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NT Agonist Regulates Expression of Nuclear High-affinity Neurotensin Receptors

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SUMMARY Neurotensin (NT) exerts multiple functions in the central nervous system and peripheral tissues. Its actions are mainly mediated by a high-affinity G-protein-coupled receptor, the NT-1 receptor. In this study we demonstrated a nuclear NT binding site in different cellular models. We first noted that a large percentage of NT-1 receptor cell body immunoreactivity was located in the nuclear soma and nuclear envelope of rat substantia nigra, a brain area rich in NT-containing axon terminals. The NT-1 receptor was also visualized in purified nuclei from CHO cells stably transfected with NT-1 receptor coupled to the enhanced green fluorescence protein by immunocytochemistry. We observed that both the nuclear envelope and the nuclear soma were labeled, and the labeling intensity significantly increased after NT agonist treatment. These results suggested that NT-1 receptors, present in both the nuclear soma and the nuclear envelope, can be modulated by the ligand. Lastly, [125I]-NT binding experiments performed on isolated nuclei from a human lung cancer cell line endogenously expressing NT-1 receptor and NT, LNM35, revealed the existence of nuclear Gpp(NH)p-sensitive binding sites. These binding sites markedly decreased when cells were chronically treated with an NT-1 receptor antagonist, SR 48692. Taken together, these data suggest that the agonist regulates the expression of nuclear NT-1 receptors. (J Histochem Cytochem 52:335–345, 2004)

KEY WORDS neurotensin NT-1 receptor G-protein-coupled receptor nuclear envelope nuclear soma agonist treatment

NEUROTENSIN (NT) is a tridecapeptide localized primarily in the brain and in specialized endocrine cells (N-cells) of the small bowel (Reinecke 1985). NT acts as a neurotransmitter in the central nervous system (CNS) and as a hormone in the periphery. Its actions are mediated through the stimulation of two specific G-protein-coupled receptors (GPCRs), the NT-1 and NT-2 receptors, exhibiting high and low affinity for NT, respectively (Tanaka et al. 1990; Chalon et al. 1996). NT also binds to a single transmembrane domain receptor, the NT-3 receptor, 100% homologous to gp95/sortilin (Mazella et al. 1998). The NT-3 receptor is predominantly localized in the trans-Golgi network but the mature protein is also present in the plasma membrane (Nielsen et al. 1999). At the cell surface, the NT-3 receptor forms heterodimers with the NT-1 receptor (Martin et al. 2002). Overexpression of the NT-3 receptor causes attenuation of NT signaling through the NT-1 receptor (Martin et al. 2002).

In the CNS and in the periphery, the vast majority of NT effects are mediated by the NT-1 receptor. Second-messenger pathways activated by NT-1 receptor have primarily been studied in cultured cell lines expressing the NT-1 receptor. When murine neuroblastoma cells, N1E-115, are challenged with NT, phosphatidyl inositols are hydrolyzed, leading to Ca\(^{2+}\) mobilization and the formation of cGMP (Gilbert and Richelson 1984; Amar et al. 1985,1987; Snider et al. 1986). More recently, it was shown that NT activated the MAP kinases, p44 and p42, leading to immediate or delayed responses to NT involving gene transcription, cell growth, death, or differentiation (Poinot-Chazel et al. 1996; Ehlers et al. 1998,2000).
The interaction between NT and the NT-1 receptor results in endocytosis of NT-1 receptor through sequestration, internalization, and trafficking of the receptor (Pierce et al. 2002). Several studies using fluorescent NT analogues showed that the ligand was localized within small vesicular organelles in the perinuclear region after its NT-1 receptor internalization (Faure et al. 1995a,b). Additional localization studies revealed the presence of the NT-1 receptor in the nuclear compartment (Dana et al. 1989; Boudin et al. 1998). This latter phenomenon is not unique because it has already been described for other GPCRs, such as angiotensin type 1, VIP, opioid, prostaglandin, and muscarinic receptors, but the function of these receptor complexes in the nuclear soma or in the nuclear envelope is unknown (Re et al. 1984; Omary and Kagnoff 1987; Tang et al. 1992; Belcheva et al. 1993; Lind and Cavanagh 1993; Bhattacharya et al. 1999). It could also be suggested that the NT-NT-1 receptor interaction is recognized as a nuclear targeting signal, as has been shown for the angiotensin type 1 and opioid receptors (Booz et al. 1992; Belcheva et al. 1995).

