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Pharmacological properties of the mouse neurotensin receptor 3. Maintenance of cell surface receptor during internalization of neurotensin

Valérie Navarro[a], Stéphane Martin[a], Philippe Sarret[a], Morten S. Nielsen[b], Claus M. Petersen[b], Jean-Pierre Vincent[a], Jean Mazella[a,*]

[a] *Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, UMR 6097, 660 route des Lucioles, 06560 Valbonne, France*
[b] *Department of Medical Biochemistry, University of Aarhus, 8000 Aarhus, Denmark*

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Abstract We recently reported the molecular identification of a new type of receptor for the neuropeptide neurotensin (NT), the neurotensin receptor 3 (NTR3), identical to sortilin, which binds receptor-associated protein. Here, we demonstrate that the cloned mouse NTR3 is expressed on the plasma membrane of transfected COS-7 cells. The mouse NTR3 is detectable by photoaffinity labeling and immunoblotting at the cell surface as a 100 kDa *N*-glycosylated protein. Biochemical analysis and confocal microscopic imaging clearly indicate that NT is efficiently internalized after binding to NTR3, and that despite this internalization, the amount of receptor present on the cell surface is maintained.

Key words: Neurotensin; Receptor; Sortilin; Internalization; Cloning

1. Introduction

Among the three identified neurotensin (NT) receptors, NTR1 and NTR2 belong to the family of G protein-coupled receptors (GPCRs) [1–3], whereas the latest cloned protein NTR3 represents a new type of neuropeptide receptor [4]. Indeed, this NTR, also named sortilin, is a typical type 1 membrane protein with a large luminal domain, a single transmembrane domain and a short cytoplasmic tail [5]. NTR3 binds both NT and the 39 kDa receptor-associated protein (RAP), an endoplasmic reticulum-resident protein [6]. The receptor is synthesized as an inactive precursor which binds ligands only after furin cleavage in late Golgi [7], and also binds lipoprotein lipase (LpL) [8].

The high affinity NT receptor (NTR1) as well as the levocabastine-sensitive NT receptor (NTR2) are both expressed on the plasma membrane (PM) of transfected cells, and their binding to NT can be demonstrated using whole cells as well as membrane homogenates [9,10]. The transduction mechanism of NTR2 has not been clearly established [2,10], whereas the coupling of the NTR1 to phospholipase C was demonstrated in several eukaryotic expression systems [9,11,12]. Both receptors are internalized after NT binding [10,13], suggesting a functional regulation of their cell surface expression. However, the sorting of internalized NTR1 and NTR2 differs since the NTR2 recycles to the PM whereas the NTR1 remains intracellularly sequestered [10,13].

The binding of NTR3 to RAP and NT was originally established using 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS)-solubilized extracts of human brain membranes and transfected COS cells [4,5]. These reports demonstrated that a large pool of NTR3 was localized in Golgi and intracellular vesicles. However, recent studies demonstrated that in response to insulin, NTR3 was translocated with the glucose transporter Glut4 to the PM in rat adipocytes [14,15], and more recently, NTR3 was shown to mediate uptake and degradation of the LpL [8]. However, nothing is known about the interaction of NT with the NTR3 present at the PM of eukaryotic cells. In this work, we isolated the mouse brain counterpart of the human NTR3. We also showed for the first time that NT is able to bind the expressed receptor when present at the PM of transfected COS-7 cells. This binding is followed by a rapid and sucrose-sensitive internalization of the ligand–receptor complex but the amount of receptor at the cell surface remained unchanged.

