THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 15, Issue of April 11, pp. 10087–10094, 1997

Printed in U.S.A.

Neurotensin Agonist Induces Differential Regulation of Neurotensin Receptor mRNA

IDENTIFICATION OF DISTINCT TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL MECHANISMS*

(Received for publication, October 11, 1996, and in revised form, January 6, 1997)

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The binding of neurotensin (NT) to specific receptors triggers the multiple functions that NT exerts in both periphery and brain. By studying the effect of the concentration and time of NT agonist exposure, two separate regulatory mechanisms were detected for the neurotensin receptor (NTR) gene in human colonic adenocarcinoma cells (HT-29).

The incubation of cells for 6 h with the NT agonist, JMV 449, resulted in an increase of 270% in NTR mRNA levels. These changes were the direct result of new NTR gene transcription, as indicated by run-on and half-life experiments. In addition, the transcriptional activation of the NTR gene was dependent on NT-receptor complex internalization and *de novo* protein synthesis.

A second response was detected with prolonged exposure to JMV 449. In this case, a decrease of 70% was detected in NTR mRNA levels. Unlike the initial phase, this change was mediated by a post-transcriptional event as the half-life of NTR mRNA from treated cells decreased by 50% as compared with control cells.

NT agonist appears to regulate the synthesis of NTR mRNA. In HT-29 cells, this feedback is exerted by a biphasic response. These phases are apparently independent and mediated by two separate mechanisms.

Neurotensin (NT)[1] is a tridecapeptide, widely distributed in the central nervous system and peripheral tissues, exerting multiple functions (1). In the central nervous system, NT is a neurotransmitter as well as a neuromodulator of other neurotransmitters such as dopamine, acetylcholine, serotonin, and noradrenaline (2, 3). NT also possesses neuroendocrine actions inducing the release of several pituitary hormones (4). In the periphery, NT is secreted from mucosal endocrine cells of the small intestine into the circulation (5). In the gastrointestinal tract, NT causes many physiological effects including the stimulation of pancreatic secretion, the facilitation of colonic motility and fatty acid translocation, and tissue growth (6).

In rat, NT actions are mediated by the stimulation of several specific receptors exhibiting high or low affinity for NT (7, 8). The high affinity neurotensin receptor (NTR) is composed of 424 amino acids and belongs to the seven-transmembrane domain receptor family coupled to the G-proteins (7). The human NTR counterpart has also been cloned from human colonic adenocarcinoma cells (HT-29) (9). When HT-29 cells are challenged with a NT agonist, phosphatidylinositols are hydrolyzed leading to Ca^{2+} mobilization (10). In contrast to N1E-115 cells, stimulation by NT in HT-29 cells is not associated with protein kinase C activation (10, 11).

In addition to triggering cellular responses by specific ligands, receptors are often themselves regulated by their own agonists. In the case of NT, several studies have shown that variations in NTR expression were caused by changes in NT levels. For example, acute agonist stimulation of NTR induces desensitization and down-regulation of receptor in primary cultures of rat forebrain and HT-29 cells (12, 13). Prolonged exposure of N1E-115 cells to NT resulted in the disappearance of most NT-binding sites, and *de novo* synthesis of NTR was required for the recovery of receptor-binding sites and function (14). When hypothalamic neurons from primary cultures were chronically exposed to forskolin and dexamethasone, an increase in NT synthesis and release into the culture media were observed. Concomitantly, a decrease in NT binding and in NTR mRNA levels was observed (15). Moreover, chronic treatment with the NTR-specific antagonist, SR 48692, produced substantial increases in NT-binding sites and in NTR mRNA levels in rat brain (16). These results suggest that endogenous NT may exert a negative control upon its own receptors.

Transcriptional and post-transcriptional regulation mechanisms have been described for several G-protein coupled receptors including, β_2-adrenergic (17), α-adrenergic (18), angiotensin (19), muscarinic (20), and thyrotropin receptors (21). The best described of these receptors is the β_2-adrenergic receptor, which was reported to be down-regulated by long-term agonist exposure via destabilization of its own mRNA (22, 23). In contrast, shorter exposure to agents that elevated cAMP levels resulted in an increase in the transcription rate of the β_2-adrenergic receptor gene (17). However, in another system, a short exposure to a serotoninergic agonist was recently shown to cause the up-regulation of 5-HT_2 receptor mRNA by a post-transcriptional mechanism (24).

The objective of the current study was to investigate the molecular mechanisms of NTR synthesis regulation in HT-29 cells. A time course using different doses of agonist was performed, while applying a quantitative RT-PCR method to measure NTR mRNA levels. We demonstrate that high doses of NT agonist induce a short-term transcriptional up-regulation of NTR mRNA requiring receptor internalization. Furthermore, a post-transcriptional down-regulation of NTR mRNA was detected upon long-term exposure to agonist. This mechanism included the destabilization of NTR mRNA, even at low agonist concentrations.

* This work was supported by a fellowship from the "Ministère de l'enseignement supérieur et de la recherche" (to F. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NT, neurotensin; HT-29, human colon cancer cell line; JMV 449, H-LysΨ(CH_2NH)Lys-Pro-Tyr-Ile-Leu-OH; NTR, neurotensin receptor(s); N1E-115, murine neuroblastoma cell line; PAO, phenylarzine oxide; RT-PCR, reverse transcriptase-polymerase chain reaction; SR 48692, 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricyclo(3.3.1.1.^{3,7})decan-2-carboxylic acid.

EXPERIMENTAL PROCEDURES

Cell Culture—HT-29 human colon adenocarcinoma cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 2 mM glutamine, in a humidified atmosphere of 5% CO_2, 95% air. At confluence, cells were routinely dispersed in trypsin-EDTA and subcultured at a 1:15 dilution. The cells were used for experiments on the fourth day after plating. The media was changed every other day. Cells were treated with 0.3 or 100 nM JMV 449 (Neosystem), a potent and stable pseudopeptide NT agonist (25). These concentrations were chosen because they represent 85 and 100% binding site occupancy, respectively, based on the determined K_i value for JMV 449 of 0.06 nM at 20 °C.[2] For some experiments cells were treated with the NTR antagonist SR 48692, with a K_i value of 24 nM (26).