We demonstrate in this study the presence of NT-1 receptors in the nuclear compartment of the substantia nigra neuron cell bodies, in nuclei isolated from LNM35 pulmonary cells (which endogenously express NT and NT-1 receptor), as well as nuclei from CHO cells stably expressing the NT-1 receptor. Our data suggest a relationship between nuclear expression of the NT-1 receptor and the presence of NT in the extracellular compartment.

Materials and Methods
Electron Microscopy and Tissue Preparation
Sprague–Dawley rats (n=3) were maintained on a 12-hr light/dark cycle and fed ad libitum. Rats were deeply anesthetized with pentobarbital (70 mg/kg) and perfused transcardially with 30 ml heparin (100 U/ml), followed sequentially by a mixture of 50 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), and by 200 ml of 2% paraformaldehyde in PB. The brains were fixed for 30 min in 2% paraformaldehyde in PB. Coronal sections (40 μm) were cut on a vibratome and collected in PB. Immunolabeling was performed by a procedure previously described (Chan et al. 1990; Boudin et al. 1998). Ultrathin sections for gold staining were collected from the surface of blocks of the substantia nigra, counterstained with lead citrate and uranyl acetate, and examined with a JEOL 100CX electron microscope.

Electron Microscopy and Quantitative Analysis
We analyzed three rat brains. For each rat, two to four sections from different blocks were systematically scanned. Microscopic fields with cell bodies were photographed at an original magnification of ×8300. All silver-enhanced gold granules were classified according to the type of element and the ultrastructure with which they were associated. For perikarya, they were classified as plasma membrane, cytosol (labeling apparently not associated with any organelle), Golgi apparatus/endoplasmic reticulum, vesicle, mitochondria, nuclear envelope, or nuclear soma. We measured the surface of the nucleus and cytoplasm of each cell body on the film negatives using a computer-based image analysis system associated with a CCD camera and the software Histograp (Biocom; Les Ulis, France). We analyzed a total of 264 micrographs representing 75 cell bodies and 1119 gold particles.

Cell Culture
Human lung cancer cells (LNM35) were grown in RPMI 1640 (Invitrogen; Cergy Pontoise, France). CHO cells were grown in MEM α medium without ribonucleosides and deoxyribonucleosides (Invitrogen). All media were supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen). For stably transfected CHO cells, 250 μg/ml of geneticin (G418) was added to maintain the transgene. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. At confluence, cells were routinely dispersed in trypsin–EDTA 0.25% and subcultured at 1:10 dilution.

Stable Cell Line Establishment
The expression plasmid pNT1-EGFP containing the rat NT-1 receptor coding region and the modified form of EGFP from Aequorea victoria, directed by the CMV promoter, was provided by Dr. Llorens-Cortes (Lenkei et al. 2000). pNT1-EGFP was stably transfected in CHO cells as described by Boudin et al. (1995). Stable transfectants were selected with G418 (1000 μg/ml) and colonies were screened for EGFP expression, using a Nikon Diaphot inverted fluorescence microscope at a ×40 magnification, and by [125I]-NT-binding assays (Boudin et al. 1995).

Isolation of Membrane-depleted Nuclei
Purified nuclei were prepared using a modification of the procedure previously described (Facy et al. 1990). Cells were washed twice in cold PBS and collected by low-speed centrifugation (300 × g for 7 min). The pellet was washed in 20 ml TKCM buffer containing 10 mM Tris-HCl (pH 7.3), 20 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.2 mM spermidine, and collected by centrifugation. The pellet was resuspended in 20 ml TKCM buffer and incubated on ice for 1 hr. Cells were homogenized twice with a Potter-Elvehjem tissue grinder in the presence of a mixture of protease inhibitors for mammalian cell and tissue extracts (Sigma-Aldrich; St-Quentin, France), and then centrifuged at 300 × g for 7 min. The cell suspension was layered onto 2 M sucrose, 50 mM Tris-HCl, pH 7.4, and centrifuged at 25,000 × g for 30 min. Nuclei were collected from the bottom of the tubes, and cell membrane homogenates were collected at the interface.

