Activation of Receptor Gene Transcription Is Required to Maintain Cell Sensitization after Agonist Exposure. STUDY ON NEUROTENSIN RECEPTOR

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Neurotensin (NT) acts through specific G protein-coupled receptors to induce effects in the central nervous system and periphery. In this study we have shown that in the human neuroblastoma cell line CHP 212, an NT agonist, JMV 449, induced high affinity neurotensin receptor (NTR) gene activation. ^{125}I-NT binding of cells challenged with JMV 449 rapidly decreased then reappeared and subsequently stabilized at 50% of the control values after 48 h of agonist exposure. These receptors, which reappeared at the cell surface, are as active as those found in control cells as demonstrated by Ca^{2+} mobilization. Furthermore, the tyrosine hydroxylase (TH) gene, a known NT target gene, remained activated after prolonged NT agonist exposure in this cell line.

In the murine neuroblastoma cell line, N1E-115, NT did not stimulate NTR gene activation but induced NTR mRNA destabilization after long term agonist exposure. In this cell line, NT binding dropped to 15% of control values and remained at this value after agonist treatment. The TH expression, which was originally activated upon NT agonist exposure, decreased to control values after prolonged agonist exposure.

These observations combined with the data obtained from a complementary study with HT-29 cells (Souazé, F., Rostène, W., and Forgez, P. (1997) *J. Biol. Chem.* 272, 10087–10094) revealed the crucial role of agonist-induced receptor gene transcription in the maintenance of cell sensitivity. A model for G protein-coupled receptor regulation induced by prolong and intense agonist stimulation is proposed.

The G protein-coupled receptor constitutes a large family of plasma membrane receptors, representing 1% of the total genome, and are implicated in a diverse variety of cellular functions (1–3). The understanding of how the expression of these receptors are regulated should provide a better comprehension regarding agonist function.

Until now, most studies have reported on the effects of de-

sensitization, receptor coupling, and second messenger stimulation of G protein-coupled receptors (4, 5). To elucidate these mechanisms, experiments were carried out on agonist-deprived cultured cells and then triggered with a rapid burst of high agonist concentrations. Under these conditions, cells would remain desensitized for long periods of time (6, 7). However, *in vivo,* cells are constantly surrounded by agonists, each possessing a characteristic basal level. In the stimulated state, receptors are triggered by "waves" of agonist such as the hormones released into the blood circulation, and the neurotransmitters released into the synaptic cleft, which are more or less intense or frequent.

The complete desensitization of a cell would have grave consequences *in vivo,* because the cell's triggering mechanisms would be essentially inoperable for long periods of time. Rather it would seem required that the cell's machinery would provide a means to remain sensitized to agonist during standard stimulated conditions. By examining the long term effect of agonist exposure on high affinity neurotensin receptor (NTR)[1] expression, we were able to clarify a potential mechanism responsible for maintain of cell sensitization to neurotensin (NT) agonist.

NT acts as a neurotransmitter and a neuromodulator in the central nervous system and as a hormone in the periphery (8–10). The actions of NT are mediated by the stimulation of several specific receptors exhibiting low or high affinity for NT (11). These receptors have recently been cloned and belong to the G protein-coupled receptor family (12, 13). Second messenger pathways activated by NTR have been studied primarily in cultured cell lines expressing NTR. When murine neuroblastoma cells, N1E-115, are challenged with NT, phosphatidylinositols are hydrolyzed leading to Ca^{2+} mobilization and the formation of cyclic GMP (14, 15). In human colonic adenocarcinoma cells (HT-29), NT also stimulates Ca^{2+} mobilization (16). In contrast to N1E-115 cells, stimulation by NT in HT-29 cells is not associated with protein kinase C activation (15, 17).

The mechanistic pathways through which NT acts are still unclear. Nevertheless, some of these pathways which provide the short or long term effects of NT are associated with the transactivation of gene transcription. Among the genes activated by NT through NTR are, c-*fos*, *Zif 268*, *tyrosine hydroxylase* (TH), and *NTR* itself (18–20). The activation of gene

[1] The abbreviations used are: NTR, neurotensin receptor; NT, neurotensin; CHP 212, human neuroblastoma cell line; HT-29, human colon cancer cell line; JMV 449, H-LysΨ(CH$_2$NH)Lys-Pro-Tyr-Ile-Leu-OH; N1E-115, murine neuroblastoma cell line; RT, reverse transcriptase; PCR, 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}; TH, tyrosine hydroxylase; PBS, phosphate-buffered saline.

transcription by NT relies on the presence, the quantity, and the coupling state of NTR at the cell membrane. Several studies have demonstrated that these receptor settings are regulated by the amount and duration to which cells are exposed to NT (16, 19–22). When cells are triggered by high concentrations of agonist, NTR becomes internalized and disappears from the cell surface (16) causing the cells to become desensitized from a few minutes to few hours (16, 20–22).

We have previously demonstrated that agonist-induced NTR expression is regulated at the transcriptional and post-transcriptional level (20) and that these mechanisms act independently of each other. In this study, our objective was to examine the effect of NTR expression variation due to NT agonist on cell sensitivity and on target gene stimulation (*e.g.* the tyrosine hydroxylase gene). For this purpose, two TH-expressing neuroblastoma cell lines, the murine-derived N1E-115 and the human-derived CHP 212, were used. These cell lines have the fortuitous advantage in that each line possesses only one of the two mechanisms previously described by Souazé *et al.* (20). In the present experiments, both cell lines were exposed to high concentrations of NT agonist for short and long term periods. We demonstrate that these cell lines react differently to NT depending on the state of NTR gene activation.

Cells which possess NTR gene activation capability remain sensitized, continue to express TH, and respond to agonist even after prolonged agonist exposure. In contrast, cells possessing only the destabilization mechanism for NTR mRNA, but lacking the transcriptional activation mechanism of NTR, lose their ability to bind NT, become desensitized, and cease to stimulate TH gene. We establish here, that the activation of receptor gene transcription is crucial to maintain cell sensitization. We propose a model of receptor expression regulation induced by intense or prolonged agonist exposure.

