Receptor Trafficking via the Perinuclear Recycling Compartment Accompanied by Cell Division Is Necessary for Permanent Neurotensin Cell Sensitization and Leads to Chronic Mitogen-activated Protein Kinase Activation*

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Most G protein-coupled receptors are internalized after interaction with their respective ligand, a process that subsequently contributes to cell desensitization, receptor endocytosis, trafficking, and finally cell resensitization. Although cellular mechanisms leading to cell desensitization have been widely studied, those responsible for cell resensitization are still poorly understood. We examined here the traffic of the high affinity neurotensin receptor (NT1 receptor) following prolonged exposure to high agonist concentration. Fluorescence and confocal microscopy of Chinese hamster ovary, human neuroblastoma (CHP 212), and murine neuroblastoma (N1E-115) cells expressing green fluorescent protein-tagged NT1 receptor revealed that under prolonged treatment with saturating concentrations of neurotensin (NT) agonist, NT1 receptor and NT transiently accumulated in the perinuclear recycling compartment (PNRC). During this cellular event, cell surface receptors remained markedly depleted as detected by both confocal microscopy and 125I-NT binding assays. In dividing cells, we observed that following prolonged NT agonist stimulation, NT1 receptors were removed from the PNRC, accumulated in dispersed vesicles inside the cytoplasm, and subsequently reappeared at the cell surface. This NT binding recovery allowed for constant cell sensitization and led to a chronic activation of mitogen-activated protein kinases p42 and p44. Under these conditions, the constant activation of NT1 receptor generates an oncogenic regulation. These observations support the potent role for neuropeptides, such as NT, in cancer progression.

G protein-coupled receptors (GPCRs) and their agonists participate in numerous aspects of cellular and tissue regulation (1). Agonist interaction instantly leads to GPCR activation, phosphorylation, sequestration, and cell desensitization. Upon receptor endocytosis, the GPCRs may return to the cell surface directly from sorting endosomes (short cycle) (2). However, a long cycle involving the passage through the perinuclear recycling compartment (PNRC), before reaching the plasma membrane, has been suggested recently (2–4). In the present study, by examining the effect of prolonged agonist exposure on neurotensin receptor (NT1 receptor) intracellular trafficking, we determined the conditions leading to cell agonist re-sensitization, along with the physiological consequences of this recovery on intracellular signalization.

Neurotensin (NT) is a brain and gastrointestinal peptide that fulfills many central and peripheral functions through its interaction with specific receptors (5, 6). Three subtypes of NT receptors have been cloned: NT1, NT2, and NT3 (7–9). NT1 and NT2 exhibit high (sub-nanomolar) and low (nanomolar) affinity for NT, respectively, and belong to the family of G protein-coupled receptors. NT3 is a single transmembrane domain receptor with 100% homology to gp95/sortilin (9). All three receptors are internalized after interaction with NT (10–12). In the periphery, the bulk of NT is released into the circulation by endocrine cells, called N-cells, localized along the luminal epithelium of the gastrointestinal tract (13). In the central nervous system, NT is released by neurons distributed throughout the neuraxis (14).

Most studies on the intracellular trafficking of GPCRs, including NT1 receptor, have been performed on naive cells and exposed to agonists for short periods ranging from a few minutes to a couple of hours. Within this time frame, most GPCRs are not recycled to the plasma membrane following ligand-induced internalization but a few GPCRs are efficiently recyc-
cells were rinsed twice with PBS, preincubated in the same buffer in phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (N1E-115) neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM). The expression of MAP kinase varied with the duration of MAP kinase stimulation, and the activation of NT1 receptor gene by NT correlated with cell resensitization. As cellular models, we studied the targeting of NT1 receptors after a prolonged agonist exposure to understand the role of NT1 in physiological or pathological conditions, because it is a key molecule in many diseases.

Our objective was to examine intracellular trafficking and targeting of NT1 receptors after a prolonged agonist exposure in relation with cell resensitization. As cellular models, we established CHO, CHI212, and N1E-115 cells, which stably expressed a fusion protein comprising the NT1 receptor and the enhanced green fluorescent protein (EGFP). By combining the use of NT1 receptor-EGFP fusion proteins and fluorescent NT, we were able to visualize intracellular trafficking of NT1 receptor and its ligand. We demonstrated that when cells were chronically exposed to high doses of NT agonist, NT1 receptors accumulate in the perinuclear recycling compartment and subsequently recycle to the cell surface. Consequently, the cells remain permanently sensitized leading to the chronic activation of MAP kinase.

MATERIALS AND METHODS

Cell Culture and Stable Cell Line Establishment—CHO cells were maintained in minimum Eagle's medium, without ribonucleosides and deoxyribonucleosides (Invitrogen). Human (CHP) 212 and murine (N1E-115) neuroblastoma cells were grown in Dulbecco's modified Eagle's medium/Nut Mix F12 (Invitrogen). All media were supplemented with 10% fetal calf serum (FCS) and 2 mM l-glutamine (Invitrogen). Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere.