2. Materials and methods

2.1. Materials

NT was from Peninsula Laboratories and NT(2–13) was synthesized by Neosystem. SR48692 was from Sanofi Recherche. ^{125}I-Tyr$_3$-NT, α-azidobenzoyl-^{125}I-Tyr$_3$-NT(2–13), and fluorescent NT were prepared and purified as described previously [16–18]. The pTARGET expression vector was from Promega. Taq DNA polymerase and reagents for PCR were from Appligene, oligonucleotides from Eurogentec, Dulbecco's modified Eagle's medium from Life Technologies Inc. CHAPS, cholesteryl hemisuccinate (CHS), gentamicin, 1,10-phenanthroline, phenylarsine oxide (PheAsO) and monensin were from Sigma France, and fetal calf serum was from Roche Diagnostics.

2.2. cDNA cloning and expression of the mouse NTR3

10^6 clones derived from the mouse cDNA library [2] were screened by hybridization with a *Hin*dIII fragment (900 bp) of the human NTR3 cDNA as previously described [2]. One hybridization-positive clone was isolated by repeated purification. Both strands were sequenced using the ABI-Prism DNA sequencing kit (Applied Biosys-

*Corresponding author. Fax: (33)-4-93 95 77 08.
E-mail: mazella@ipmc.cnrs.fr

Abbreviations: NTR, neurotensin receptor; NT, neurotensin; PheAsO, phenylarsine oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHS, cholesteryl hemisuccinate; RAP, receptor-associated protein; PAGE, polyacrylamide gel electrophoresis; LpL, lipoprotein lipase; PM, plasma membrane; PCR, protein-coupled receptor; HDM, high density microsomes; LDM, low density microsomes; Cyto, cytosol

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tems, Foster City, CA). The 2.5 kb corresponding to the coding region of the mouse NTR3 was subcloned after PCR amplification into the eukaryotic expression vector pTARGET.

Transient transfections were performed with 1–5 μg of recombinant vector by the DEAE-dextran precipitation method [19]. Binding and internalization assays were performed either on cells plated in 12 mm cell culture dishes or on CHAPS-solubilized extracts prepared approximately 60 h after transfection.

2.3. Solubilization of mNTR3 and binding assay

Cell homogenates were solubilized as previously described [4]. CHAPS-solubilized extracts (25 μg of protein) were incubated with ^{125}I-Tyr$_3$-NT (2000 or 50 Ci/mmol) for 1 h at 0°C in 250 μl of 25 mM Tris–HCl (pH 7.4), 0.1% CHAPS (w/v) and 0.02% CHS (w/v) (CHAPS buffer). Bound ligand was separated from free ligand by filtration on GF/B filters pretreated with 0.3% polyethyleneimine [9]. Non-specific binding was determined in the presence of 1 μM unlabeled NT. Crude and CHAPS-solubilized homogenates from non-transfected COS-7 cells were totally devoid of specific ^{125}I-Tyr$_3$-NT binding.

2.4. Binding experiments to transfected cells

Kinetics experiments were performed on cells plated in 12 mm cell culture dishes. Cells (2×10^5) were equilibrated for 10 min in an Earle's Tris–HEPES buffer, pH 7.5, supplemented with 0.1% glucose and 0.1% bovine serum albumin in the presence or in the absence of 0.45 M sucrose or 10 μM PheAsO, an internalization blocker [17]. The equilibration buffer was then replaced by 250 μl of binding buffer containing 0.4 nM ^{125}I-Tyr$_3$-NT in the presence of 0.8 mM 1,10-phenanthroline for various periods of time at 37°C. At the end of incubation, cells were washed twice with 0.5 ml of equilibration buffer or with 0.5 ml of the same buffer containing 0.5 M NaCl (pH 4) twice for 2 min to remove non-sequestered radioactivity. Cells were harvested with 0.1 N NaOH and counted in a γ counter. Non-specific binding was determined with 1 μM unlabeled NT.

Saturation experiments were performed in the presence or in the absence of 25 μM monensin, a recycling inhibitor [17], using concentrations from 0.5 to 10 nM ^{125}I-Tyr$_3$-NT (200 Ci/mmol). In parallel experiments, cells were also incubated as above in the presence of 0.45 M sucrose which inhibited clathrin-dependent sequestration. At the end of incubation, cells were washed, harvested and counted as described above.