EXPERIMENTAL PROCEDURES

Cell Culture—Human (CHP 212) and murine (N1E-115) neuroblastoma cells were grown in Dulbecco's modified Eagle medium/F-12 (Life Technologies, Inc.). All media were supplemented with fetal calf serum (10% for N1E-115 and 15% for CHP 212) and 2 mM glutamine in a humidified atmosphere of 5% CO_2-95% air. To induce the differentiation of N1E-115, cells were incubated in media containing 1.5% FCS and 1% dimethyl sulfoxide for 48 h. Cells were treated with 1 μM of JMV 449 (Neosystem), a potent and stable pseudopeptide NT agonist (23).

Determination of the $[Ca^{2+}]_i$—Determination of intracellular calcium was performed as described previously by Hermans *et al.* (24). Briefly, cells grown on glass coverslips were incubated for 1 h with 5 μM fura-2-AM and then examined using an inverted epifluorescent Nikon microscope. The $[Ca^{2+}]_i$ was estimated from 4–5 cells, excited with the 340/380-nm wavelength pair. Emitted fluorescence intensity was measured at 510 nm.

Animals and Treatments—Adult male Wistar rats (200–250 g) (IFFA Credo, France) were maintained on a 12-h light/12-h dark cycle with lights on at 8:00 a.m. and were given food and water *ad libitum*. Rats were killed by decapitation, and the different brain structures or peripheral tissues were removed and immediately frozen in liquid nitrogen and stored at −80 °C until use. Brain structure dissections were performed according to the stereotaxic atlas of Paxinos and Watson (25). For chronic treatment experiments, animals received a daily intraperitoneal injection of SR 48692 at a dose of 1 mg/kg (Sanofi Recherches, Toulouse, France) or vehicle (1% Tween) for 15 days and were decapitated 24 h after the last injection.

Binding Studies—Radioligand binding studies were carried out either on membranes or on cultured cells. Membranes were prepared as described previously in Boudin *et al.* (26). Binding studies were performed as follows, 50 or 100 μg of protein were 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) for 1 h at room temperature. Binding assays on cultured cells were carried out in 12-well culture plates containing 10^5 cells/well. After the various treatments, the binding procedure was followed as described by Scarcériaux *et al.* (22).

To verify that JMV 449 was completely washed away before the

binding was performed, cells were incubated for 30 min on ice with 1 μM JMV 449. Cells were washed three times with cold phosphate-buffered saline, membrane preparations or binding assays on cultured cells were performed as described above. Under these conditions, the recovery of binding from cells incubated with JMV 449 was 85 ± 8% compared with control cells.

RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted from cells by the acidic phenol/chloroform guanidine thiocyanate method (27). Additional details are described in Refs 20, 28, and 29. Quantitative RT-PCR was carried out according to the conditions previously described in Refs. 28 and 29. Total CHP 212 or N1E-115 RNA was quantified by using cRNAΔ96 (rat NTR coding region deleted by 96 base pairs between the site *Hin*CII-*Nco*I) as internal control, and total rat brain structures and peripheral tissues were quantified by using cRNAΔ34 (rat NTR coding region deleted by 34 base pairs between the site *Nco*I-*Nhe*I) as internal control (28, 29). 0.5–5 μg of the total RNA and various dilutions of internal control were reverse transcribed for 1 h at 37 °C with 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 1 unit/ml of RNasin, 50 pmol of the specific primer (RT-NTR), and 1 mM concentration of each dNTP in a 30-μl final volume. The PCR amplification was performed on 1:5 v/v of the RT reaction in a mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each dNTP, 25 pmol of each primer (NTR-sense and NTR-antisense), 1×10^6 cpm of a 5′-end-labeled [γ-^{32}P]ATP NTR-AS and 1 unit of *Taq* polymerase (Perkin-Elmer Cetus). 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 26–30 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 Cetus). For the quantitative assay, total RNA was mixed with an exact number of internal control molecules, which were previously estimated from the titration assay and reverse transcribed, for details see Refs. 20, 28, 29. A series of 3-fold dilutions from RT reaction were amplified under the same conditions as described above. In all experiments, the difference between the internal control, cRNAΔ96 or cRNAΔ34, and NTR mRNA never exceeded 1.5-fold, providing an accuracy of at least 90% (28).
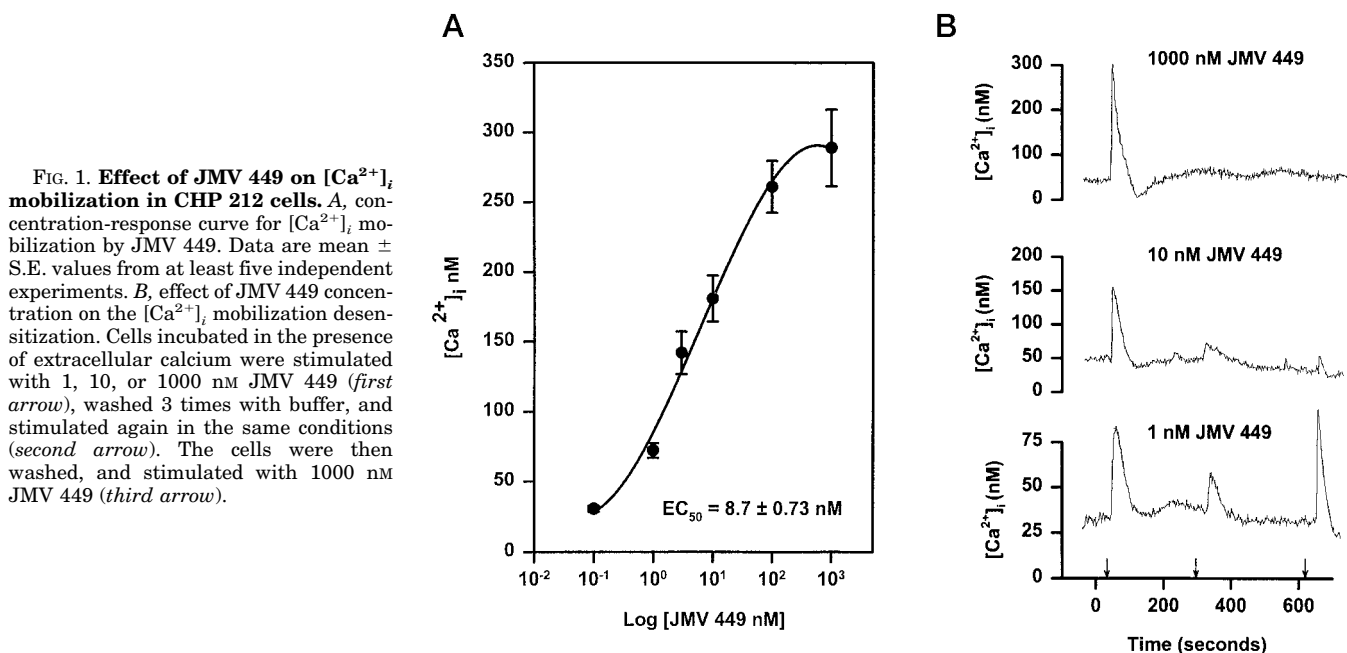
Western Blotting—N1E-115 and CHP 212 cell extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis according to Laemmli procedure (30). After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad) (31). Following electroblotting, nitrocellulose membrane was probed with a polyclonal anti-TH antibody (Jacques Boy, France) for CHP 212 and a monoclonal anti-TH antibody (Boehringer Mannheim) for N1E-115. The antigen-antibody complex was visualized with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies and the ECL system (Amersham Pharmacia Biotech). Relative amounts of proteins were quantified 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