N1E-115 differentiation was induced with 1.5% FCS and 1% Me₂SO for 48 h (28). Transfection of N1E-115 and CHP 212 cell lines with pNT1-EGFP, a plasmid expressing rat NT1 receptor fused at the C terminus with the EGFP coding sequence, was performed using the calcium phosphate coprecipitation method for 16 h at 37 °C of pDNA3-CAT per well, for 16 h at 37 °C. After transfection, the media were removed, and cells were treated with or without 1 μM JMV 449 for 2 h. Washed three times with ice-cold PBS, the cells were incubated, for 45 min at room temperature, with a rabbit anti-goat Texas Red-transferrin (Molecular Probes) was added to the culture medium without fetal calf serum at a concentration of 25 μg/ml for labeling of the perinuclear recycling compartment (32). Cells were then washed three times with ice-cold PBS and fixed with 4% formaldehyde for 20 min. Cells were mounted on glass slides with Aquamount and examined by confocal microscopy.

Fluo-NT Traffic in CHO-NT1-EGFP Cells—Cells were plated on Lab-Tek chambered coverglasses (Naïge Nunc) and were incubated for 24 h (treatment) and 3 h (wash) with 50 μM NT in the same culture medium, at 37 °C, or with 10 μM SR 48692 (Sanofi-Synthelabo), a NT1 receptor antagonist, for 3 or 6 h. Cells were then directly processed for confocal microscopy.

Binding Studies—Cell membrane preparations were obtained from CHO-NT1-EGFP and N1E-115-NT1-EGFP cells using the procedure of Boulin et al. (33). Cells were washed three times with cold PBS in order to remove agonist used for treatment. Under these conditions, the binding recovery of cells incubated with JMV 449 was 85 ± 15% as compared with control cells (23). Radioligand binding studies were performed as follows: 15 μg of protein were incubated with 1 nM [3H]-NT in a final volume of 250 μl of buffer A (50 mM Tris, pH 7.4, 0.2% BSA, 1.8 mM 1,10-phenanthroline, 1 mM sodium azide, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate) supplemented with 100 μM unlabeled NT. Binding assays were terminated by the addition of ice-cold 50 mM Tris-HCl, pH 7.4, followed by filtration through glass-microfiber filters (GF/B, Whatman) pre-incubated in 0.2% polyethyleneimine. After washing three times with 5 ml of ice-cold buffer, the radioactivity retained on the filters was counted in a γ-counter (Wallac model 1470 Wizard).

Confocal Microscopy—Confocal microscopic analysis was carried out using a TCS SP Leica microscope (Lasertechnik, GmbH) equipped with a 63× objective (plan apo; NA = 1.4). A focal series was collected for each specimen every 0.5 μm. CHO-NT1-EGFP and N1E-115-NT1-EGFP cell lines and every 1 μm for the N1E-115-NT1-EGFP cell line. Each confocal image shown here corresponded to the middle image from a 10-section series. For each optical section, double fluorescence was simultaneously acquired using a krypton-argon mixed gas laser adjusted to 488 nm for GFP and to 588 nm for TRITC. The variable center of spectrophotometers was adjusted to recover green (500–550 nm) and red (580–630 nm) fluorescence. The signal was treated with line averaging to integrate the signal collected over 4 lines in order to reduce signal noise. Selected paired sections were then processed to produce a single overlay image (color merged) using a personal computer equipped with Photoshop software (version 6).