Chromatin Preparation
Nuclei were resuspended in 1 ml of 1 mM Tris-HCl, pH 7.9. The nuclear suspension was incubated on ice for 1 hr before homogenization. The chromatin was separated from
the nuclear membranes by centrifugation at 12,000 × g for 30 min.

Control of the Purity of Membrane-depleted Nuclei
CHO–NT1 cells were labeled with a mix containing 7.5 μCi/ml of L-[35S]-methionine and L-[35S]-cysteine (Amersham Biosciences; Orsay, France) for 16 hr. Membrane homogenates and nuclei were prepared as described above and the radioactivity of each fraction was counted. In parallel, we prepared an unlabeled cell suspension. Before the ultracentrifugation step, we added either 3 × 10^6 cpm of labeled nuclei or 7 × 10^7 cpm of labeled membrane. The mixture was layered onto a sucrose layer and centrifuged as described above. The radioactivity was counted in the membrane homogenates and in the nuclear fraction from both samples.

Enzymatic Markers
The purity of the nuclear membranes was assessed by assaying selected enzymatic marker activity for each cell fraction. The activity of 5’ nucleotidase was performed using a Sigma kit based on the method developed by Arkesteijn (1976). The ouabain-sensitive Na^+-K^+-ATPase was assayed by the method of Gache et al. (1976). Ouabain-sensitive Na^+-K^+-ATPase activity was defined as the activity inhibited by 1 mM ouabain. The K^+-EGTA ATPase activity was measured as an enzymatic marker of the endoplasmic reticulum membranes (Gache et al. 1976).

Binding Studies
Nuclei, cell membrane homogenates, and chromatin were washed with 50 mM Tris-HCl, pH 7.4, and were immediately used for [125I]-NT-binding and enzymatic assays. Protein contents were estimated by the method of Bradford using bovine serum albumin as standard (Bradford 1976). Radioligand binding studies were carried out either on cell membrane homogenates or on nuclei. Binding studies were performed as follows: 40 μg of protein from cell membrane homogenates or 80 μg of protein from nuclei was incubated with 0.5 nM [125I]-NT in a final volume of 250 μl of buffer A (50 mM Tris, pH 7.4, 0.2% BSA, and 0.8 mM 1.10-phenanthroline, 1.6 mM MgCl₂) for 45 min at room temperature (RT). Nonspecific binding was measured in the presence of 1 μM unlabeled NT. The competition binding was carried out under the same conditions, using a range of unlabeled NT concentrations (10^-6 to 10^-11 M). Binding assays were terminated by addition of ice-cold 50 mM Tris-HCl (pH 7.4) supplemented with 0.2% BSA, followed by filtration through glass microfiber filters (GF/B; Whatman, Maidstone, UK) preincubated in 0.2% polyethyleneimine (Sigma–Aldrich). After washing three times with 5 ml ice-cold buffer, the radioactivity retained on the filters was counted in a y-counter (Wallac model 1470 Wizard). Binding affinities and densities (Kd and Bmax) were estimated by the Ligand EBDA program.

Immunofluorescent Staining of Nuclei for NT-1 Receptor
Fifty μl of purified nuclei were sedimented onto slides with a Cytospin centrifuge. Nuclei were fixed in 2% paraformaldehyde for 30 min. Fixed nuclei were washed three times with PBS at RT before proceeding to immunocytochemistry. Nonspecific binding was blocked with 0.1% Triton X-100/ PBS containing 10% normal rabbit serum (NRS) for 1 hr at RT. Nuclei were then washed three times with PBS and incubated for 1 hr at RT with a goat polyclonal anti-NT-1 receptor antibody (Santa Cruz Biotechnology; Santa Cruz, CA) diluted 1:25 in PBS containing 0.1% Triton X-100 and 3% NRS (buffer A). Nuclei were then rinsed three times with PBS and incubated for 45 min at RT with rabbit anti-goat–Texas Red secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA) diluted 1:300 in buffer A. After three final rinses in PBS, slides were mounted in Glycergel (DAKO-Cytomation; Trappes, France). Appropriate controls devoid of NT-1 receptor antibody were performed in parallel to determine nonspecific staining.