Binding Studies—Radioligand binding studies were carried out on membranes prepared as described previously in Boudin *et al.* (27). Binding studies were performed as followed, 60 μg of protein was incubated with 0.1 nM ^{125}I-NT in a final volume of 250 μl of buffer A (50 mM Tris, pH 7.4, 0.2% bovine serum albumin, and 0.8 mM 1,10-orthophenanthroline). Nonspecific binding was measured in the presence of 1 μM unlabeled NT. Binding assays were performed for 60 min at 4 °C and terminated by centrifugation at 4 °C for 4 min at 12,000 × *g*. The supernatant was removed, the membrane pellets were rinsed twice with 500 μl of buffer A and centrifuged again. The pellets were counted in a γ-counter (Wallac model 1470 Wizard). The saturation experiments were carried out under the same conditions, using a range of ^{125}I-NT concentrations (0.015–1 nM). The saturation kinetics of ^{125}I-NT binding was analyzed by Scatchard plot and the apparent K_d and B_{max} were estimated.

To verify that JMV 449 was completely washed away before the membrane was prepared, cells were incubated for 30 min on ice with 100 nM JMV 449. Cells were washed three times with cold phosphate-buffered saline, membrane preparation and binding were performed as described previously. Under these conditions, the recovery of binding from cells incubated with JMV 449 was 85 ± 15% compared with the control cells.

RNA Extraction—Total RNA was extracted from cells by the acidic phenol/chloroform guanidine thyocianate method (28). An additional ethanol precipitation was performed in NET buffer (150 mM NaCl, 15 mM Tris-HCl, pH 7.5, 1 mM EDTA). The RNA pellet was resuspended in 50 μl of sterile deionized diethyl pyrocarbonate-treated H_2O. Aliquots were prepared and stored at −80 °C. Total RNA recovery was measured by spectrophotometric absorbance at 260 nm.

Internal Control (cRNA) Preparation—Neurotensin receptor cDNA was kindly supplied by Dr. Nakanishi (Kyoto University, Japan). The plasmid pΔ96 was constructed by deleting a 96-nucleotide fragment (*Hinc*II-*Nco*I) from the rat NTR cDNA (−7 to 1301), which had been previously inserted into the *Sma*I-*Bam*HI site of pT7/T3α18. An oligonucleotide containing poly(dA)$_{45}$ was inserted at the *Sal*I-*Bam*HI site. The internal control used in this study, cRNAΔ96, was prepared by *in vitro* transcription of the linearized plasmid pΔ96 at the *Sal*I site with T7 RNA Polymerase (Life Technologies, Inc.) and then purified on oligo(dT) columns (Sigma) (29). After elution from oligo(dT) columns, the cRNAΔ96 was ethanol precipitated, then diluted in diethyl pyrocarbonate/H_2O containing 1 unit/μl RNasin (Promega). The quality of cRNAΔ96 was checked by electrophoresis (30) and the concentration estimated by spectrophotometric absorbance at 260 nm. The cRNAΔ96 solution was diluted to 1 × 10^7 molecules/μl in diethyl pyrocarbonate/H_2O containing 0.5 unit/μl of RNasin, aliquoted, and stored at −80 °C.

Primer Labeling—Fifty pmol of antisense PCR primer were 5′-^{32}P-end-labeled with 20 units of T4 polynucleotide kinase (New England Biolab, 10,000 units/ml) in a final volume of 50 μl of buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol) containing 100 pmol of [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) at 37 °C, for 30 min. The end-labeled oligonucleotide was subsequently purified on a Sephadex G50–150 spin column (30), and 1 μl of eluent was counted on a GF/C filter (Whatman) in 3 ml of dry extract scintillation fluid (Optiphase 178 HiSafe 178 2, Wallac-Pharmacia).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Quantitative RT-PCR was carried out as in the conditions described by Souazé *et al.* (31). The primer RT-NTR (5′-GCTGACGTAGAAGAG-3′) was used for reverse transcription of endogenous and internal control molecules. The primers S-NTR (5′-CCTTCAAGGCCAAGACCCTC-3′) and AS-NTR (5′-CAGCCAGCAGACCACAAAGG-3′) were used in PCR, giving a PCR product of 349 nucleotides for the internal control, cRNAΔ96, and 433 nucleotides for endogenous NTR mRNA. The assay consisted of two steps. In the first step, the estimation of mRNA molecules in each group was made by a titration assay. A 100 ng of total HT-29 RNA and various dilutions of cRNAΔ96 were reverse transcribed for 1 h at 37 °C with 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies) in a mixture containing 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 unit/μl RNasin, 50 pmol of the specific primer (RT-NTR), and 1 mM of each dNTP in a 30-μl final volume. The reaction was terminated by heating at 95 °C for 5 min and the samples were quick-chilled on ice. The PCR amplification was performed on 1:5 (v/v) of the RT reaction in a mixture containing 16 mM Tris-HCl, pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each dNTP, 25 pmol of each primer (NTR-S and NTR-AS), 1 × 10^6 cpm of a 5′ end-labeled [γ-^{32}P]ATP NTR-AS, and 1 unit of *Taq* polymerase (Perkin Elmer). The amplification profile consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min 30 s. The 26 cycles of PCR were preceded by denaturation at 95 °C for 5 min and were followed by a final extension at 72 °C for 10 min. Amplification was performed in a DNA thermal cycler 480 (Perkin Elmer). In the second step, a precise quantification was performed using the quantitative assay (32). Depending on the level of NTR mRNA, 100 or 500 ng of total RNA was mixed with an exact number of cRNAΔ96 molecules which were previously estimated from the titration assay. This mixture was reverse transcribed and six tubes of a 3-fold dilution of this reaction were amplified by PCR under the same conditions as described above. In all experiments, the difference between the internal control, cRNAΔ96, and NTR mRNA never exceeded 1.5-fold, providing an accuracy of at least 90% (31).