2.5. Measurement of cell surface receptor translocation

Transfected COS-7 cells were incubated for various times at 37°C with 100 nM of NT. Free peptide and peptide remaining bound to the cell surface were removed by a series of washes with ice-cold buffers: three washes with Earle's buffer, pH 7.5, two washes with 150 mM NaCl, 5 mM acetic acid, and three washes with the Earle's buffer. Finally, the amount of cell surface receptors was measured as described above in the presence of the internalization blocker PheAsO.

2.6. Photoaffinity labeling on whole cells

α-Azidobenzoyl-^{125}I-Tyr$_3$-NT(2–13) (0.25 nM) was incubated in the absence or in the presence of 10 μM PheAsO for 30 min in the dark at 37°C with whole COS-7 cells transfected with the mNTR3 cDNA. Non-specific labeling was determined in the presence of 10 μM NT. Cells were then washed twice for 2 min either with ice-cold binding buffer or with the same buffer containing 0.5 M NaCl (pH 4) and directly irradiated on ice for 10 min with a 6 W UV lamp (253 nm). Cells were scraped and analyzed by SDS–PAGE as previously described [13]. Radiolabeled bands were finally detected using a Phosphorimager.

2.7. Subcellular fractionation and Western blot analysis

Cells transfected with the cDNA of the mNTR3 were either left in the basal state or treated with 100 nM NT for 30 min and homogenized in 0.25 M sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM PMSF, then subjected to differential centrifugation as previously described [20]. Proteins of each fraction were assayed for Na^+K^+ATPase activity. A six-fold enrichment in enzyme activity was found in the PM fraction by comparison with whole cell homogenates whereas the other fractions were devoid of Na^+K^+ATPase activity. Proteins recovered from different fractions were separated on an 8% polyacrylamide gel according to Laemmli [21], and transferred on nitrocellulose membranes. Immunoblotting was performed as previously described [5] using a 1:1000 dilution of anti-NTR3 antibody, and revealed by the Lumilight enhanced chemiluminescence method (Roche Diagnostics).

2.8. Confocal microscopic studies

Cells transfected with the mNTR3 were grown on glass microscope slides pretreated with polylysine (10 μg/ml) and incubated for 30 min at 37°C in Earle's buffer, pH 7.5, containing 20 nM α-BODIPY-NT(2–13). Cells were washed with the hypertonic acid buffer as above and air-dried before confocal microscopy. For immunocytochemistry, after incubation with 100 nM NT for 30 min at 37°C, cells were fixed for 20 min at room temperature with 4% paraformaldehyde in phosphate-buffered saline (PBS). For conditions allowing permeabilization, cells were treated in PBS containing 10% horse serum and 0.05% Triton X-100 for 20 min at room temperature before incubation with the NTR3 antibody (1:300) in PBS containing 5% horse serum and 0.05% Triton X-100 for 1 h at room temperature. Finally, cells were incubated with the secondary Texas red donkey anti-rabbit antibody (1:500) (Jackson Immunoresearch Laboratories, Inc.) for 1 h at room temperature in the buffer used above. To visualize the cell surface labeling (without permeabilization), cells were treated in identical conditions in the absence of Triton X-100. Permeabilized and non-permeabilized cells were mounted in Mowiol (Sigma) and examined under a Leica inverted microscope equipped with an argon/krypton laser. Images of cells were acquired as single optical sections taken through the middle of the cells and averaged over 32 scans/frame.

3. Results

The screening of a mouse brain cDNA library [2] allowed us to isolate a single clone of 8.5 kb. The largest open reading frame of this cDNA encodes a protein of 825 amino acids (GenBank, AAF22639) which shares 95% and 92% identity with the rat and human NTR3, respectively [4,5,14]. All the structural elements described for human NTR3 were preserved in the mouse counterpart including the furin cleavage site.