RNA Extraction and Reverse Transcription-polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from LNM35 cells using the acidic phenol/chloroform guanidine thiocyanate method (Chirgwin et al. 1979). One hundred ng of total LNM35 RNA was reverse-transcribed in a 30-μl reaction mixture containing 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 1 mM each of dNTP, 24 U RNasin (Promega; Charbonnière, France), 1 μg/μl of each oligo (dN) and oligo (dT) (Amersham Biosciences; Orsay, France) for NT transcript, or primer RT-NT1 (5’-GCTGACGTAGAGAAG-3’) for NT-1 receptor transcript, and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen; Cergy Pontoise, France) at 37°C for 1 hr. The PCR amplification was performed on 1:5 (v/v) dilution of the RT reaction in a mixture containing 16 mM Tris-HCl, pH 8.3, 40 mM KCl, 1 mM MgCl₂, 0.2 mM of each dNTP, 25 pmol of the sense primer, 25 pmol of the antisense primer and 1 U Taq polymerase (Applied Biosystems; Les Ulis, France) in a final volume of 50 μl. The PCR primers had the following sequences: S-NT1 5’-CGTGGAGCTGTACAACTTCA-3’ and AS-NT1 5’-CAGCCAGCAGACCACAAAGG-3’ for NT-1 receptor; S-NT 5’-AAGCACATGTTCCTCTTCTT-3’ and AS-NT 5’-CATACAGTCCGTTTACAG-3’ for NT (Invitrogen). The amplicon sizes of NT-1 receptor, and NT were 590 and 446 nucleotides, respectively. The amplification profile was divided into 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 45 sec, and extension at 72°C for 45 sec. PCR products were electrophoresed on 1% agarose gels in 90 mM Tris borate and 2 mM EDTA buffer. We routinely introduced a 100 bp DNA ladder (Invitrogen) as a size marker. Gels were stained with ethidium bromide and photographed under a UV lamp.

Confocal Microscopy
Confocal microscope analysis was carried out using the TCS SP Leica microscope (Lasertechnik) equipped with a ×63 objective (plan apo; NA 1.4). A focal series was collected for each specimen every 0.5 μm for nuclei from the CHO–NT1–EGFP cell line. Each confocal image shown here corresponded to the middle of seven optical serial sections. For each optical section, double fluorescence was simultaneously acquired using a krypton–argon mixed-gas laser adjusted to
488 nm for GFP and to 568 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 four lines in order to reduce signal noise. Selected paired sections were then processed to produce a single overlay image (color merged) using a PC computer equipped with Photoshop software (version 6.0) (Adobe; Tucson, AZ). To quantify the intensity of nuclear labeling, Leica TCS-NT software was used. For each experiment, the middle optical sections of 10–15 nuclei from control cells and 10–15 nuclei from treated cells were studied with the same confocal acquisition parameters. An ellipse was drawn around each nucleus to select the region to analyze and the activated pixel intensity was measured. The results provided include the area and the mean intensity of the selected region.

**Results**

**Nuclear Distribution of NT-1 Receptor Immunoreactivity in Neuron Cell Bodies of Rat Substantia Nigra**

Substantia nigra (SN) contains a dense network of NT-containing axon terminals interspersed among dopaminergic neurons, which highly express NT-1 receptor (Boudin et al. 1998). In SN neurons, NT-1 receptor immunoreactivity is mostly intracellular and is primarily located on dendrites, axons, and axon terminals. NT-1 receptor immunoreactivity found on nerve cell bodies represents 10.6% of total labeling (Boudin et al. 1998). Using electron microscopy, we examined the detailed distribution of NT-1 receptor immunoreactivity in SN cell bodies (Figure 1) and observed that 30% of the gold particle grains were associated with the nuclear compartment. In the nucleus, 87 ± 4.4% of the gold grains were found inside of the nucleus, whereas 13 ± 4.6% were distributed on the nuclear envelope. The significant proportion of NT-1 receptors inside the nucleus led us to study additional cell models to determine if the intracellular localization was related to the presence of the agonist in the extracellular medium.