PCR Product Analysis—In both titration and quantitative assays, 20 μl of PCR samples were electrophoresed on 5% polyacrylamide gels in 90 mM Tris borate, 2 mM EDTA buffer. We routinely introduced a 100-base pair DNA ladder (Life Technologies, Inc.) size marker. Gels were stained with ethidium bromide and the bands cut out from the gel and counted in a β-scintillation counter (Beckman, Model LS6000SC) with 3 ml of scintillation fluid. The amount of radioactivity (cpm) recovered from the excised gel bands was plotted against the number of known cRNAΔ96 control molecules or the quantity of total RNA. Linear regressions of both curves were calculated and the absolute number of target molecules (number of NTR mRNA molecules) was estimated by extrapolating the value of 1 μg of total RNA to the internal control. Results are expressed as number of target molecules/μg of total RNA.

Controls—A negative control was routinely introduced for all titration and quantitative assays to confirm the absence of contamination. For these controls, RNA was omitted from the RT reaction mixture and the reverse transcription was carried as described above. The PCR amplification was performed in the same conditions as the samples and the radioactivity present at the equivalent position of the positive band was counted. The radioactivity in this band, guided by ethidium bromide (EtBr) staining, was used as background. The experiment was rejected if the negative control contained visible bands or background greater than 100 cpm. The absence of contaminating DNA in the cRNA preparation was tested by performing a PCR on 1 × 10^7 cRNAΔ96 molecules under standard conditions.

Estimation of mRNA Stability—To estimate the stability of the NTR mRNA, HT-29 cells were exposed to 100 nM JMV 449 for 1, 3, 6, or 72 h before the addition of 5 μg/ml actinomycin D. Total cellular RNA was extracted at each time point and the level of NTR mRNA measured by the quantitative RT-PCR assay.

Nuclear Run-on Assays—Nuclei were isolated according to the alternate protocol described by Greenberg and Bender (33). Isolated nuclei were aliquoted by 8 × 10^7 in 200 μl of glycerol buffer (50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and frozen in liquid nitrogen. To detect nascent transcripts, 200 μl of nuclei preparation in glycerol buffer were added to 200 μl of a reaction buffer containing 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 mM KCl, 5 mM dithiothreitol, 0.5 mM unlabeled GTP, ATP, and CTP each, 1 μM UTP, and 40 μl of [α-^{32}P]UTP (400 Ci/mmol) for 30 min at 30 °C. Twenty nmol of each dNTP was then added to the reaction for 15 min at 30 °C. The transcription mixture was digested with 50 μg of RNase-free DNase followed by 200 μg of proteinase K. Newly transcribed labeled RNA was extracted and subsequently hybridized for 65 h at 45 °C with a fragment (−7 to 1301) of NTR cDNA (3 μg/slot) or α-tubulin (1 μg/slot) immobilized on nitrocellulose. After hybridization, each sample was washed twice with 5 × SSC containing 50% formamide, 0.1% SDS for 45 min at 50 °C and twice with 2 × SSC for 15 min at room temperature. The samples were then treated with 200 μg of RNase A for 45 min at

[2] Dr. P. Kitabgi, personal communication.

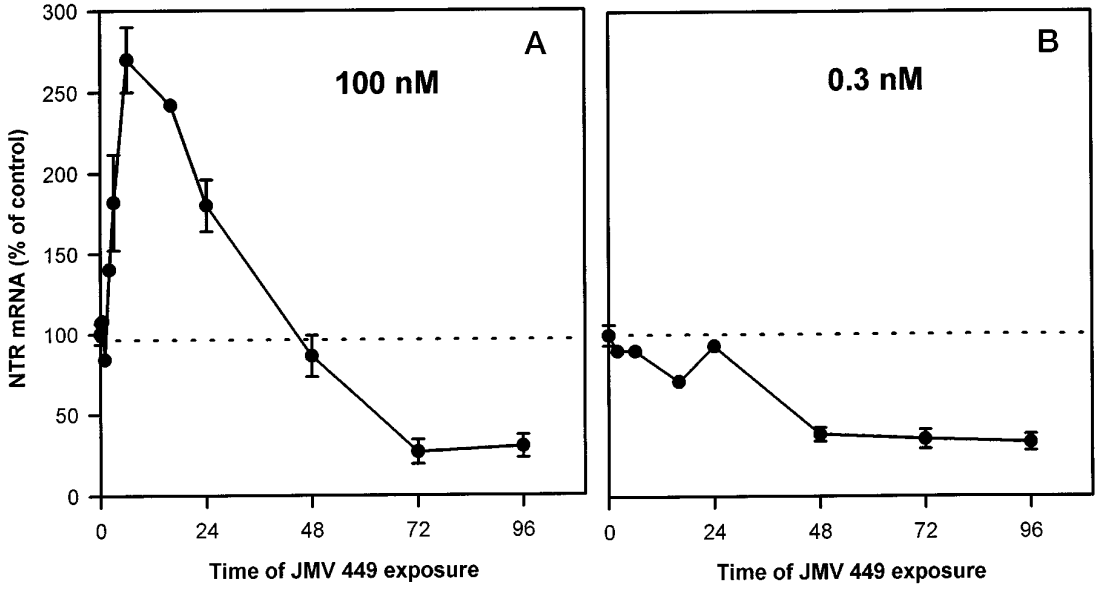

FIG. 1. **Time course of the effect of JMV449 on NTR mRNA levels.** HT-29 cells were treated with either 100 nM (*panel A*) or 0.3 nM (*panel B*) JMV 449 for 1–96 h. Fresh media containing the same treatment was changed every 24 h. The concentration of NTR mRNA fluctuates based on the exposure time to JMV 449. In control cells, NTR mRNA expression is equivalent to $8 \pm 0.5 \times 10^6$ molecules/μg of total RNA.

50 °C, followed by a wash with 1 × SSC containing 0.1% SDS at 50 °C for 30 min.

The filters were dried and subjected to autoradiography for 24 h with an intensifying screen. Relative changes in transcription were assessed from autoradiograms which were analyzed by scanning densitometry using the software program RAG (Biocom France).

Statistics—Statistical analysis were performed using the Student's *t* test. Data are expressed as the mean ± S.E.