NTR Binding Characteristics in CHP 212 and N1E-115 Cells—Binding studies were performed on membrane homogenates of N1E-115 and CHP 212 using ^{125}I-NT. The two cell lines exhibited a specific and saturable binding. In differentiated N1E-115, Scatchard analysis revealed a K_d value of 66.3 ± 26.2 pM and a B_{max} value of 52.8 ± 7.5 fmol/mg of protein membrane. These results are very similar to those found in the same cell line by Bozou *et al.* (32). Binding characteristics in CHP 212 cells revealed a K_d value of 153 ± 30.6 pM, and a binding capacity value of 15.1 ± 7.6 fmol/mg of membrane was detected. The K_d values were similar to those obtained when Chinese hamster ovary cells were transfected with the human high affinity NTR (33).

NT Agonist Stimulates Intracellular Ca_{2+} Mobilization in CHP 212—In contrast to N1E-115, in which second messenger pathways have been extensively studied, no data are available for the transduction pathways associated with NTR in CHP 212. For this reason, we confirmed that NTR was efficiently coupled to G protein in CHP 212. When CHP 212 cells were

A

B

FIG. 1. **Effect of JMV 449 on $[Ca^{2+}]_i$ mobilization in CHP 212 cells.** *A,* concentration-response curve for $[Ca^{2+}]_i$ mobilization by JMV 449. Data are mean ± S.E. values from at least five independent experiments. *B,* effect of JMV 449 concentration on the $[Ca^{2+}]_i$ mobilization desensitization. Cells incubated in the presence of extracellular calcium were stimulated with 1, 10, or 1000 nM JMV 449 (*first arrow*), washed 3 times with buffer, and stimulated again in the same conditions (*second arrow*). The cells were then washed, and stimulated with 1000 nM JMV 449 (*third arrow*).

triggered with 1 μM of NT agonist, JMV 449, a rapid and large increase in the [Ca2+]$_i$ was observed after 15 s. As shown in Fig. 1*A*, the peak of [Ca2+]$_i$ is concentration dependent. A significant response was detected with 1 nM JMV 449 and a maximal stimulation was observed with 100 nM JMV 449. The EC_{50} value (8.7 ± 0.73 nM) was in the same range as those observed in primary cultured neurons and PC-12 expressing NTR cells (34, 35).

The desensitization of NT to Ca^{2+} mobilization was studied. As shown in Fig. 1*B*, after stimulation with 1000 nM JMV 449, $[Ca^{2+}]_i$ rapidly returns to basal levels. No Ca^{2+} mobilization was seen when cells were washed and exposed to a second JMV 449 stimulation. This desensitization phenomenon is partial, depending on the agonist concentration employed (Fig. 1*B*). Cells first induced with 1 and 10 nM of agonist showed a second $[Ca^{2+}]_i$ peak after an equivalent challenge of agonist (Fig. 1*B*). A further challenge by 1000 nM of agonist did not elicit any response in those cells already treated with medium to high concentrations of agonist (10 and 1000 nM). Cells initially treated with 1 nM of agonist were responsive to this challenge. The desensitization of NTR is usually observed in cell lines that naturally express NTR in primary cell culture (34).

NTR mRNA Regulation Induced by Agonist Treatment Is Distinct in Both Neuroblastoma Cell Lines—The level of NTR mRNA was studied after short term (5 h) and long term (72 h) NT agonist treatment (1 μM JMV 449). The number of molecules of NTR mRNA was measured by quantitative RT-PCR as described by Souazé *et al.* (28). The basal level of NTR mRNA in both cells is very low, 204,000 ± 33,000 molecules/μg of total RNA in N1E-115, and 263,000 ± 58,000 molecules/μg of total RNA in CHP 212. Exposure of CHP 212 cells to JMV 449 resulted in a large increase (232%) of NTR mRNA after 5 h of treatment. This increase (281%) persisted after prolonged exposure with JMV 449 (Fig. 2). In N1E-115 the NTR mRNA regulation was very distinct. As shown in Fig. 2, no changes in NTR mRNA levels were detected after 5 h of treatment. A prolonged treatment (72 h) showed a decrease of 39% of NTR mRNA level.