p242p44 MAP Kinase Phosphorylation Assay—Differentiated and non-differentiated CHO-NT1-EGFP cells and N1E-115-NT1-EGFP cells were treated with 1 μM JMV 449 for 10 or 20 min or 24 h at 37 °C. After treatment, cells were washed twice with ice-cold PBS. Cells were detached by gentle scraping and centrifuged at 1200 × g for 10 min. Cell pellets were resuspended in 1 ml of PBS, transferred into an ice-cold microtube, and centrifuged at 1200 × g for 10 min. Cell pellets were resuspended in 0.5 ml of RIPA buffer containing 0.5% Nonidet P-40, 1% sodium azide, 1% SDS, 1% Nonidet P-40, 0.5% deoxycholate) supplemented with 100 μg/ml phenylmethylsulfonyl fluoride (Sigma) and 1 μg/ml aprotinin (Roche Applied Science). The samples were then batched twice for 15 s, incubated in ice for 30 min, and centrifuged at 12,000 × g, at 4 °C for 20 min. The supernatants were assayed using the MAP kinase assay (Pierce) for recombinant protein concentration. Equal amounts of protein were loaded on a 10% SDS-PAGE. The gel was run for 3 h at 50 mA and then transferred overnight, at 200 mA, onto nitrocellulose (Bio-Rad) in transfer buffer (25 mM Tris, pH 8.1, 192 mM glycine). Activation of MAP kinase was determined by measuring the phosphorylation state of the MAP kinase, using a phospho-specific antibody.
rylation state of p42 MAP kinase and p44 MAP kinase using an anti-active p42/p44 MAP kinase polyclonal antibody (Santa Cruz Biotechnology). To assess activated p42/p44 MAP kinase, the nitrocellulose blot was incubated in blocking buffer (PBS 1X; 0.05% Tween 20; 5% nonfat dry milk) for 2 h at room temperature and then probed with mouse polyclonal antibody to phosphorylated p42/p44 MAP kinase (Santa Cruz Biotechnology), diluted 1:750 in blocking buffer overnight at 4 °C. The blot was washed three times for 5 min each with PBST (PBS 1X, 0.05% Tween 20) and then probed with sheep anti-mouse horseradish peroxidase-linked secondary antibody (1:2000 dilution in blocking buffer (Sigma) for 1 h at room temperature. After three final washes, the immunoreactive bands were revealed with the enhanced chemiluminescence system (Amersham Biosciences). The blot was then stripped with stripping buffer (100 mM citric acid, pH 1.5) for 20 min at room temperature and re-probed with antibody to total p42/p44 MAP kinase (0.4 µg/ml) in blocking buffer for 1 h at room temperature, followed by incubation with goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:2000) (Sigma) as described above.

RESULTS

Upon Exposure to a High Dose of Agonist, NT1 Receptor and NT Transiently Accumulate in a Juxtanuclear Compartment—The agonist-induced intracellular trafficking of NT1 receptor was studied in CHO cells stably transfected with a plasmid expressing the rat NT1 receptor fused at the C terminus with the EGFP coding sequence (29). Binding characteristics were studied on the chosen clone, CHO-NT1-EGFP. Prepared membranes exhibited a K_d of 0.43 ± 0.05 nM and a B_max of 1590 fmol/mg of protein. The binding affinity of NT1 receptor-EGFP is identical to the NT1 receptor because prepared membranes from CHO-NT1 cells expressing the rat NT1 receptor revealed a K_d of 0.44 ± 0.03 nM (33). Signal transduction was shown to be operational in CHO-NT1-EGFP cells, because inositol phosphate production was obtained with the same EC_{50} value as in cell lines expressing the endogenous gene (29, 34).

In the absence of agonist, NT1 receptors were localized at the cell surface (Fig. 1A, unstimulated). In order to study agonist-induced receptor trafficking, CHO-NT1-EGFP cells were incubated for 0–24 h at 37 °C with 100 or 0.1 nM of a weakly degradable NT agonist, JMV 449 (31), corresponding to saturating and non-saturating binding conditions, respectively. Confocal microscopic examination of CHO-NT1-EGFP cells revealed that after 3 h of exposure to 100 nM JMV 449, EGFP was accumulated in a distinct juxtanuclear compartment (Fig. 1A).

This phenomenon was transient; the localization peaked at 6 h of treatment, declined at 8 h, and disappeared completely at 24 h (Fig. 1A). In contrast, when cells were treated with 0.1 nM JMV 449, the perinuclear localization was not observed at 3, 6, and 8 h of JMV 449 treatment. At 24 h of 100 nM NT agonist treatment, the intensity of EGFP fluorescence at the cell surface was identical to that observed in unstimulated cells (Fig. 1A). The dose response inducing this cellular phenomenon was studied after 6 h of JMV 449 treatment. As shown in Fig. 1B, only saturating concentrations of JMV 449 (10 nM and 1 µM), providing 99.9 and 100% binding site occupancy, respectively, induced EGFP accumulation in the perinuclear region. The same observation was made with 1 µM NT, 1 µM NT1-13, and 1 µM of the NT analog ESAI 11D348 (data not shown). No EGFP accumulation was observed in the perinuclear region under non-saturating agonist concentrations of 0.1 (Fig. 1B), 0.3 (Fig. 1B), and 1 nM (not shown) providing for 43, 69, and 89% site occupancy. As expected, 1 µM of NT-11 did not affect NT1 receptor trafficking (data not shown).

To verify that the accumulation of the NT1 receptor in the juxtanuclear compartment was not fibroblast-specific, the experiments were repeated in CHP 212 cells, a human neuroblastoma cell line that endogenously expresses NT1 receptor (24), and in N1E-115 cells, a murine neuroblastoma cell line (28). Both cell lines were stably transfected with the pNT1-EGFP plasmid (29). In unstimulated CHP212-NT1-EGFP and N1E-115-NT1-EGFP cells, the green fluorescence was located at the cell surface (Fig. 2, Unstimulated). Also, as seen in CHO-NT1-EGFP cells, EGFP accumulation was detected in the perinuclear region of both cell types, following a 6-h treatment with 1 µM JMV 449 (Fig. 2A). We confirmed by immunocytochemistry that the NT1 receptor was still fused to EGFP during the trafficking process, using an antibody directed against the rat C-terminal portion of NT1 receptor. As shown in Fig. 2A, a perfect overlap between EGFP labeling (green) and NT1 receptor immunoreactivity (red) was apparent (Fig. 2A, Overlay).

