergic pathways in the central nervous system: basic neurobiology and implications for the pathogenesis and treatment of schizophrenia. *Psychoneuroendocrinology* **11:**15–37.

Nouel D, Sarret P, Vincent JP, Mazella J, and Beaudet A (1999) Pharmacological, molecular and functional characterization of glial neurotensin receptors. *Neuroscience* **94:**1189–1197.

Petersen CM, Nielsen MS, Nykjaer A, Jacobsen L, Tommerup N, Rasmussen HH, Roigaard H, Gliemann J, Madsen P, and Moestrup SK (1997) Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. *J Biol Chem* **272:**3599–3605.

Pettibone DJ, Hess JF, Hey PJ, Jacobson MA, Leviten M, Lis EV, Mallorga PJ, Pascarella DM, Snyder MA, Williams JB, et al. (2002) The effects of deleting the mouse neurotensin receptor NTR1 on central and peripheral responses to neurotensin. *J Pharmacol Exp Ther* **300:**305–313.

Pierce KL, Maudsley S, Daaka Y, Luttrell LM, and Lefkowitz RJ (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc Natl Acad Sci USA* **97:**1489–1494.

Poinot-Chazel C, Portier M, Bouaboula M, Vita N, Pecceu F, Gully D, Monroe JG, Maffrand JP, Le Fur G, and Casellas P (1996) Activation of mitogen-activated protein kinase couples neurotensin receptor stimulation to induction of the primary response gene Krox-24. *Biochem J* **320 (Pt 1):**145–151.

Portier M, Combes T, Gully D, Maffrand JP, and Casellas P (1998) Neurotensin type 1 receptor-mediated activation of krox24, c-fos and Elk-1: preventing effect of the neurotensin antagonists SR 48692 and SR 142948. *FEBS Lett* **432:**88–93.

Remaury A, Vita N, Gendreau S, Jung M, Arnone M, Poncelet M, Culouscou JM, Le Fur G, Soubrié P, Caput D, et al. (2002) Targeted inactivation of the neurotensin type 1 receptor reveals its role in body temperature control and feeding behavior but not in analgesia. *Brain Res* **953:**63–72.

Richard F, Barroso S, Martinez J, Labbé-Jullié C, and Kitabgi P (2001) Agonism, inverse agonism and neutral antagonism at the constitutively active human neurotensin receptor 2. *Mol Pharmacol* **60:**1392–1398.

Rioux F, Quirion R, St-Pierre S, Regoli D, Jolicoeur FB, Bélanger F, and Barbeau A

(1981) The hypotensive effect of centrally administered neurotensin in rats. *Eur J Pharmacol* **69:**241–247.
Rostène W and Alexander MJ (1997) Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* **18:**115–173.
Sarret P, Gendron L, Kilian P, Nguyen HM, Gallo-Payet N, Payet MD, and Beaudet A (2002) Pharmacology and functional properties of NTS2 neurotensin receptors in cerebellar granule cells. *J Biol Chem* **277:**36233–36243.
Sarret P, Perron A, Stroh T, and Beaudet A (2003) Immunohistochemical distribution of NTS2 neurotensin receptors in the rat central nervous system. *J Comp Neurol* **461:**520–538.
Schotte A, Leysen JE, and Laduron PM (1986) Evidence for a displaceable non-specific [^{3}H]neurotensin binding site in rat brain. *Naunyn Schmiedeberg's Arch Pharmacol* **333:**400–405.
Tanaka K, Masu M, and Nakanishi S (1990) Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* **4:**847–854.
Tyler BM, Jansen K, McCormick DJ, Douglas CL, Boules M, Stewart JA, Zhao L, Lacy B, Cusack B, Fauq A, et al. (1999) Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. *Proc Natl Acad Sci USA* **96:**7053–7058.
Vincent JP (1995) Neurotensin receptors: binding properties, transduction pathways and structure. *Cell Mol Neurobiol* **15:**501–512.
Vincent JP, Mazella J, and Kitabgi P (1999) Neurotensin and neurotensin receptors. *Trends Pharmacol Sci* **20:**302–309.
Vita N, Laurent P, Lefort S, Chalon P, Dumont X, Kaghad M, Gully D, Le Fur G, Ferrara P, and Caput D (1993) Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor. *FEBS Lett* **317:**139–142.
Vita N, Oury-Donat F, Chalon P, Guillemot M, Kaghad M, Bachy A, Thurneyssen O, Garcia S, Poinot-Chazel C, Casellas P, et al. (1998) Neurotensin is an antagonist of the human neurotensin NT2 receptor expressed in Chinese hamster ovary cells. *Eur J Pharmacol* **360:**265–272.
Yamada M, Lombet A, Forgez P, and Rostène W (1998) Distinct functional characteristics of levocabastine sensitive rat neurotensin NT2 receptor expressed in Chinese hamster ovary cells. *Life Sci* **62:**PL375–PL380.
Yamauchi R, Sonoda S, Jinsmaa Y, and Yoshikawa M (2003) Antinociception induced by beta-lactotensin, a neurotensin agonist peptide derived from beta-lactoglobulin, is mediated by NT2 and D1 receptors. *Life Sci* **73:**1917–1923.

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