3.1. Specificity of the cloned mouse NTR3

The binding properties of the mNTR3 were first characterized on CHAPS-solubilized extracts of COS-7 cells transiently transfected with the cloned cDNA. The abilities of NT agonists or antagonists and of unrelated peptides to inhibit the binding of ^{125}I-Tyr$_3$-NT to the CHAPS-solubilized mNTR3 and whole cells were compared to data obtained using the solubilized human NTR3 [4]. As shown in Table 1, the pharmacological profiles were similar since NT and Trp$_{11}$NT were more potent than NN and D-Trp$_{11}$NT. The non-peptide antagonist SR48692 and levocabastine were unable to inhibit ^{125}I-Tyr$_3$-NT binding to solubilized extracts as well as to whole cells at concentrations below 10 μM (Table 1). We also tested the ability of RAP to inhibit the binding of NT on whole COS cells expressing mNTR3; RAP competed with iodinated NT with an IC_{50} of 100 ± 8 nM ($n=2$), a value close to that obtained for RAP binding to purified soluble minireceptors secreted by CHO cells [7].

3.2. Binding and internalization of the mouse NTR3 expressed in eukaryotic cells

Kinetics of the ^{125}I-Tyr$_3$-NT association to transfected COS-7 cells at 37°C showed that a large fraction of bound ligand (about 75% at 60 min) was resistant to the hypertonic acid wash (Fig. 1A) and then represented internalized ligand. In the presence of hyperosmotic sucrose (0.45 M), conditions which block the clathrin-dependent endocytosis, the hyperton-

Table 1
Compared IC_{50} values of NT and its related compounds in competition experiments with ^{125}I-Tyr$_3$-NT on CHAPS-solubilized extracts from COS-7 cells expressing human [4] or mouse NTR3 and on whole cells expressing the mNTR3

	Solubilized hNTR3	Solubilized mNTR3	Whole cells mNTR3
NT	17 nM	45 ± 8 nM	40 nM
Trp$_{11}$-NT	17 nM	10 ± 5 nM	60 ± 14 nM
Neuromedin N	63 nM	> 1 μM	600 ± 100 nM
D-Trp$_{11}$-NT	72 nM	> 1 μM	1 μM
Xenin	> 1 μM	219 ± 5 nM	215 ± 30 nM
Levocabastine	> 10 μM	> 10 μM	> 10 μM
SR48692	3 μM	> 10 μM	> 10 μM

The values represent the mean ± S.E.M. from at least three independent experiments performed in duplicate.

ic acid wash totally dissociated ^{125}I-Tyr$_3$-NT bound to cells (Fig. 1A). Experiments performed in the presence of PheAsO or at 4°C showed kinetic profiles identical to those obtained in sucrose (not shown).

The specific binding of ^{125}I-Tyr$_3$-NT at 37°C to COS-7 cells expressing the mNTR3 was concentration-dependent and saturation was obtained at concentrations up to 200 nM (Fig. 1B). The K_d value was about 40 nM and the maximal binding capacity (B_{max}) reached 500 fmol/mg protein, a value similar to that observed in neurons (250 fmol/mg [17]). However, in the presence of hyperosmotic sucrose, which makes it possible to determine the amount of receptor initially present at the cell surface, the B_{max} was 100 fmol/mg protein, whereas the K_d value was not affected (36 ± 7 nM). In the presence of the recycling inhibitor monensin, the B_{max} was 250 fmol/mg protein, a value indicating that part of the binding measured at 37°C in the absence of drug was due to recycling of internalized receptor.