For control purposes, we confirmed that the C-terminal rat antibody did not recognize the endogenously expressed human NT1 receptor in CHP 212 cells (data not shown). Furthermore, it should be noted that in N1E-115-NT1-EGFP cells, endogenous murine NT1 receptor is only expressed in differentiated cells, whereas the N1E-115-NT1-EGFP cells used in this ex-

FIG. 1. Intracellular localization of NT1 receptor-EGFP with the dose and the time of NT agonist exposure. A, CHO-NT1-EGFP cells were seeded on Lab-Tek chambered coverslip and treated with either 100 or 0.1 nM JMV 449, at 37 °C. Confocal images were acquired successively as indicated in the figure. B, CHO-NT1-EGFP cells control or incubated as indicated with JMV 449, for 6 h, at 37 °C. Images were obtained from living cells with a confocal microscope as described under “Materials and Methods.”
NT1 receptor only in the differentiated state with a
agonist. H9262 NT1 receptor-EGFP cells were treated or not with 1
nM of JMV 449, at 37 °C, for 6 h. CHO-NT1-EGFP (upper panels), CHP212-NT1-EGFP (middle panels), and N1E-115-NT1-EGFP (lower panels). The cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with goat polyclonal anti-NT1 receptor followed by Texas Red-conjugated rabbit anti-goat to detect NT1 receptor. The unstimulated and 6-h JMV 449 columns correspond to the EGFP, and its recovered green fluorescence. The NT1 receptor column shows the NT1 receptor immunocytochemistry and its recovered red fluorescence. The overlay column displays the overlay from the confocal images from the treated cells recovered in green and red; colocalization is identified as yellow. B, wild type differentiated N1E-115 and wild type CHP 212 cells, unstimulated or treated for 6 h with 1 μM JMV 449, were fixed as described above. NT1 receptor immunocytochemistry was performed. Confocal images were acquired and treated as described under “Materials and Methods.”

The NT1 Receptor Accumulates in the PNRC—The PNRC is an organelle formed by tubulo-vesicular structures located near the centrioles and the Golgi apparatus (35), which serves as a compartment for controlling the recycling of membrane proteins. For example, accumulation in the PNRC is the rate-limiting step in the trafficking of internalized transferrin (32).

By using labeled transferrin as a marker for PNRC, several studies have shown that internalized G protein-coupled receptors may accumulate in the PNRC. The intracellular localization of NT1 receptors was compared with transferrin accumulation, in both CHP212-NT1-EGFP and N1E-115-NT1-EGFP cells, following incubation with 1 μM of JMV 449 for 6 h, followed by the addition of Texas Red-labeled transferrin 20 min before the end of the treatment. As shown in Fig. 4A, the bulk of NT1 receptor was found in the juxtanuclear compartment in which transferrin has accumulated.

Rab11, a small GTP-binding protein, has been found to be mainly associated with the PNRC (36). As illustrated in Fig. 4B, Rab11 immunocytochemistry performed on CHP212-NT1-EGFP cells, treated for 6 h with JMV 449, demonstrated an extensive colocalization of Rab11 immunoreactivity and NT1 receptor.

Accumulated NT1 Receptors in the PNRC Are Internalized Receptors—We have shown previously that NT1 receptor gene transcription was stimulated in the presence of saturating NT agonist concentration for 6 h (23). In order to determine the transcriptional or the endocytotic origin of NT1 receptors accumulated in PNRC, we analyzed CHO-NT1-EGFP cells treated with 1 μM JMV 449 for 6 h in the presence of nocodazole, a microtubule-depolymerizing agent, or actinomycin D, a general transcriptional inhibitor. First, we checked if the 125I-NT binding capacity of membranes was in agreement with the NT1 receptor trafficking. As expected, when cells were treated with saturating NT agonist concentration (1 μM), membrane-binding capacity was strongly diminished after 6 h of treatment (Fig. 5A). The addition of nocodazole or actinomycin D during JMV 449 treatment did not significantly alter the binding capacity of the cells compared with JMV 449 alone (Fig. 5A). Treatment with nocodazole or actinomycin D alone had no effect on the binding capacity (Fig. 5A).

Then we studied the traffic of NT1 receptor following 6 h of agonist exposure in the presence of nocodazole. Under this treatment, NT1 receptor was internalized but formed small clusters disseminated throughout the cytoplasm (Fig. 5B). By contrast, in the presence of actinomycin D and JMV 449 for 6 h, NT1 receptor was internalized and accumulated in the PNRC. Application of nocodazole or actinomycin D alone was without effect on receptor trafficking (Fig. 5B).