RESULTS

NT Receptor mRNA Regulation Is Dependent on Time and Concentration of Agonist Exposure—The number of molecules of NTR mRNA was measured by quantitative RT-PCR as described by Souazé *et al.* (31). Chronic exposure of HT-29 cells to the NT agonist, JMV 449, resulted in a biphasic response as detected by the variations in NTR mRNA. As shown in Fig. 1, the levels of receptor mRNA increased to a maximum of 270% between 6 and 8 h of treatment for cells chronically treated with 100 nM JMV 449. This effect on NTR mRNA was equally observed at 10 nM and 1 μM JMV 449 (data not shown).

NTR mRNA expression returned to control values after 48 h of continuous treatment with 100 nM JMV 449 (Fig. 1*A*). Continued exposure to JMV 449 for up to 96 h caused a decrease of 70% in the quantity of NTR mRNA molecules, as compared with NTR mRNA level observed under basal conditions (Fig. 1*A*). In contrast, when cells were treated with nonsaturating concentrations of JMV 449, 0.3 nM, no changes in receptor mRNA expression was detected during the initial 24 h. However, prolonged exposures between 48 and 96 h resulted in a similar decrease of NTR mRNA, as was seen with treatment at 100 nM (Fig. 1*B*). As shown in Fig. 2, treatment with JMV 449 concentrations as low as 3×10^{-12} M was sufficient to induce the decrease in NTR mRNA levels observed after 72 h of exposure, indicating that this down-regulation can be produced by the activation of a small number of NT-binding sites.

To determine if the two phases of NTR mRNA expression involved independent mechanisms, NTR mRNA augmentation was induced during the down-regulation period. As shown in Table I, the up-regulation observed with 100 nM JMV 449 was still obtained under conditions where maximal NTR mRNA down-regulation was produced. This increase, however, was lower in the pretreated cells (173%) as compared with the non-pretreated cells (270%).

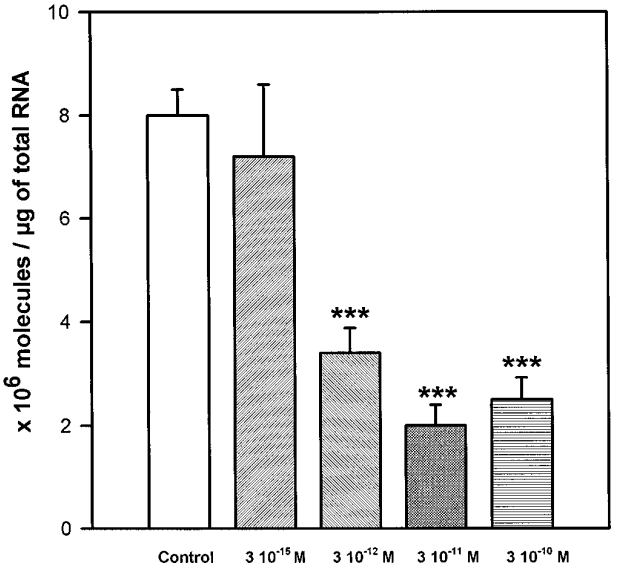

FIG. 2. **Effect of different doses of JMV 449 on NTR mRNA levels.** HT-29 cells were treated for 72 h with increasing concentrations of JMV449 under similar conditions as Fig. 1. There was no significant difference between 3×10^{-10} and 3×10^{-12} M. ***, $p < 0.001$ *versus* control.

TABLE I
Effect of high concentration of JMV 449 on NTR-mRNA levels
Mean values ± S.E. of *n* experiments.

Treatment	10^6 molecules of NTR mRNA/μg of total	Respective control
		%
None	8.0 ± 0.5 (n = 13)	100 ± 6.2
100 nM JMV 449 (6 h)	20.8 ± 1.4 (n = 8)[a]	270 ± 20
0.3 nM JMV 449 (48 h)	2.93 ± 0.33 (n = 3)	100 ± 11.4
0.3 nM JMV 449 (48 h) followed by 100 nM JMV 449 (6 h)	5 ± 0.13 (n = 3)[a]	173 ± 16.8[b]

[a] $p < 0.001$ *versus* respective control.
[b] $p < 0.01$ *versus* % of up-regulation at 100 nM.

To further identify any potential relationship between these apparently separate NTR mRNA responses, cells were concomitantly treated with JMV 449 and the NTR antagonist, SR

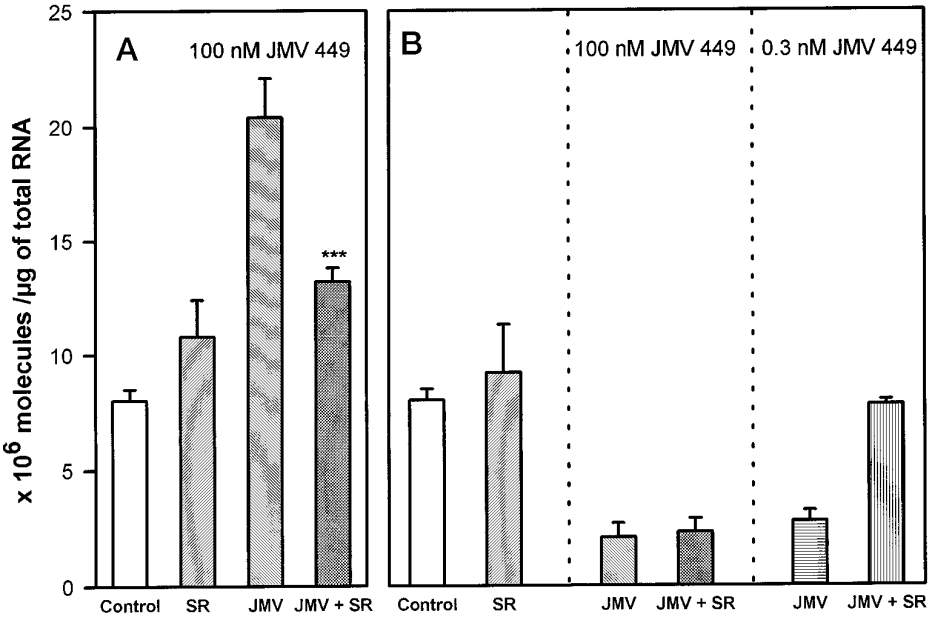

FIG. 3. **Effect of the NTR antagonist, SR 48692, on NTR mRNA variations induced by JMV 449 in HT 29 cells.** Cells were treated with 100 or 0.3 nM JMN 449, 1 μM SR 48692, or both: *panel A* for 6 h, *panel B* for 72 h. Fresh media containing the same treatment was changed every 24 h. ***, $p < 0.001$ relative to JMV 449-treated cells at 6 h.