To determine whether the decrease in NTR mRNA observed in N1E-115 cells was induced via the second messenger pathway, cells were treated with 1 μM JMV 449 for 0.5, 1, and 2 h, washed three times with PBS, and incubated with fresh me-
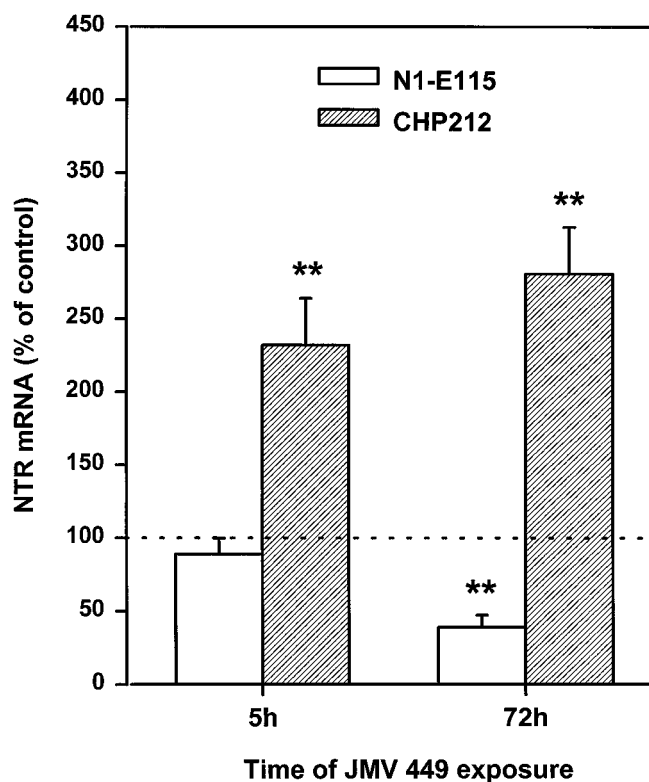

FIG. 2. **Effect of JMV449 on NTR mRNA levels.** N1E-115 or CHP 212 cells were treated with 1 μM JMV 449 for 5 or 72 h. Fresh medium containing the same treatment was renewed every 24 h. Values are the mean ± S.E. of four separate experiments. **, $p < 0.01$ relative to control.

dium for 48 h. Under these conditions, 30 min of JMV 449 treatment is sufficient to induce 48 ± 5.5% decrease of NTR mRNA 48 h later (Fig. 3). This finding indicates that the decrease of NTR mRNA levels after prolonged agonist treatment is the result of early cellular events occurring after the stimulation of the N1E-115 by NT agonist.

Mechanisms Underlying NTR mRNA Regulation Is Transcriptional in CHP 212 and Post-transcriptional in N1E-115—
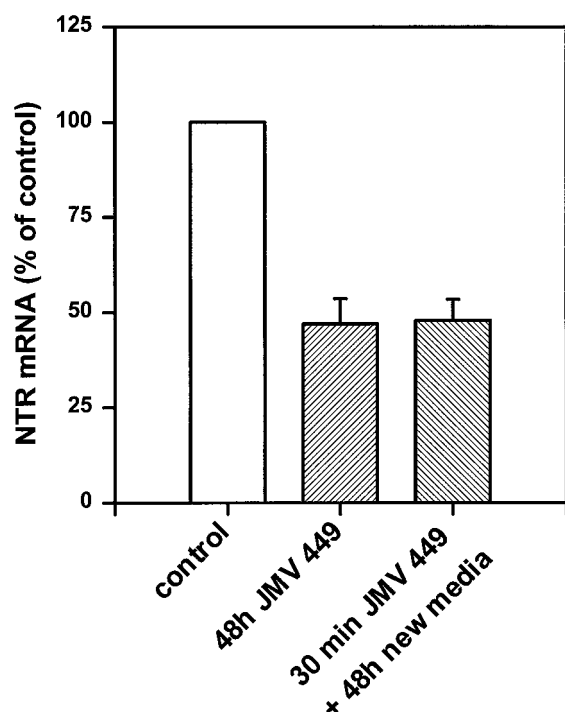
FIG. 3. **Effect of a brief exposure to JMV 449 on NTR mRNA level.** N1E-115 cells were treated for 48 h or 30 min with 1 μM JMV 449, then new medium was added after washing 3 times with PBS. Values are the mean $\pm$ S.E. of three separate experiments.
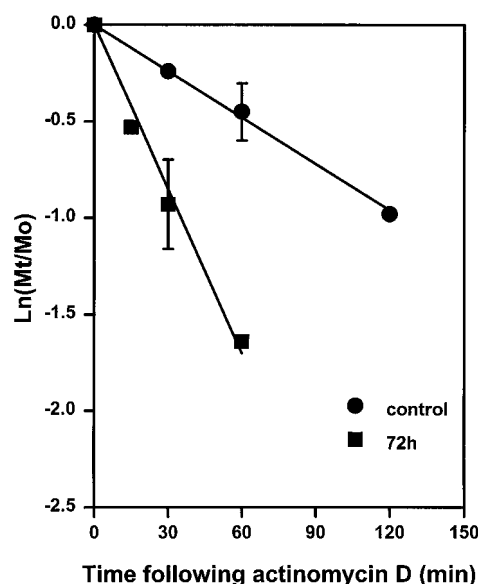

FIG. 4. **Determination of NTR mRNA stability in control and NT-agonist treated N1E-115 cells.** Cells were incubated in the absence ( ) or presence of 1 μM JMV 449 for 72 h (■). At each respective time point, actinomycin D (5 μg/ml) was added to the culture medium. The cells were further incubated for the indicated times, harvested, and total cellular RNA was prepared. The decay of NT 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 = receptor mRNA levels at given time after addition of actinomycin D, and Mo = receptor mRNA levels at time 0. Values are the mean $\pm$ S.E. of two to four separate experiments.