**Purity and Characterization of the Subcellular Fractions Isolated from CHO–NT1 Cell Line**

Several detection procedures were employed to ensure purity of the subcellular fractions isolated from the CHO–NT1 cell line. First, unlabeled cell suspensions were purposely contaminated with labeled nuclei or labeled membrane homogenates purified from cell cultures treated with L-[35S]-methionine and L-[35S]-cysteine, as described in the experimental procedures. This technique revealed a slight contamination (4%) of the nuclear fraction by cytosolic and plasma membranes (Table 1). Our approach to prepare nuclei has been to adopt a gentle homogenization method and to avoid detergents. This technique resulted in a large loss of nuclei but permitted the subsequent use of binding assays. Equally, the sucrose concentration employed in the cell suspension fractionation determined the quality and the quantity of purified nuclei. To quantitatively diminish the membrane contamination within the nuclear fraction, we increased the sucrose molarity, resulting in a low quantity of sedimented nuclei.

The purity of the nuclear fraction was also assessed by assaying the enzymatic activity for selected markers in the cellular fractions. Table 2 shows the activity of marker enzymes in nuclei prepared from CHO–NT1 cells. Activity of the ouabain-sensitive Na⁺/K⁺-ATPase and K⁺-EGTA ATPase was undetectable in the

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**Table 1** Estimation of contamination between subcellular fractions

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<thead>
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<th>Presence of radioactive nuclei (%)</th>
<th>Presence of radioactive membranes (%)</th>
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<tr>
<td>Membrane homogenates</td>
<td>17</td>
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<td>Nuclear fraction</td>
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**Figure 1** Electron micrograph showing the immunoreactivity of immunogold-labeled NT-1 receptor in neuron cell bodies of the substantia nigra. Gold particles were seen in association with the nuclear envelope (NE) (arrows) and were also present inside the nucleus (N2) (arrowheads). Some immunogold particles were located on the plasma membrane (PM). Others were located in the cytoplasm in association with mitochondria (M), and others in vesicles (V). N1, nucleolus. Bar = 2.3 μm.
nuclear fraction. A second marker for the plasma membrane, 5'-nucleotidase, revealed an average 6% contamination of the nuclei by plasma membrane components. Taken together, we concluded that the nuclear preparations were only marginally contaminated with 5% of plasma membrane, based on the average of the two procedures.

NT-1 Receptors and Neurotensin-binding Sites Are Found in Purified Nuclei from Cell Lines Overexpressing NT-1 Receptor

Cytoplasmic membranes obtained from the CHO–NT1 cell line were previously shown to exhibit a $K_d$ of 0.44 ± 0.01 nM and a $B_{\text{max}}$ of 1960 fmol/mg protein (Boudin et al. 1995). [125I]-NT binding analyses carried out on cell membrane homogenates, purified nuclei, and chromatin from CHO–NT1 cells exhibited a high specific binding of 98 ± 0.6%, 94.6 ± 1.5%, and 74.6 ± 4.2%, respectively. Scatchard analyses were performed on cell membrane homogenates and purified nuclei (Figure 2A). Calculations from Scatchard plots showed a lower affinity in the purified nuclei fraction than in the cell membrane homogenates, with a $K_d$ of 1.2 ± 0.3 nM vs 0.3 ± 0.2 nM, respectively. The Hill coefficients from nuclear and cell membrane fractions were 1 ± 0.1 and 1.1 ± 0.3, respectively, indicating a single population of binding sites. The binding capacity of the cell membrane homogenates fraction was 3808 ± 788 fmol/mg protein. Surprisingly, the $B_{\text{max}}$ of purified nuclei appeared to be very high, 1836 ± 406 fmol/mg protein. However, binding experiments performed in the presence or absence of Gpp(NH)p showed that the nuclear NT-1 receptor population was not sensitive to Gpp(NH)p and therefore was not coupled to G-protein (Figure 2B). In contrast, the cell membrane fraction is coupled to G-protein because it is sensitive to Gpp(NH)p (Figure 2B). Significant specific binding was also present in the chromatin fraction, but the amount of recovered material did not permit further analysis (data not shown).