48692 (26). At a concentration of 100 nM agonist, the NTR mRNA peak habitually detected at 6 h was completely inhibited with 1 μM NTR antagonist (Fig. 3*A*), whereas SR 48692 had no effect on the diminution of NTR mRNA observed at a longer exposure (Fig. 3*B*). These experiments suggest that the NTR mRNA changes are mediated through two different intracellular and independent mechanisms. Nevertheless, as expected according to the difference of K_i of the two components (see "Experimental Procedures"), the decline of NTR mRNA observed with 0.3 nM at 72 h was completely antagonized by 1 μM SR 48692 (Fig. 3*B*). Those results confirmed that the effects caused by JMV 449 treatment act through NTR.

Destabilization of NTR mRNA by Long-term Exposure to JMV 449—NTR mRNA turnover was studied to determine the molecular mechanisms underlying the variations observed in NTR mRNA levels. Transcription was inhibited with actinomycin D in control cells or cells preincubated with agonist for various durations. A similar NTR mRNA half-life was observed in cells treated for 6 h with 100 nM JMV 449 (56.1 ± 6.9 min) and in control cells (58.8 ± 10.8 min). In addition, cells treated with 100 nM JMV 449 also had the same half-life at 1 and 3 h (data not shown). In contrast, pretreatment of cells with 100 nM JMV 449 for 72 h resulted in a rapid decrease in NTR mRNA half-life (24.8 ± 2.2 min). This effect was also observed with 0.3 nM JMV 449 treatment (data not shown). A semi-logarithmic plot of the data revealed that JMV 449 treatment for 72 h decreased the half-life of NTR receptor mRNA by approximately 60% (Fig. 4). Thus, a post-transcriptional event is directly implicated in the down-regulation of the NTR mRNA induced by long-term NT agonist treatment, whereas, mRNA stabilization is not responsible for the NTR mRNA induction.

Transcriptional Activation of the NTR Gene—To confirm this hypothesis, nuclear run-on assays were performed on cells to evaluate the cause of NTR mRNA induction. The transcription rate of control cells was compared with the rate determined from cells pretreated with 100 nM JMV 449 for 4 h. As shown in Fig. 5, a 220% increase of newly synthesized mRNA was detected in JMV 449-treated cells as compared with control cells. Therefore, the increase in NTR mRNA observed after short-term exposure to 100 nM JMV 449 is mediated by changes in the NTR transcription rate.

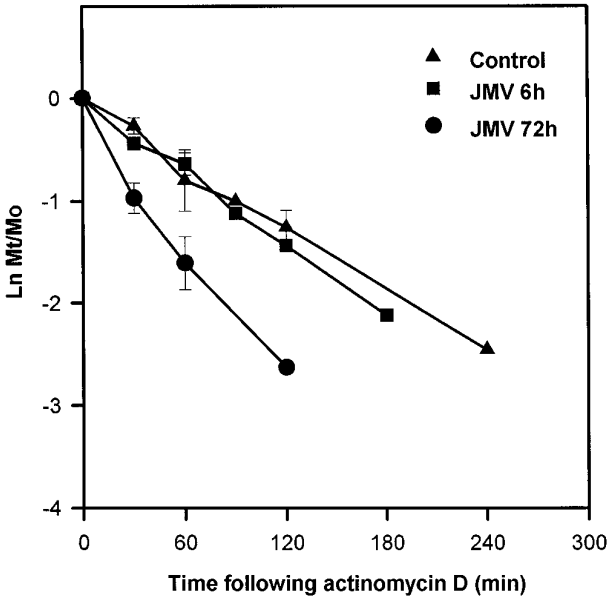

FIG. 4. **Determination of NTR mRNA stability in control and JMV 449-treated cells.** Cells were incubated in the absence (▲) or presence of 100 nM JMV 449 for 6 h (■) or 72 h (●). At each respective time, actinomycin D (5 μg/ml) was added to the culture medium. The cells were further incubated for the specified times, harvested, and total cellular RNA was prepared. The decay of NTR receptor mRNA values was detected by quantitative RT-PCR. Receptor mRNA half-lives were calculated by linear regression of ln(*Mt*/*Mo*) *versus* time, where *Mt* is receptor mRNA levels at a given time after addition of actinomycin D, and *Mo* is receptor mRNA levels at time 0. Values are the mean ± S.E. of two to four separate experiments.

NTR Gene Activation Requires Protein Synthesis—In an effort to further discern the nature of the NTR gene activation caused by treatment with 100 nM JMV 449, HT-29 cells were treated for 3 h with 100 nM JMV 449 in the presence of the protein inhibitor synthesis, cycloheximide. Incubation with 2.5 μg/ml cycloheximide alone resulted in an increase in NTR mRNA levels ($15.2 \times 10^6 \pm 1.3$) equivalent to those caused by incubation with JMV 449 alone ($14.3 \times 10^6 \pm 1.6$). JMV 449 had no effect in the presence of cycloheximide, since cotreat-