In addition, we verified that NT agonist had no effect on the cytomegalovirus promoter directing the NT1 receptor-EGFP coding sequence in the plasmid used to generate the stable cell lines. Luciferase activity did not change in a construct containing cytomegalovirus promoter associated with luciferase transiently transfected in CHO-NT1-EGFP cells and treated with
concentrations of JMV 449 ranging from 0.1 nM to 1 μM, over a time course of 2–24 h (data not shown). Second, no change was observed on NT1 receptor mRNA levels, when CHO-NT1-EGFP cells were treated for 2, 4, and 6 h with 1 μM JMV 449 (data not shown). NT1 receptor gene transcription activation by NT agonist is not necessary for NT1 receptor accumulation in PNRC, suggesting that NT1 receptor accumulated in the PNRC is mainly internalized receptor.

**NT1 Receptor Is Recycled from the PNRC to the Cell Surface**—In a previous study (23, 24), we showed that under prolonged and saturating agonist exposure, neuroblastoma CHP 212 and colon adenocarcinoma HT-29 cells recovered a fully functional NT1 receptor after 24 h. In contrast, N1E-115 cells, in which endogenous NT1 receptor expression was induced by differentiation, were not re-sensitized (24). In order to localize the receptor in the cell, we examined NT1 receptor traffic in N1E-115-NT1-EGFP, under non-differentiated and differentiated states. Analysis of NT1 receptor localization after 24 h of agonist treatment showed that in non-differentiated cells, NT1 receptor returned to the cell surface, whereas in differentiated cells, NT1 receptors remained located in the PNRC (Fig. 6A).

Binding analysis performed on membranes of N1E-115-NT1-EGFP challenged with JMV 449 for 6 h showed that as observed in CHO-NT1-EGFP cells, N1E-115-NT1-EGFP cells are totally desensitized in both differentiation states (data not shown) (Fig. 5A). After prolonged agonist exposure (24 h), 125I-NT binding capacity was restored to 55% of control values in non-differentiated cells, but remained below 15% of control values in differentiated cells (Fig. 6B). A similar observation was made in wild type NT1 receptor expressing cells. As shown in Fig. 6C, when differentiated N1-E115 cells were exposed with JMV 449 for 24 h, NT1 receptors remained in the PNRC, whereas in CHP 212 cells NT1 receptors returned to the cell surface.

We questioned if the disappearance of NT1 receptor from the PNRC was due to its degradation or its targeting to the plasma membrane. In order to access this information, CHP212-NT1-
Fig. 5. Effect of nocodazole and actinomycin D on NT1 receptor-EGFP cell binding capacity and traffic. A, effect on $^{125}$I-NT binding capacity of CHO-NT1-EGFP cells. CHO-NT1-EGFP cells were incubated with 1 μM JMV 449 in the presence or in the absence of 10 μM nocodazole for 6 h (top) or 5 μg/ml actinomycin D (bottom). $^{125}$I-NT binding was performed on cell membranes as described under “Materials and Methods.” B, living cells were visualized by confocal microscopy as described under “Materials and Methods.” Unstimulated CHO-NT1-EGFP cells or cells treated with 1 μM JMV 449, at 37 °C, for 6 h are shown (upper panel). CHO-NT1-EGFP cells treated with 10 μM nocodazole alone or with 1 μM JMV 449 for 6 h are shown (middle panel). CHO-NT1-EGFP cells treated with 5 μg/ml actinomycin D alone or with 1 μM JMV 449 for 6 h are shown (lower panel). Data are expressed as percent of control. Values are expressed as the mean ± S.E. of at least three separate experiments.

Fig. 6. Cell resensitization is dependent on cell differentiation. A, non-differentiated and differentiated N1E-115-NT1-EGFP cells, control or treated with 1 μM JMV 449, for 24 h are shown. B, $^{125}$I-NT binding was performed on cell membranes from non-differentiated (black bar) or differentiated (gray bar) N1E-115-NT1-EGFP cells treated with 1 μM JMV 449, at 37 °C, for 24 h. Results are expressed as percentage of the respective control cells. Data represent the mean ± S.E. of three separate experiments. C, wild type differentiated N1E-115 and wild type CHP 212 cells, unstimulated or treated for 24 h with 1 μM JMV 449, were fixed and treated as described in Fig. 2. NT1 receptor immunocytochemistry was performed. Confocal images were acquired and treated as described under “Materials and Methods.”
EGFP cells were treated with 1 μM JMV 449, and lysosome-associated membrane glycoproteins, LAMP2, immunocytochemistry was performed (39). After a 6-h treatment, NT1 receptor EGFP is accumulated in the juxtanuclear compartment, whereas the immunoreactivity corresponding to lysosome vesicles (red) are dispersed in the cytosol. From 8–16 h of JMV 449 treatment, the NT1 receptor is seen being removed from the PNRC but never colocalized with LAMP2. After 24 h of agonist exposure, NT1 receptor localized near and at the plasma membrane, but no overlap with LAMP2 labeling was detected (Fig. 7A). In differentiated N1E-115-NT1-EGFP, treated for 24 h with 1 μM JMV 449, NT1 receptor remained blocked in PNRC, with no detectable colocalization of lysosomal particles (Fig. 7B). These data indicate that NT1 receptor is never associated with lysosomal vesicles.