To visualize localization of the NT-1 receptor in the nuclei, we used a CHO–NT1-EGFP cell line expressing the rat NT-1 receptor fused with the EGFP (Lenkei et al. 2000). Purified nuclei were exposed to confocal microscopy and an image gallery was sequentially acquired. Similarly to what was detected by immunocytochemistry in rat SN, Figure 3 illustrates that the NT-1 receptor–EGFP was found at the nuclear membrane and in the nuclear soma. NT-1 receptor–EGFP was detected at the center of the nucleus, slices 2, 3, and 4.

### Effects of NT Agonist Treatment on NT-1 Receptor Nuclear Localization

We hypothesized that the presence of the NT-1 receptor in SN cell nuclear bodies was related to the dense NT innervations of this brain area. We therefore investigated the role of an NT agonist, JMV 449, on the NT-1 receptor nuclear localization in CHO–NT1-EGFP cells treated with 1 μM JMV 449 for 6 hr. Nuclei from treated and untreated CHO–NT1-EGFP cells were labeled for NT-1 receptor immunoreactivity and visualized by confocal microscopy. First, we confirmed that the NT-1 receptor is still fused to EGFP by immunocytochemistry by using an antibody directed against the N-terminal portion of the NT-1 receptor. As shown in Figure 4A, (above), an overlap between EGFP labeling (green) and NT-1 receptor immunore-
activity (red) was observed. These results confirm the presence of the NT-1 receptor inside the nucleus and in the nuclear envelope. In nuclei from cells treated with the agonist, we observed that both EGFP (green) and NT-1 receptor (red) labelings were always more intense compared to control nuclei (Figure 4A). Quantification of EGFP intensity was performed on the central slice after each image acquired by confocal microscopy. Figure 4B shows that, after JMV 449 treatment, the EGFP (green) intensity is increased by twofold, resulting in an accumulation of NT-1 receptors both in nuclear soma and in the nuclear envelope.

NT-binding Sites from Purified Nuclei of Cells Endogenously Expressing NT and NT-1 Receptor

The result observed in CHO–NT1-EGFP cells confirmed the hypothesis that NT-1 receptor nuclear localization was the consequence of NT stimulation. We then investigated localization of the NT-1 receptor in a cell line having a native NT autocrine regulation. The human lung cancer cell line LNM35 exhibits high expression of both NT and the NT-1 receptor, as suggested by the PCR amplicon intensity for NT and NT-1 receptor transcripts (Figure 5A). Binding studies performed on LNM35 crude membrane extracts exhibited an 80 ± 4.8% specific binding for [125I]-NT. Scatchard analysis showed a single population of sites (Figure 5B). The Kd value and the Hill coefficient (0.22 ± 0.07 nM and 1.04 ± 0.06 nM, respectively) were similar to those obtained for cloned and endogenous human high affinity NT-1 receptors (Vita et al. 1993; Najimi et al. 1998). The NT binding capacity found in LNM35 crude membrane extracts was 98.3 ± 23.4 fmol/mg protein. To identify the presence of the nuclear NT-1 receptor in LNM35 cells, subcellular fractionation was carried out as described in the experimental procedures. [125I]-NT binding analyses were performed on cell membrane homogenates, purified nuclei, and chromatin. Specific [125I]-NT-binding was detected in cell membrane homogenates (80.6 ± 4%), in the nuclear fraction (32.4 ± 2.4%), and in chromatin (72.7 ± 3.5%), indicating the presence of NT-1 receptor-specific sites in these cell fractions. Unfortunately, the recovery of purified nuclei from LNM35 was very poor, and the level of NT nuclear sites was low (16,210 ± 3690 cpm/mg protein). Therefore, proper Scatchard analyses could not be performed on purified nuclei. Nevertheless, to further characterize the NT-1 receptor localized in LNM35 purified nuclei, binding experiments were done in the presence or absence of Gpp(NH)p. As shown in Figure 5C, the specific binding was affected by the non-hydrolysis analogues of GTP in the nuclear fraction, indicating that the nuclear sites were coupled to G-protein. The cell membrane homogenate fraction