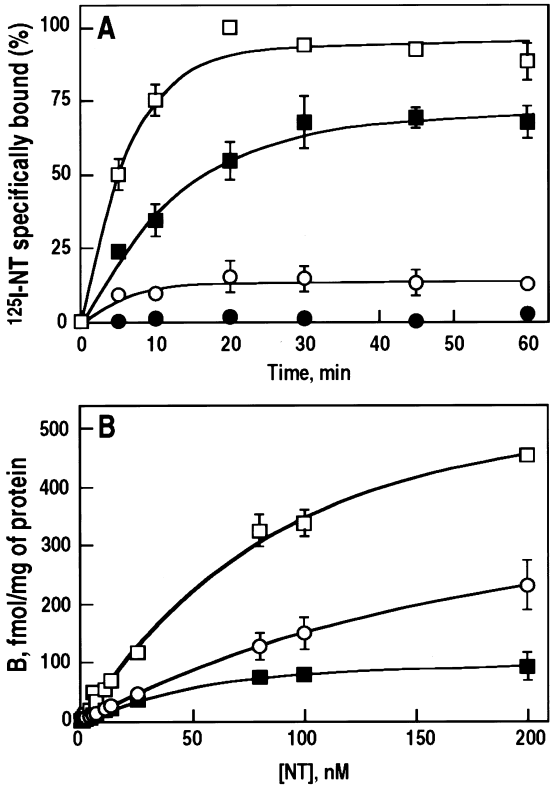

Fig. 1. Binding properties of the mouse NTR3 on whole COS-7 cells. A: Kinetics for ^{125}I-Tyr$_3$-NT binding to whole COS-7 cells expressing the mouse NTR3. Incubations were performed in the presence (circles) or in the absence (squares) of 0.45 M sucrose, followed (closed symbols) or not (open symbols) by an acid NaCl wash. Values are expressed as the percent of total cell-associated ligand at each time. Each point represents the mean ± S.E.M. of at least three different experiments with duplicate determination. B: Saturation of specific ^{125}I-Tyr$_3$-NT binding to whole COS-7 cells expressing the mouse NTR3. Experimental details are described in Section 2. Briefly, cells were incubated at 37°C with increasing concentrations of ^{125}I-Tyr$_3$-NT in the absence (open squares) or in the presence of 25 μM monensin (open circles) or 0.45 M sucrose (closed squares). Results are expressed as the mean ± S.E.M. from three different experiments.

3.3. Photoaffinity labeling experiments

COS-7 cells transfected with the mNTR3 were covalently labeled with ^{125}I-Tyr$_3$-α-azido-NT(2–13) under various experimental conditions (Fig. 2). Autoradiogram revealed that a single protein with a M_r of about 100 kDa was specifically labeled at 37°C (Fig. 2). The labeling of the 100 kDa protein remained intense after hypertonic acid treatment which dissociates the surface-bound ligand. In the presence of PheAsO, the same 100 kDa protein was labeled but with a weaker intensity. This labeling corresponded to the labeling of the

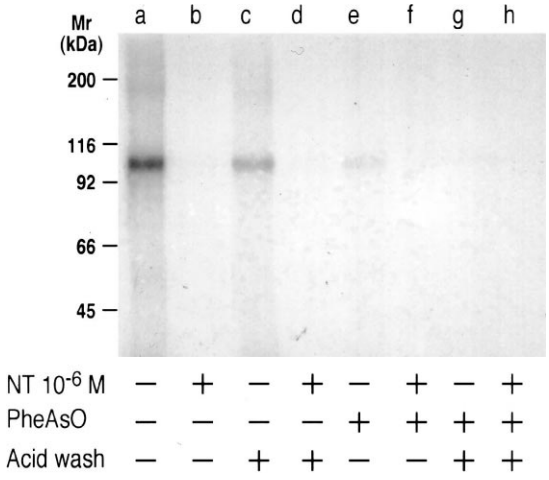

Fig. 2. Photoaffinity labeling of COS-7 cells expressing the mouse NTR3 using α-azidobenzoyl-^{125}I-Tyr$_3$-NT(2–13). Cells were incubated in the dark with 0.25 nM of α-azidobenzoyl-^{125}I-Tyr$_3$-NT(2–13) for 30 min at 37°C in the presence (e–h) or in the absence (a–d) of 10 μM PheAsO. The non-specific labeling was obtained in the presence of 10 μM NT. Cells were washed with either ice-cold buffer (a, b, e, f) or hypertonic acid buffer (c, d, g, h) and irradiated by a UV lamp for 10 min at 4°C. Samples were analyzed by SDS–PAGE and radiolabeled bands were detected with a Phosphorimager.

cell surface receptor since it totally disappeared after the acid wash (Fig. 2). No labeling was observed on non-transfected cells (not shown).