In order to determine the origin of NT1 receptor restored at the cell surface, we set up the following experiment: N1E-115-NT1-EGFP cells were pre-treated for 6 h with 1 μM JMV 449 in order to accumulate NT1 receptor in the PNRC, and then the treatment was continued in the presence or the absence of the protein synthesis inhibitor cycloheximide (CHX). Arrowheads indicate NT1 receptors reaching the cell surface. An additional 3 h of JMV 449 in the presence or in the absence of cycloheximide, NT1 receptor was partially removed from the PNRC, and many clusters of small dispersed vesicles were detected in the cytoplasm (data not shown). NT1 receptor was totally removed from the PNRC and dispersed in small clusters close to the cell surface, following an additional 3-h treatment and independent of the presence or absence of cycloheximide (Fig. 8).

**Conditions for Cell Surface NT1 Receptor Recovery**—One particularity of in vitro neuronal differentiation, promoted by low serum concentration, is the delay caused to cell division. We questioned if the lack of NT1 cell surface receptor recovery in differentiated cells (N1E-115-NT1-EGFP) resulted from neuronal differentiation per se or from cell division arrest. To address this issue, we followed NT1 receptor trafficking in CHO-NT1-EGFP cells cultured either in 10 or 2.5% FCS. The latter concentration is the lowest FCS concentration in which CHO cells remain alive after 24 h. As shown in Fig. 9, A and B, cells were exposed to 1 μM JMV 449 or NT for 24 h. The NT1 receptors were removed from PNRC when cells were cultured in 10% FCS, and they remained in the PNRC when cells were grown in 2.5% FCS. According to 125I-NT binding was restored to 65% of control values in cells grown in 10% FCS but remained below 15% of control values in cells grown in 2.5% FCS (Fig. 9C). A similar binding capacity...
profile was observed when cells were treated with the natural agonist (data not shown).

Inhibition of the MAP kinase pathway for a prolonged period of time, with the inhibitor PD 98059, arrests the cell cycle at G1 (38, 39). When N1E-115-NT1-EGFP cells were cotreated for 24 h with NT agonist and PD 98059, NT1 receptor remained in the PNRC independently of the differentiation state. When cells were treated with PD 98059 alone, NT1 receptor remained at the cell surface (Fig. 10A). Cultured N1E-115-NT1-EGFP cells, in the presence of the MAP kinase inhibitor, displayed an accumulation of NT1 receptor in PNRC similar to that of N1E-115-NT1-EGFP cultured in low serum concentrations to induce differentiation (compare Fig. 6A and Fig. 10A). Accordingly, the binding capacity of N1E-115-NT1-EGFP cells treated with PD 98059 and JMV 449 was not restored (Fig. 10B). Similar observations were made in CHO-NT1-EGFP cells cultured in 10% FCS and concomitantly treated with PD 98059 and JMV 449; NT1 receptors remained in the PNRC (Fig. 10C). Addition of a MAP kinase inhibitor and JMV 449, for 24 h, to cells cultured with 10% FCS resulted in a similar receptor localization as in cells cultured with 2.5% FCS (compare Fig. 9A and Fig. 10C).

Additional experiments were performed using lovastatin, a potent inhibitor of the enzyme hydroxymethylglutaryl-CoA reductase, which also acts as an antimitogenic agent by arresting cells in the G1 phase of the cell cycle (40, 41). In CHO-NT1-EGFP, this inhibitor blocked the receptor in the PNRC, because the juxtanuclear EGFP staining was still visible after 24 h of concomitant treatment with JMV 449 and lovastatin (Fig. 10D).

In order to evaluate if cell cycle completion is necessary for NT1 receptor recovery at the plasma membrane, the cell cycle was arrested asynchronously in G2/M phase by microtubule depolymerization with nocodazole (42). As we described above, disruption of the microtubule network prevented the accumulation of NT1 receptor in the PNRC (Fig. 5, A and B). CHO-NT1-EGFP cells were first treated with JMV 449 for 6 h to induce the accumulation of NT1 receptor in the PNRC, and then concomitantly treated with JMV 449 and nocodazole for 24 h (Fig. 10D). NT1 receptor returned at the plasma membrane after 24 h of agonist treatment, even in the presence of nocodazole (Fig. 10D), suggesting that the signals triggering receptor recycling was specifically present in G1 phase.