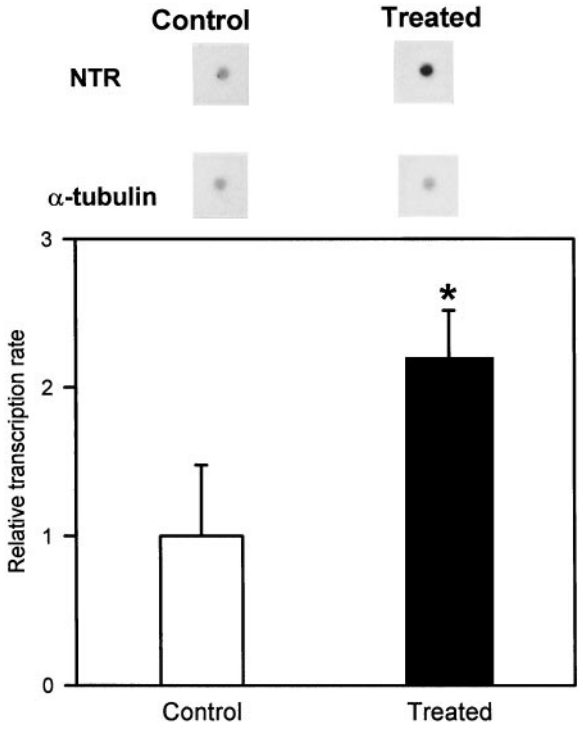

FIG. 5. **Relative transcription rate of the NTR gene as assessed by nuclear run-on transcription assay in control or JMV449-treated cells.** Cells were incubated for 4 h with or without 100 nM JMV 449. Nuclei were prepared and incubated in the presence of 400 μCi of [α-^{32}P]UTP. The [^{32}P]RNAs were isolated and hybridized to nitrocellulose membranes containing 3 μg of NTR cDNA or 1 μg of α-tubulin cDNA. Following autoradiography, the level of the hybridized transcript in each spot was quantified by densitometric scanning. *Top*, a representative autoradiogram of NTR and α-tubulin run-on analysis; *bottom,* quantification of the result, expressed as percentage of control (mean ± S.E. of three independent experiments). *, $p < 0.05$, relative to untreated cells.

TABLE II
Effect of cycloheximide on JMV 449 induced NTR-mRNA levels

HT-29 cells were treated for 3 h with 100 nM JMV 449 and/or 2.5 μg/ml cycloheximide. Values ± S.E. of n experiments.

Treatment	10^6 molecules of NTR mRNA/μg of total RNA
None	8.1 ± 0.5 (n = 4)
JMV 449	14.3 ± 1.6 (n = 4)[a]
Cycloheximide	15.2 ± 1.3 (n = 3)
JMV 449 + cycloheximide	14.3 ± 1.0 (n = 3)

[a] $p < 0.001$ *versus* control (untreated cells).

TABLE III
Effect of PAO and concanavalin A on JMV 449 induced NTR-mRNA levels

HT-29 cells were treated for 3 h with 100 nM JMV 449 and/or 10^{-5} M PAO or 250 μg/ml concanavalin A. Values ± S.E. of n experiments.

Treatment	10^6 molecules of NTR mRNA/μg of total RNA
None	7.2 ± 0.7 (n = 4)
JMV 449	13.7 ± 1.7 (n = 4)[a]
PAO	6.5 ± 0.46 (n = 3)
Concanavalin A	8.4 ± 1.1 (n = 3)
JMV 449 + PAO	6 ± 0.65 (n = 3)
JMV 449 + concanavalin A	7.7 ± 1.3 (n = 3)

[a] $p < 0.001$ *versus* control (untreated cells).

ment with both agents did not result in any further increases in NTR mRNA levels (14.3 10^6 ± 1.0) (Table II). Thus, protein synthesis is required prior to the induction of NTR gene transcription by JMV 449.

NTR Gene Activation Requires Receptor Internalization—It is known that NT induces the internalization of NTR in a number of cell lines including HT-29 (12, 14). Previous experiments have shown that in rat basal forebrain slices and in septal neuroblastoma cells (SN17), NT-NTR internalized complex is transported from the cell periphery to the perinuclear region by endosomes (34, 35). This result suggested that NTR internalization may play a role in NT signaling. To determine if the internalization process was important for NTR gene activation, cells were treated with phenylarzine oxide (PAO) or concanavalin A, components which have previously been shown to inhibit the sequestration of β_2-adrenergic receptors (36, 37). As seen in Table III, concomitant exposure of JMV 449 and PAO or JMV 449 and concanavalin A for 3 h strongly inhibited the transcriptional activation of the NTR gene. The level of NTR mRNA in the presence of these inhibitors was equal to that found in control cells. Interestingly, the steady state level of NTR mRNA was not modified by PAO or concanavalin A treatment. In parallel, it was confirmed that PAO and concanavalin A inhibit NTR internalization by performing the experiments as described by Chabry *et al.* (38). When cells were incubated with 0.1 nM ^{125}I-NT for 30 min, 70 ± 5% of total NT was internalized. When cells were preincubated with PAO 92 ± 1% of the radioactivity remained bound to the membranes and could be completely washed away with phosphate-buffered saline, pH 2.5, indicating that PAO completely inhibited NTR internalization. When the same experiment was repeated with concanavalin A, a similar result was seen. However, only 62 ± 5% of the bound radioactivity could be washed away suggesting that concanavalin A is less effective, compared with PAO, in inhibiting NTR internalization.

Effect of NTR mRNA Variations on NT Receptor Density—To place the functional significance of NTR mRNA variations into context, the NT binding was analyzed. When cells were challenged with 100 nM JMV 449, ^{125}I-NT binding rapidly decreased. This effect was maximal between 1 and 8 h and corresponded to 85% of the control values (Fig. 6*A, inset*). After a prolonged exposure to JMV 449, membranes exhibited a 60% ^{125}I-NT binding recovery after 24 h as compared with the control values (Fig. 6*A*). Saturation experiments carried out with ^{125}I-NT at 4 °C on HT-29 cell membranes demonstrated a single population of high-affinity binding sites, with an apparent dissociation constant (K_d) of 0.70 ± 0.20 nM and a maximal number of sites (B_{max}) of 269 ± 35 fmol/mg of protein (Table IV). These binding characteristics were equivalent to those previously described for the same cell line (10). The reduced binding capacity of the HT-29 membranes caused by a 6-h exposure of 100 nM JMV 449 corresponded to a decrease of 70% in the B_{max} value compared with the control, without any significant changes in the K_d values. The recovery of ^{125}I-NT binding after prolonged treatment with JMV 449 similarly corresponded to an increase of 40% in the number of NTR sites between 6 and 72 h with no change in the affinity for ^{125}I-NT (Table IV). This increase of NT-binding sites immediately ensued the transcription peak (at 6 h) suggesting that the restoration of NT-binding sites is the consequence of *de novo* protein synthesis. However, only a partial restoration of NTR was detected at the cell membrane. This result can be explained by a dynamic situation, where newly synthesized NTR is made available to the cell membrane while the NTRs are internalized due to the continued agonist exposure.