NTR mRNA turnover was studied to determine the molecular mechanisms underlying the variations observed in NTR mRNA levels. For this purpose, transcription was inhibited with actinomycin D in control cells or in cells pretreated with JMV 449. As shown in Fig. 4, the half-life of NTR mRNA in N1E-115 was reduced by approximately 4 times when cells were treated for 72 h with JMV 449. This result indicates that post-transcriptional events are directly implicated in the down-regulation of NTR mRNA. In contrast, in CHP 212 a similar half-life was displayed in control cells and cells treated with JMV 449 for 5 h and 72 h (Table I). This result suggests that NTR mRNA stabilization was not responsible for NTR mRNA induction. The extremely low expression of NTR in CHP 212 limits the feasibility of a nuclear run-on study. Therefore, we could not directly demonstrate that the induction of NTR mRNA is because of the activation of the NTR gene. Nevertheless, concomitant treatment with JMV 449 and actinomycin D for 5 h prevents the increase of NTR mRNA levels indicating that a transcriptional event initiates the induction of NTR mRNA (control cells treated with actinomycin D 9,329 $\pm$ 1,832 molecules, cells treated with JMV 449 for 5 h, and actinomycin D 7,584 $\pm$ 1,887 molecules). We conclude that NTR mRNA is regulated by two distinct molecular mechanisms, transcriptional in CHP 212 and post-transcriptional in N1E-115.

NTR Binding upon Prolonged NT Agonist Treatment Is Different in CHP 212 and N1E-115—The time course of NT agonist treatment was studied on ^{125}I-NT binding in the two cell lines. As shown in Fig. 5A, changes in ^{125}I-NT binding occurred when cells were treated with JMV 449. When both cell lines were challenged with 1 μM JMV 449, ^{125}I-NT binding rapidly decreased. After 2 h of treatment, 90% of ^{125}I-NT binding was lost. Surprisingly, after a prolonged exposure to JMV 449, CHP 212 exhibited a 50% ^{125}I-NT binding recovery, whereas in N1E-115, the ^{125}I-NT binding remained at 10% of the control value. No additional changes in ^{125}I-NT binding were detected after stabilization at 48 h (Fig. 5A). Similar experiments were performed on membrane homogenates and identical results

TABLE I
Effect of JMV 449 on NTR mRNA half-life

For CHP 212, the half life of NTR mRNA was estimated in cells incubated in the absence or in presence of 1 μM JMV 449 for 5 or 72 h. Actinomycin D (5 μg/ml) was added in the medium for 1 hour. The decay of NTR mRNA value was estimated by quantitative RT-PCR. Values shown are the mean $\pm$ S.E. of three separate experiments. For N1E-115 NTR mRNA half-life was calculated from the linear regression extrapolated from the data shown in Fig. 4.

	CHP 212	N1E-115
Control	79 $\pm$ 22	86.5
5 h	77 $\pm$ 6	ND[a]
72 h	73 $\pm$ 10	24.4

[a] ND, not determined.

were observed (data not shown). The loss of ^{125}I-NT binding was also detected when N1E-115 cells were treated with 3 nM JMV 449 or NT (data not shown).

In CHP 212 cells, the 50% ^{125}I-NT binding recovery is not in agreement with the level of NTR mRNA, which is increased approximately two-fold after prolonged JMV 449 exposure (Fig. 2). The degradation rate of JMV 449 is notably lower than NT (36), therefore, the continuous presence of agonist in cell media, even in small amounts, may mask the detection of a receptor exposed at the membrane surface. To answer this question, CHP 212 cells were incubated with 1 μM JMV 449 for 48 h, washed three times with cold PBS, then incubated with fresh media at 37 °C for different periods of time. The result of this experiment is shown in Fig. 5B. The recovery of ^{125}I-NT binding, compared with the control value, is a long process because a 36-h incubation period is necessary to reach the control value. This result suggests that a regulatory mechanism limits the number of receptors exposed at the cell surface. The presence of receptor pools localized in vesicles inside the membrane and not accessible by the agonist was checked. For this purpose, binding experiments were performed on cells treated with 1 μg/ml of the detergent digitonin. As shown in Fig. 5C, the
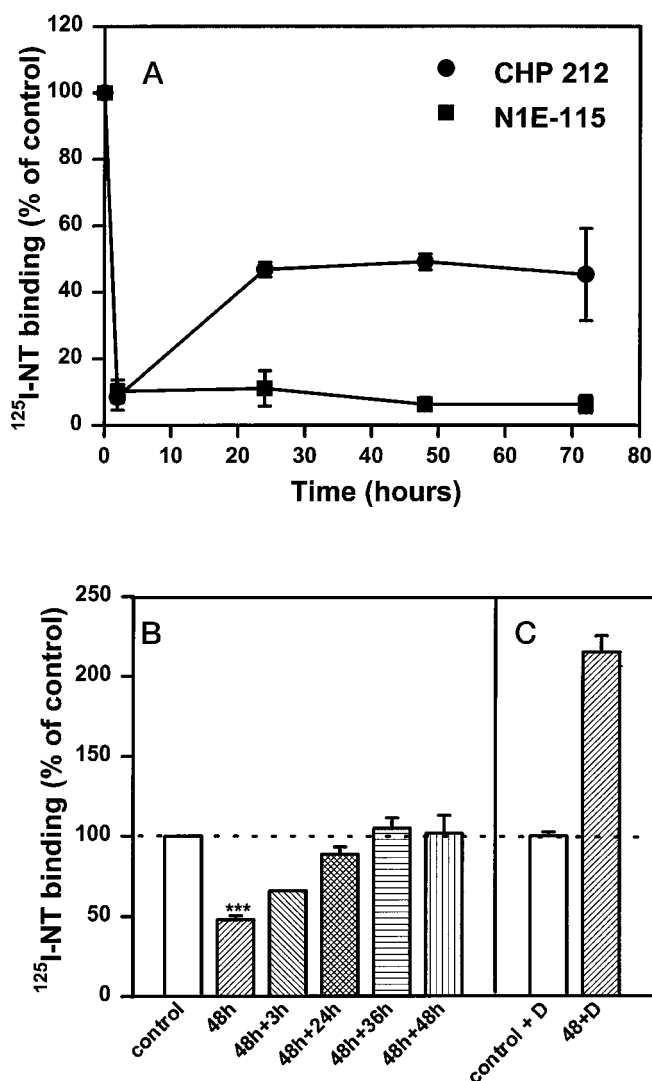
FIG. 5. **Effect of JMV 449 on ^{125}I-NT binding on CHP 212 and N1E-115 cells.** *Panel A,* time course of JMV 449 on ^{125}I-NT binding. CHP 212 or N1E-115 were exposed to 1 μM JMV 449 for 2–72 h. ^{125}I-NT binding was performed as described under "Experimental Procedures." *Panel B,* time course of ^{125}I-NT binding recovery after JMV 449 exposure. CHP 212 cells were treated for 48 h with 1 μM JMV 449, washed 3 times with cold PBS, and incubated for 3, 24, 36, or 48 h with new medium. Binding assays were then performed as described under "Experimental Procedures." *Panel C,* effect of digitonin on ^{125}I-NT binding after JMV 449 exposure in CHP 212. Cells were incubated for 48 h with 1 μM JMV 449, washed 3 times with cold PBS, and the binding assay was performed in the presence of 1 μg/ml of digitonin (*D*). Data, expressed as percent of control binding in untreated cells, are the mean ± S.E. of three to five independent experiments performed on separate cells. ***, $p < 0.001$ relative to control