**NT1 Receptor Recovery Results in a Persistent Activation of MAP Kinases**—It has been shown that NT activated MAP kinase p42 and p44 in CHO and in NCM460 cells stably transfected with NT1 receptor (43, 44). We analyzed the phosphorylation status of p42 and p44 in differentiated and non-differentiated N1E-115-NT1-EGFP and CHO-NT1-EGFP cells after short and long term agonist exposure. As seen in Fig. 11, A and B, activation of p42 and p44 was detected when N1E-115-NT1-EGFP cells were exposed to JMV 449 for 20 min. In differentiated cells, the levels of pp42 and pp44 returned to control

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**Fig. 9.** Cell resensitization is dependent on cell proliferation. A, CHO-NT1-EGFP grown in 10 or 2.5% FCS containing media control or 1 μM JMV 449, for 24 h. Visualization was performed directly by fluorescence microscopy at ×40 lens. B, CHO-NT1-EGFP grown in 10 or 2.5% FCS containing media control or 1 μM NT, for 24 h. Living cells were visualized by confocal microscopy as described under "Materials and Methods.” C, 125I-NT binding was performed on cell membranes from CHO-NT1-EGFP cells grown in 10% (black bar) or 2.5% (gray bar) FCS and treated with 1 μM JMV 449, at 37 °C, for 24 h. Result is expressed as percentage of the respective control cells. Data represent the mean ± S.E. of three separate experiments.

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**Fig. 10.** A, CHO-NT1-EGFP grown in 10 or 2.5% FCS containing media control or 1 μM JMV 449, for 24 h. Visualization was performed directly by fluorescence microscopy at ×40 lens. B, CHO-NT1-EGFP grown in 10 or 2.5% FCS containing media control or 1 μM NT, for 24 h. Living cells were visualized by confocal microscopy as described under "Materials and Methods.” C, 125I-NT binding was performed on cell membranes from CHO-NT1-EGFP cells grown in 10% (black bar) or 2.5% (gray bar) FCS and treated with 1 μM JMV 449, at 37 °C, for 24 h. Result is expressed as percentage of the respective control cells. Data represent the mean ± S.E. of three separate experiments.

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**Fig. 11.** A and B, activation of p42 and p44 was detected when N1E-115-NT1-EGFP cells were exposed to JMV 449 for 20 min. In differentiated cells, the levels of pp42 and pp44 returned to control.
levels with prolonged (24 h) NT agonist exposure (Fig. 11A). In contrast, in non-differentiated cells, a persistent activation of p42 and p44 was detected after a JMV 449 exposure for 24 h, indicating cell re-sensitization (Fig. 11B).

In CHO-NT1-EGFP cells cultured in 10% FCS, the MAP kinase activation was not detected after 10 min of JMV 449 treatment (Fig. 11C). This was expected, because the experimental conditions needed to observe the MAP kinase activation by NT required the removal, for at least 24 h, of FCS from the culture media (43). In CHO-NT1-EGFP cells, cultured in 10% FCS and treated with JMV 449 of 24 h, a strong MAP kinase activation was observed (Fig. 11C). In summary, we observed that under persistent and high concentration of NT agonist exposure, dividing cells restored binding capacity to NT. The cells became re-sensitized which led to a persistent activation of MAP kinase p42 and p44.

**DISCUSSION**

We report here on how cells maintain sensitivity despite being in the state of continuous agonist stimulation. By following the intracellular routing of internalized NT1 receptors, we were able to clarify the cellular mechanism underlying this process and to show that in dividing cells, constant cell sensitization is associated with a chronic activation of MAP kinases.

Following prolonged stimulation with saturating doses of agonist, NT1 receptors are internalized and transiently accumulate in the PNRC using the microtubule network (Figs. 1 and 4). This recycling compartment constitutes an intermediate step in the recycling of transferrin receptors to the cell surface (32). Accumulation in the PNRC is not particular to NT1 receptors, because other GPCRs, such as chemokine receptor, CCR5 (3), the endothelin type A receptor (4), and the V2 vasopressin receptor (2) are also targeted to this cellular compartment after agonist stimulation. However, what distinguishes NT1 receptor from other documented cases is the time required for recycling. NT1 receptor requires 6 h of agonist induction to reach the PNRC as compared with 1 h for CCR5.

In CHO-NT1-EGFP cells cultured in 10% FCS, the MAP kinase activation was detected after 10 min of JMV 449 treatment (Fig. 11C). This was expected, because the experimental conditions needed to observe the MAP kinase activation by NT required the removal, for at least 24 h, of FCS from the culture media (43). In CHO-NT1-EGFP cells, cultured in 10% FCS and treated with JMV 449 of 24 h, a strong MAP kinase activation was observed (Fig. 11C). In summary, we observed that under persistent and high concentration of NT agonist exposure, dividing cells restored binding capacity to NT. The cells became re-sensitized which led to a persistent activation of MAP kinase p42 and p44.