When cells were treated with 0.3 nM JMV 449, the ^{125}I-NT binding profile was altered and significantly shifted in time. A 45% decrease in ^{125}I-NT binding was observed after 1 h of treatment. ^{125}I-NT binding stabilized after 6 h of JMV 449 treatment at 80% of the control value (Fig. 6*B, inset*). This is in

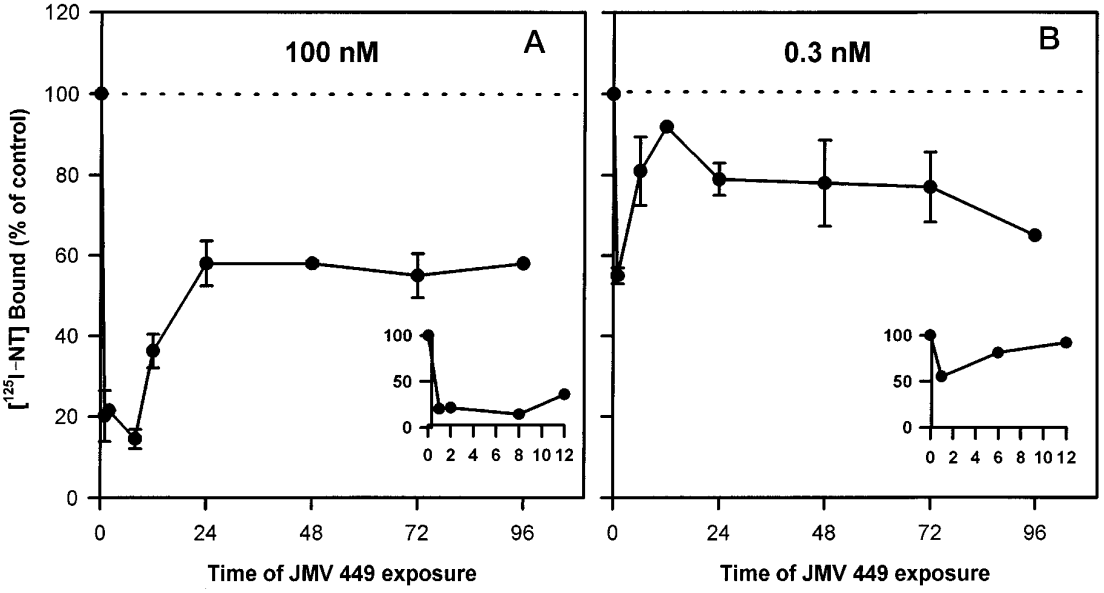

FIG. 6. **Time course of the effect of JMV 449 on ^{125}I-NT binding.** HT-29 cells were exposed to JMV 449, 100 nM (*panel A*) or 0.3 nM (*panel B*) for 1–96 h. Membranes were prepared and ^{125}I-NT binding was performed as described under "Experimental Procedures." Data expressed as percent of control binding in untreated cells are the mean ± S.E. of three to five independent experiments. *Inset*, expanded view of time course for 1–12 h.

TABLE IV
Effect of JMV 449 on ^{125}I-NT specific binding

HT-29 cells were treated for 6 or 72 h at 37 °C with the indicated compound, then assayed for ^{125}I-NT binding on membrane homogenates at 4 °C. Values shown are the mean values ± S.E. of 3 experiments performed in triplicate.

	^{125}I-Neurotensin binding	
	K_d	B_{max}
	nM	*fmol/mg*
Control	0.70 ± 0.20	269 ± 35
100 nM JMV 449 (6 h)	0.80 ± 0.17	82.5 ± 6.5
100 nM JMV 449 (72 h)	0.65 ± 0.12	115 ± 18[a]

[a] $p < 0.05$ *versus* 6 h JMV 449-treated group.

contrast with incubations at 100 nM JMV 449 where HT-29 cells required 24 h for the stabilization of ^{125}I-NT binding.

For prolonged agonist exposures (48–96 h), a decrease of 40 or 20% in NT binding was detected when cells were incubated with either high 100 or 0.3 nM JMV 449 (Fig. 6, *A* and *B*). NTR mRNA destabilization was activated during this period and could explain why the level of receptor at the cell surface remained below control values.

DISCUSSION

In this study we have shown that the binding of NT agonist induced a biphasic response in the regulation of NTR mRNA. Initial exposure to 100 nM agonist generated a large increase in NTR mRNA (~270%). This initial response was optimal at approximately 6 h of agonist exposure, as shown in Fig. 1*A*. Another separate response occurred with continued NT agonist exposure. This response stabilized at 72 h, corresponding to a net decrease of 70% in NTR mRNA quantities.

These two phases were mediated by distinct and apparently independent mechanisms. NTR mRNA induction was the direct result of transcriptional activation as determined by both run-on and half-life experiments (Figs. 4 and 5). The decrease in NTR mRNA is primarily mediated through a post-transcriptional mechanism as determined by the change in its half-life during this second phase. These two phases appear to be independent because it is possible to induce new transcription during the NTR mRNA destabilization phase. Experiments with the NTR-specific antagonist, SR 48692, corroborate this hypothesis because concomitant exposure of 100 nM JMV 449 and SR 48692 resulted in a marked inhibition of NTR mRNA induction. In contrast, the same treatment had no effect on the decrease of NTR mRNA when compared with incubation of JMV alone. These results would suggest that the two types of NTR mRNA responses, detected in this study, originate from the same receptor but require different degrees of stimulation.

It has been previously described that high doses of agonist exposure leads a transient increase in receptor mRNA levels, as is the case for the β_2-adrenergic and 5-HT$_2$ receptors. In both cases the result was due to the transcriptional activation of the cognate receptor gene (17, 41). In the case of NTR mRNA, the activation of NTR gene expression could only be induced with high concentrations of NT agonist. Indeed, the effect occurring at 99% site occupancy (10 nM) was not observed when 83% of NTR sites were occupied (0.3 nM). Apparently, maximal receptor stimulation is required to produce this effect.