Figure 3  Image gallery from confocal microscopy of purified nuclei isolated from CHO–NT1-EGFP cells. A focal series of seven optical sections was collected for each nucleus every 0.5 μm. Bar = 1.7 μm.
was also sensitive to Gpp(NH)p. The specific \(^{[125]I}\)-NT binding decreased by threefold in comparison to control (Figure 5C). These results argue for the presence of G-protein-coupled NT-1 receptor in purified LNM35 nuclei. To evaluate the relationship between NT transmission and the NT-1 receptor localization in the nuclear compartment, LMN35 cells were treated for 48 hr with 10\(^{-5}\) M SR 48692, an NT-1 receptor antagonist (Gully et al. 1993). By blocking the neurotensinergic transmission, nuclear expression of the NT-1 receptor strongly declined in the LNM35 cells (Figure 5D). Indeed, when cells were treated with SR 48692, the NT-binding capacity in the purified nuclei and in the cell membrane homogenates was considerably decreased, by 72% and 90%, compared to their respective controls (Figure 5D).

**Figure 4**  Effect of an NT agonist on the nuclear NT-1 receptor. CHO-NT1-EGFP cells were treated or not with 1 μM JMV 449 at 37°C for 6 hr and subcellular fractions were prepared. (A) Nuclei were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with goat polyclonal anti-NT-1 receptor, followed by Texas Red-conjugated rabbit anti-goat to detect NT-1 receptors as described in Materials and Methods. Images were acquired by confocal microscopy and each picture corresponds to the central section of a serial xy optical section of 0.5 μm. The NT-1 receptor column shows the NT-1 receptor immunocytochemistry and its recovered red fluorescence. The third column displays the overlay from the confocal images of nuclei from control or treated cells recovered in green and red; co-localization is identified as yellow. The fourth panel represents an xz optical section perpendicular to the xy optical section. Bar = 1.7 μm. (B) Quantitative analysis of the nuclear labeling in nuclei from CHO-NT1-EGFP cells treated or not with JMV 449 for 6 hr. **p<0.01 vs control.
Discussion

GPCRs are cell membrane receptors that mediate signal transduction as a consequence of an interaction with their respective ligands. A few years ago, several studies described the presence of GPCR inside of the nucleus and at the nuclear envelope (Dana et al. 1989; Tang et al. 1992; Belcheva et al. 1993). However, to date the events that generate this phenomenon remain unknown.

In this study, in vivo and in vitro cell models were used to demonstrate the physiological relevance of the agonist-induced localization of the NT-1 receptor to the nuclear envelope and the nuclear soma. First, we studied rat SN neuronal cells, which express NT-1 receptors in neural cell bodies and dendrites (Boudin et al. 1998). This brain area is also innervated by a dense network of NT-containing axon terminals, and is consequently exposed to frequent NT stimulation (Jennes et al. 1982). We observed that NT-1 receptors were found within the nuclear soma of rat SN neuron cell bodies (Figure 1).

In a complementary approach, experiments with CHO–NT1-EGFP cells showed a direct increase in the nuclear localized NT-1 receptor due to agonist exposure. These results were confirmed in a cell line that endogenously expresses NT and the NT-1 receptor, LNM35. We further demonstrated the effect of an NT antagonist, SR 48692, to extensively inhibit the binding capacity of the NT-1 receptor from LNM35 purified nuclei. These data support the hypothesis that NT acts as the inductive agent for accumulation of the NT-1 receptor in the nuclear compartment.