**DISCUSSION**

We report here on how cells maintain sensitivity despite being in the state of continuous agonist stimulation. By following the intracellular routing of internalized NT1 receptors, we were able to clarify the cellular mechanism underlying this process and to show that in dividing cells, constant cell sensitization is associated with a chronic activation of MAP kinases.
and V2 vasopressin receptors and 15 min for endothelin type A receptor.

To evaluate the role of the receptor traffic and follow the receptor route in living cells, we purposely used non-expressing NT1 receptor cells stably transfected with NT1 receptor coupled with EGFP. Thus subsequently to its accumulation in the PNRC, we observed that the NT1 receptor was removed and redistributed in the cytosol and at the cell surface (Fig. 8). In a previous study, we have shown that in the human colon adenocarcinoma cells, HT-29, as well as in human neuroblastoma cells, CHF 212, functional NT1 receptors returned to the cell surface. We had correlated this phenomenon with a massive internalization of NT1 receptor and NT1 receptor gene transcription activation by NT agonist (23, 24). The present results conform to our earlier observation; under saturating conditions the intense receptor internalization is followed by the restoration of cell sensitivity. Contrary to what we had originally postulated, NT1 receptor gene activation by NT agonist is not necessary for cell resensitization, because we observed here that NT agonist had no effect on the promoter directing the transgene. Nevertheless, in cells where the NT1 receptor gene activation is persistent, as in CHF 212 (24), it is likely that both recycled NT1 receptors and de novo synthesis NT1 receptors resulting from the gene activation participate in establishing cell re-sensitization.

Prior to this report, it was generally held that activated NT1 receptors were not recycled to the plasma membrane but were targeted to early and late endosomes. Those results were supported by experiments performed with cells exposed to short agonist stimulation (30 min to 1 h), where recycling was observed after agonist removal and cell wash (45–47). Our data demonstrate that when the agonist exposure is prolonged additional cellular mechanisms and the cell responses are initiated to restore the receptor to the cell surface. Both mechanisms are likely to be utilized in vivo depending on the physiological circumstances. In the synaptic cleft where released NT is rapidly degraded, the receptor probably follows the degradation route, as suggested by the experiments with neurons described previously (48). In cancer cells where NT expression is increased and NT1 receptor de-repressed, receptor recycling and permanent cell sensitization would occur.

Among the signaling pathways mediating cellular transformation, recent attention has focused on neuropeptides, which for most are also known as gut peptides (49). The receptors for these neuropeptides are members of the GPCRs. Numerous studies demonstrated that the neuropeptides stimulate the extracellular signal-regulated kinase members of the MAP kinase family (49). Despite this, the impact of these peptides in tumorigenesis remains to be clarified. Permanent sensitivity to GPCR ligand resulting in a chronic activation of MAP kinase, as demonstrated here (Fig. 11), implies that these peptides may be involved in cancer progression. In particular, in cells with atypical expression of GPCR, autocrine or paracrine regulation would become oncogenic. Regarding NT, an abundance of data supports this concept. For example, while searching for a relevant gene in different cancer types, Elek et al. (50) identified from the Cancer Gene Anatomy Project data base a hit for NT1 receptor. NT1 receptor autoradiography and NT1 receptor mRNA experiments revealed the NT1 receptor expression in human tumor of ovary, pancreas, and prostate, Ewing’s sarcoma, meningiomas, and astrocytomas (21, 50–52). Furthermore, it was shown that pro-NT1/N mRNA is expressed in cancer tissues and cell lines from gut, lungs, pancreas, and prostate (17–20). In addition, it has been shown that the size of the tumor of human colon cancer cell or small cell lung cancer xenografted nude mice is increased by NT and inhibited by a NT1 receptor-specific antagonist, SR 48692 (53, 54). Expression of NT1 receptor and NT in the same type of tumor strongly suggests the presence of an oncogenic neuropeptidase regulation. In this paper, we also observed that cell resensitization is discernible in actively dividing cells. This was concluded based on the action caused by diverse inhibitors, which interfere with the cell cycle when they are used for more than a few hours in culture. In cancer cells the G1 phase is shorter due to the active cell division, and the mechanism of NT1 receptor cell resensitization described here will therefore become crucial.

In this study, we demonstrated how, under specific circumstances, cells maintain sensitivity despite the presence of continual agonist pressure, leading to the permanent stimulation of MAPK. Ligands, such as neuropeptides, act as oncogenes under these circumstances. Consequently, greater attention should be given to the maintenance of receptor sensitivity, because the judicious use of GPCR ligands could provide an effective recourse in cancer therapy.

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