^{125}I-NT binding of digitonin-treated cells exposed for 48 h with JMV 449 is increased by 200% as compared with digitonin-treated control cells. This result indicates that the NTR mRNA increase is correlated with an increase of NTR synthesis and that the new receptors are inaccessible to the agonist, thus representing a reservoir of receptors.

Effect of NTR Regulation on NT Agonist Stimulates $[Ca^{2+}]_i$ in CHP 212—We verified that the NTR present at the cell surface after 48 h of NT agonist exposure was coupled to G protein and that second messenger can be activated. For this purpose, the ability of cells treated for 48 h with 1 μM JMV 449 to mobilize the $[Ca^{2+}]_i$ was checked. The basal level of $[Ca^{2+}]_i$ was similar in control cells and cells treated 48 h with JMV 449, 95.8 ± 18.4 nM and 108 ± 26.4 nM respectively. When cells

were washed three times with PBS then stimulated with 1 μM JMV 449, a peak of $[Ca^{2+}]_i$ reached 220 ± 60 nM in control cells and 279 ± 51 nM in JMV 449-treated cells. We concluded that prolonged treatment with NT agonist did not alter the coupling of NTR. Even when the ^{125}I-NT binding was reduced by half, the capacity of the cell to mobilize the $[Ca^{2+}]_i$ remained identical.

Tyrosine Hydroxylase Activation by NT Agonist Is Dependent on the Regulation of NTR Binding—We have shown that prolonged exposure to agonist in the two neuroblastoma cell lines induced differential expression of NTR mRNA as well as differences in agonist binding capacity. To evaluate if these findings generate consequences on the expression of a target gene stimulated by the interaction of NT with NTR, expression of TH was studied on both neuroblastoma cell lines after JMV 449 treatment. These results are shown in Fig. 6. As expected, TH is stimulated in both cell lines after 5 h of JMV 449 treatment. When cells were treated with JMV 449 for 72 h, TH returned to control levels in N1E-115 cells and increased up to 200% in CHP 212 cells. These results show that the regulation of TH gene expression, activated by NT though NTR, is dependent on the type of NTR regulatory mechanism present in the cell.

Both Modes of NTR mRNA Regulation Are Present in Vivo—We have previously shown that both types of NTR mRNA regulation, activation of NTR gene transcription and destabilization of NTR mRNA, could be present in the same cell line (20). It could be surmised that *in vivo* the cells possess one or both NTR mRNA regulatory mechanisms. To evaluate the role of NT on NTR expression and on NTR mRNA levels, the level of NTR mRNA was studied after blockade of the neurotensinergic transmission with the NTR-specific antagonist, SR 48692. Chronic treatment for 15 days with this antagonist, which crosses the blood-brain barrier (37), was studied. Changes in NTR mRNA levels in various peripheral and brain tissues were observed. In the brain, two types of responses were detected as shown in Fig. 7. A significant increase of NTR mRNA was detected in the ventral mesencephalon (+77%), the prefrontal cortex (+72%), and the hypothalamus (+54%) as compared with controls. In contrast, no significant changes were detected in the striatum or cerebellum. In peripheral tissues, the colon displayed an up-regulation effect of NTR mRNA similar to the effect found in the ventral mesencephalon, the hypothalamus, and the prefrontal cortex (Fig. 7). A marked reduction of 40 and 30% was detected in the duodenum and the pancreas, respectively. These results indicated that *in vivo* NT regulated the level of NTR mRNA under mechanisms that are similar to those described in cell lines.

DISCUSSION

In this study, we used two neuroblastoma cell lines, N1E-115 and CHP 212, which exhibited different NTR regulation mechanisms induced by agonist exposure. By analyzing the levels of NTR mRNA expression and NT binding capacity during agonist exposure, we established the key role of receptor gene transcription in the maintenance of cell sensitization following long term agonist exposure.

In a previous study, we demonstrated the presence of two distinct modes of NTR expression upon treatment with high concentrations of NT agonist in HT-29 cells. The initial mechanism detectable after 6 h of agonist exposure, resulted in the activation of the NTR gene transcription. The second mechanism could only be detected after prolonged agonist exposure (72 h), and was post-transcriptional, resulting in the destabilization of NTR mRNA (20). Similar results are also found for the β2-adrenergic receptor where a short term agonist exposure stimulated the rate of the receptor gene transcription and long term agonist exposure induced the destabilization of the