The physiological effects produced from the activation of G-protein coupled receptors are the direct result of events ensuing from signal transduction in the second messenger pathways. One important class of responses is the modification of homologous receptor mRNA signals. For example, activation of the cAMP system by agonist exposure induces the stimulation of the β_2-adrenergic receptor transcription rate (17, 39). Likewise, protein kinase C activation resulting from the stimulation of 5-HT$_{2a}$ receptor by serotonin binding is responsible for the stabilization of 5-HT$_{2a}$ receptor mRNA (24). In the case of NTR, gene activation was not detectable at 6 h when cells were treated for 1 h of agonist (data not shown). Therefore, transcriptional activation of the NTR gene did not occur at the outset of agonist binding. These observations connote that second messenger activation by NT agonist is not sufficient to induce NTR transcription activation, and additional events requiring a longer exposure to agonist are implicated.

NTR mRNA up-regulation only occurred after at least 2 h of JMV 449 treatment (Fig. 1). During this period, ^{125}I-neurotensin binding decreased to 20% of the control. Previous results have shown that NTR internalization requires continued exposure to agonist and, in HT-29 cells, internalization is a protracted process since only 25% of NTRs are internalized after 30 min of agonist exposure (13). Moreover, we noticed that

when cells were treated with 0.3 nM JMV 449 the phase corresponding to the stage of receptor internalization is very short (1 h) compared with 100 nM JMV 449 treatment (8 h). The decrease of ^{125}I-neurotensin binding is also less extended, 45% for 0.3 nM *versus* 85% for 100 nM. At a concentration of 0.3 nM JMV 449, NTR gene activation is not observed indicating that a threshold must be reached to turn on the transcription process. Therefore, we were interested to test the hypothesis that the trigger for NTR gene activation was the internalization of NTR, as the time required to generate NTR gene activation corresponded to the delay necessary for total receptor internalization.

Two internalization inhibitors, PAO and concanavalin A, were employed to validate this premise (36, 37). As shown in Table III, a 3-h treatment with either inhibitor completely blocked the increase of NTR mRNA induced by JMV 449. This effect could have been due to the blockade of receptor internalization or to a secondary effect. However, it was previously shown that PAO did not alter the binding characteristics of the NTR receptor (38) and therefore did not affect agonist-receptor interaction. Furthermore, in the case of angiotensin II receptor, the initial phospholipase C-mediated signaling event was not affected by PAO (19), suggesting that this inhibitor does not disturb this early transduction response. In addition, a truncated NTR possessing diminished internalization capacity, maintained the ability to activate phospholipase C (40). The inhibition of NTR gene activation observed with PAO and concanavalin A treatment appeared to be the direct consequence of blocking NTR internalization.

Protein synthesis was blocked with cycloheximide to determine if the factors responsible for NTR gene activation were already present in the cells, or required *de novo* synthesis, when activated by NT agonist treatment. NTR mRNA levels detected after cotreatment with JMV 449 and cycloheximide is in the same range as cells treated with cycloheximide alone, indicating that newly transcribed factors are necessary to activate NTR gene. Similar results have also been noted for the up-regulation of 5-HT$_2$ receptor mRNA caused by agonist treatment in smooth muscle cells (41). Cycloheximide alone also induced an increase in NTR mRNA levels. However, this phenomenon has been previously detected and is believed to occur through the inhibition of the synthesis of labile proteins engaged in the natural turnover of the mRNA (42).

The second phase of NTR mRNA regulation was seen after chronic exposure to agonist and was the result of a post-transcriptional event, since the half-life of NTR mRNA was decreased. Decrease in mRNA stability after prolonged exposure to agonist has already been documented for β_2-adrenergic receptor (23). A 35,000-kDa protein displaying an ARE binding activity of β_1- and β_2-adrenergic receptor mRNA was reported following treatment with β-adrenergic agonist (43). Further studies on proteins responsible for NTR mRNA destabilization would be necessary to determine if mRNA destabilization is a common mechanism to alter NTR mRNA levels after long-term agonist exposure.

Interestingly, we have observed that the decline of NTR was observed at an extremely low concentration of agonist, 3 pM, corresponding to 5% site occupancy. This result implies the existence of a very high affinity site in HT-29 cells which could be localized on the cloned "high NTR affinity site" (9), or in an unknown receptor subtype. In either case, the detection of this low abundant and supposed site are beyond the sensitivity limits of binding experiments and easily explains why this site has never been described. Previous studies from our laboratory showed that *in vivo* and *in vitro*, NT exerts a negative control upon its own receptors (15, 16). A regulatory mechanism such as the destabilization of NTR mRNA, induced by extremely low concentration of agonist, might be expected to have an effect on receptor synthesis *in vivo* where the concentration of agonist is limited by the short half-life of endogenous NT (44). Further characterization of the proteins involved in the degradation of NTR mRNA in HT-29 cells and in the tissue extracts will determine whether this mechanism is pertinent *in vivo*.

In summary, the present study demonstrates that high doses of a NT agonist activate NTR gene transcription, an effect linked to the internalization of the receptor and *de novo* protein synthesis. Long-term agonist exposure induces a post-transcriptional response resulting in the down-regulation of NTR mRNA. A dynamic process can be observed between NTR mRNA and ^{125}I-NT-binding sites because both regulatory events are associated with changes in ^{125}I-NT binding. These regulatory events most likely participate in maintaining a precise level of NTR at the cell surface dependent on the quantity of NT released.

Acknowledgments—We express our many thanks to Dr. Neil Insdorf for his precious help in writing the manuscript and for helpful discussions. We also thank Dr. D. Pélaprat for helpful discussions on binding studies, Dr. D. Gully for providing SR 48692 (Sanofi Recherches), Dr. Christian Gespach for providing the HT-29 cells, and Anne Marie Lhiaubet for providing ^{125}I-neurotensin.

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Neurotensin Agonist Induces Differential Regulation of Neurotensin Receptor mRNA: IDENTIFICATION OF DISTINCT TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL MECHANISMS

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J. Biol. Chem. 1997, 272:10087-10094.
doi: 10.1074/jbc.272.15.10087