Agonist-induced targeting of GPCRs at the nuclear envelope and the nuclear soma has been previously observed. In the case of angiotensin type 1 receptor, receptor immunoreactivity was strongly increased in
the presence of angiotensin II and in a dose-dependent manner. This effect was blocked by an angiotensin type 1-specific antagonist (Lu et al. 1998). In a similar finding, nuclear upregulation of opioid binding sites in the nuclear envelope and nuclear soma of NG108-15 cells was observed after a long agonist exposure (Belcheva et al. 1995). The localization of GPCRs to the nucleus in cells exposed to agonist stimulation suggests that the function of the nuclear NT-1 receptor could be related to the cell response evoked by intense and persistent agonist stimuli. In the case of NT, the latter phenomenon may occur in various pathological situations, such as in chronic pancreatitis or in tumors with endocrine differentiation, where NT plasma or tumor tissue concentration is exceedingly increased (Theodorsson–Norheim et al. 1983; Nustede et al. 1991; Meggiato et al. 1996).

The preparation of purified nuclei is a delicate task because the nuclei are fragile and difficult to keep intact. Moreover, contamination with plasma membrane is expected during nuclear preparative purification. We obtained nuclei having 95% of purity, and we estimated that the cross-contamination could represent at most 20% of the total specific cpm of the nuclear fraction. Therefore, a large majority of the specific binding was generated by the nucleus-localized receptor. We also observed that the nuclear NT-1 receptor increased in the presence of the agonist and that a specific NT-1 receptor antagonist reduced the nuclear binding capacity in cells endogenously expressing NT. We can rule out that the diminished binding capacity of the nuclear fraction was due to site occupancy by the receptor antagonist, because we have previously demonstrated that SR 48692 does not internalize with the NT-1 receptor. Therefore, down-regulation of NT-1 receptors within the nuclei occurred either via blockade of receptor trafficking or blockade of the transduction signal from the cell membrane. However, we did not detect any effect of the NT agonist on the transgene promoter used to generate the stable cell lines (data not shown). These data imply that the accumulated nuclear NT-1 receptor would correspond to internalized receptor, sequestered in the nuclear compartment.

We found that the NT-1 receptor was coupled to G-protein in the nuclear fraction of LNM35 cells. This observation is in agreement with other GPCRs, such as angiotensin type 1, VIP, opioid, prostaglandin, and muscarinic receptors found in the nuclei or in the nuclear envelope (Booz et al. 1992; Belcheva et al. 1993; Ventura et al. 1998; Bhattacharya et al. 1999). At the opposite, nuclear NT-1 receptor from CHO-NT1 was not sensitive to Gpp(NH)p. In CHO-NT1, the Bmax of purified nuclei appeared to be equivalent to the Bmax observed in plasma membrane (Boudin et al. 1995), suggesting an abnormal NT-1 receptor overexpression in the nuclear fraction. In this fraction, coupled NT-1 receptors would probably be undetectable, and they would be hidden by the response of the uncoupled receptors. For the same reason, the high expression of NT-1 receptor-EGFP in the nuclei of control CHO–NT1-EGFP cells is probably abnormally increased.

The activation of renin, angiotensinogen, opioid peptide, or inducible NO synthase gene transcription was previously reported in experiments in which purified nuclei bearing angiotensin type 1, opioid receptors, or the prostaglandin E2 receptor EP3 were exposed to their respective agonists (Eggena et al. 1993; Ventura et al. 1998; Bhattacharya et al. 1999). If an additional biological role for nuclear receptor GPCRs is to activate specific genes, the question remains about how the agonists reach the nucleus. In the case of NT, it was previously demonstrated that NT was internalized with its receptor and was retrieved from the perinuclear region within small vesicular organelles (Faure et al. 1995a,b). Moreover, further studies indicated that NT was recruited to the trans-Golgi network from late or recycling endosomes (Vandenbulcke et al. 2000). It would therefore be consistent to suggest that internalized NT could stimulate nuclear NT-1 receptors to activate specific gene transcription.

The present data reveal that nuclear localization of NT-1 receptor is related to agonist exposure. However, further investigations are necessary to define the specific intracrine biological role of receptor NT-1 localized in the nuclear envelope and nuclear soma. These findings, in addition to similar observations made with several receptors from the same family, suggest an additional function for GPCRs related to hormonal regulation.

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