FIG. 6. **Effect of JMV 449 on expression of tyrosine hydroxylase.** N1E-115 or CHP 212 cells were treated with 1 μM JMV 449 for 5 or 72 h. Fresh medium containing the same treatment was renewed every 24 h. Expression of TH was estimated from Western blots from cell extracts obtained as described under "Experimental Procedures." Values are the mean ± S.E. of three separate experiments. **, $p < 0.01$ relative to control.
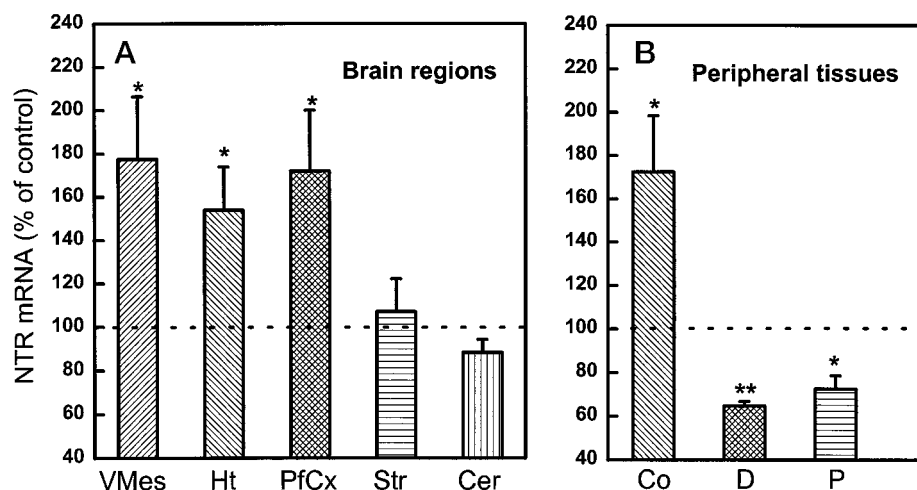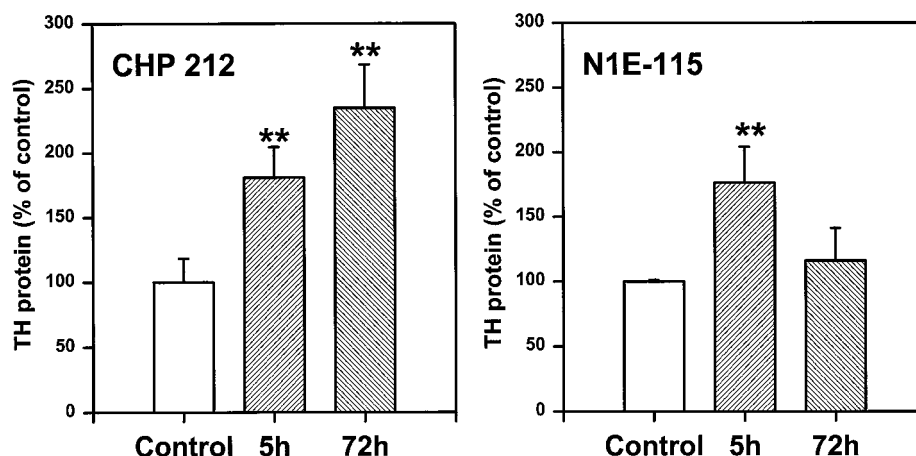

FIG. 7. **Effect of SR 48692 on NTR gene expression.** SR 48692 or 1% Tween was administered intraperitoneally at a daily dose of 1 mg/kg for 15 days. Total RNA was extracted, and NTR mRNA quantities were determined as described under "Experimental Procedures." Data are expressed as a function of controls (100%). *vMes,* ventral mesencephalon; *Ht,* hypothalamus; *pfCx,* prefrontal cortex; *Str,* striatum; *Cer,* cerebellum; *Co,* colon; *D,* duodenum; *P,* pancreas. Confidence levels were determined using the Student's *t*-test; *, $p < 0.05$; **, $p < 0.01$.

receptor mRNA (38, 39). In relation to this observation, one of the two mechanisms are detected independently for other G protein-coupled receptors in different cell lines. Indeed, the activation of receptor gene transcription after high concentration of agonist exposure was detected in the expression of 5-HT2 and β1-adrenergic receptors (40, 41). In a similar vein, receptor RNA destabilization is observed after prolonged agonist exposure in α1-adrenergic and M1 muscarinic receptors (42, 43).

The ability to separate transcriptional up-regulation from post-transcriptional destabilization in HT-29 cells peaked our interest previously, because these experiments suggested that these mechanisms were entirely independent (20). In this paper, we confirm this interpretation because only one of the two mechanisms is present in the two cell lines. In CHP 212, only the activation of the receptor gene transcription mechanism is active, whereas in N1E-115 only the mRNA destabilization mechanism is present. In the latter cell line, only 30 min of agonist exposure was necessary to induced NTR mRNA destabilization at 48 h. This destabilization is therefore an early event occurring after the stimulation of the receptor by agonist, suggesting that it is the result of the activation of the second messenger pathway. In contrast, as described for HT-29 cells, the activation of NTR gene transcription mechanism required a high concentration and duration of agonist exposure, indicating that this reaction is triggered by different physiological pathways (20).

To establish that similar mechanisms exist *in vivo,* the regulation of NTR expression by NT was studied by chronic blockade of the neurotensinergic transmission with an NTR antagonist. This treatment resulted in increases or decreases in NTR

mRNA levels depending on the brain structures or peripheral tissues studied (Fig. 7). These data indicated that, *in vivo,* distinct mechanisms inducing up and down NTR mRNA regulation are present and that they could potentially be the same mechanisms described in the cell lines.

Prolonged agonist exposure produced marked differences in the behavior of NTR in CHP 212 and N1E-115 cell lines. In the cell line capable of agonist-induced NTR gene activation, the binding of NT, after a rapid decline because of the down-regulation process, reappeared and stabilized at 50% of the control value. In contrast, N1E-115 cells lose NT binding capacity long before the RNA destabilization sets in. When both mechanisms are present, as in HT-29 cells, the binding stabilized at 50% of the control value after long term agonist exposure (20). These results strongly suggest that transcriptional gene activation is a required step for the reappearance of a receptor at the cell membrane in CHP 212 cells. Therefore, the lack of transcriptional activity leads to the desensitization of N1E-115 cells.

In CHP 212 cells, the apparent discrepancy between NTR mRNA levels and the NT binding capacity after long term agonist exposure (Figs. 2 and 5A) was noticed. In addition, the restoration of cell binding capacity was required 36 h after removal of agonist from the media and never exceeded control values (Fig. 5B). This discrepancy was because of the storage of NTR in intracellular compartments as confirmed by digitonin treatment of CHP-212 cells (Fig. 5C). Similar intracellular pools of receptors have been reported for the thrombin receptor, which is not recycled after agonist stimulation (5). The presence of these intracellular receptor pools suggested that new receptor molecules were synthesized from the NTR mRNA

FIG. 8. **Proposed model of the chronological events involved in the maintenance of cell sensitization to agonists.** *Left*, basal level of agonist; *right*, stimulated state.

▲ Agonist
Rnase
Receptor
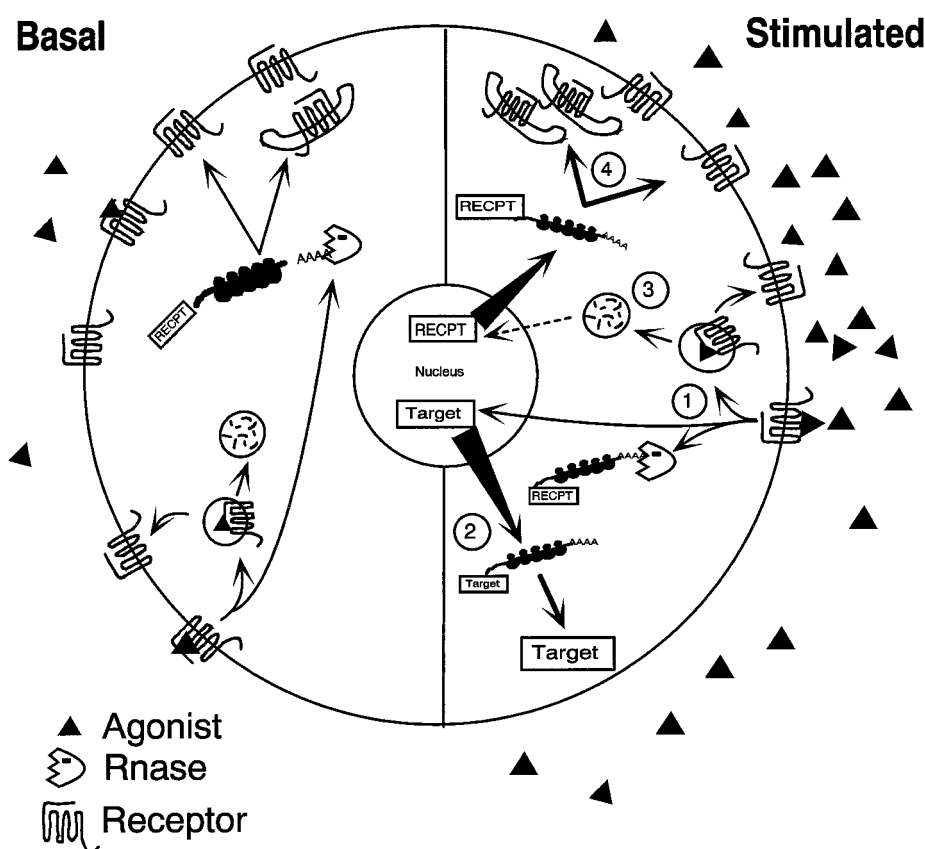
molecules proceeding the activation of NTR gene transcription.

The primordial role of NTR gene transcription mechanism in the resensitization of the cells was a surprise finding and was established by two lines of evidence. First, Ca^{2+} mobilization is similar in CHP 212 cells treated with agonist for 48 h and in control cells. Additionally, we analyzed the expression levels of an NT target gene as a function of cell binding capacity and agonist exposure. The CHP 212 and N1E-115 lines were originally chosen for these studies because of their cathecholaminergic phenotype and the fact that one of the transduction effects resulting from the binding of NT to NTR is TH gene stimulation (19). As expected, TH was activated after short term NT agonist exposure in both N1E-115 and CHP 212 cell lines. However, after prolonged agonist exposure, TH expression persisted and was enhanced in CHP 212 cells where NTR gene transcription was active (Fig. 6). In contrast, TH expression is no longer stimulated in N1E-115 cells after prolonged NT exposure. Therefore, the receptors that reappeared at the cell surface of the CHP 212 cells 48 h after NT agonist exposure were functional and able to trigger NT message. Thus, receptor gene transcription is directly implicated in maintaining agonist action on a target gene and in the maintenance of the cell-sensitized state.

TH gene transcription is also activated after the stimulation of G protein-coupled receptors such as angiotensin II, vasoactive intestinal polypeptide, nicotine, and muscarinic receptors. Two major promotor elements, an AP1 and a cyclic AMP/calcium regulatory element which can be independently activated, were implicated (47, 50). In the case of NTR, the increase of TH level in the cells is mediated by an AP1 element in a PKC dependent pathway (data not published).

The results drawn from this article and other studies enable us to propose a model for NTR expression and regulation (4–6, 20). An illustration representing how cells maintain agonist sensitivity is shown in Fig. 8. At basal agonist levels (*left*),

receptor expression is limited by the destabilization of receptor mRNA. In the stimulated state (*right*), the following chronological events occur. 1) The transduction pathway is activated leading to the activation of the agonist target genes. 2) The target proteins are produced. 3) If the agonist exposure is very intense, persistent, or chronic, receptor gene transcription is stimulated. 4) New receptors are synthesized; some restore the sensitivity of the cells, and others are stored in submembrane pools. During this process the mechanism of RNA destabilization is probably still active, because we showed that both mechanisms are independent. In the proposed model the cells are never desensitized, except under extreme agonist stimulation. The mechanisms, which cause the accumulation of the receptors in submembrane pools and the destabilization of mRNA, most likely protect the cell from a hyper-agonist stimulation.

In conclusion, we demonstrate the importance of receptor gene transcription in maintaining the cell sensitivity. The mechanism inducing this activation is still unclear, though it seems that the triggering of the second messenger is not the sole pathway involved.

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