3.4. Western blot analysis of subcellular fractions of COS-7 cells expressing the mNTR3

To quantify the amount of mNTR3 present in the PM and in other subcellular fractions, cells transfected with the mNTR3 were incubated in the absence or in the presence of NT and fractionated to separate the PM, the high and low density microsomes (HDM and LDM), the nuclear fraction and the cytosol (Cyto). On total cell extract, two proteins with M_r of 90 and 100 kDa, respectively, were detected with the anti-luminal NTR3 antibodies (Fig. 3A, lane 1). Treatment of cells with tunicamycin prevented the formation of the 100 kDa protein (Fig. 3A, lane 2) suggesting, in agreement with previous observations [7], that a large portion of the mNTR3 was *N*-glycosylated. Only the glycosylated form of mNTR3 (100 kDa) was present on the PM (Fig. 3B, lanes 1 and 2). In the fraction of LDM and HDM, the two 90 and 100 kDa forms were visualized, the larger (i.e. the glycosylated) form

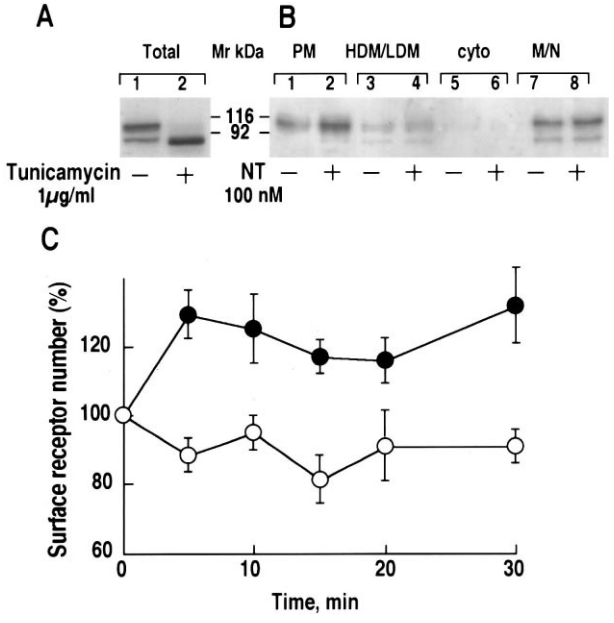

Fig. 3. Western blot analysis of subcellular distribution of mNTR3 in transfected COS-7 cells. A: Cells were incubated (overnight at 37°C) with (lane 2) or without (lane 1) 1 μg/ml tunicamycin, and total cell extracts were separated by SDS–PAGE and immunoblotted for mNTR3. B: Cells were incubated in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of 100 nM NT for 30 min at 37°C. Subcellular fractions were obtained as described in Section 2 and 30 μg of proteins corresponding to PM, HDM and LDM, Cyto, and mitochondria/nuclei (M/N) were separated and analyzed as described in A. Each experiment was carried out three times. Results from one representative experiment are shown. C: Determination of the cell surface number of NTR3. Cells transfected with the mNTR3 were incubated for various times at 37°C with 100 nM NT (closed symbols) or with 100 nM RAP (open symbols). After extensive washes, ^{125}I-Tyr$_3$-NT binding was performed at 37°C in the presence of 10 μM PheAsO to prevent membrane trafficking (internalization, recycling). Values are expressed as percent of the amount of receptor measured in the absence of treatment and are means ± S.E.M. from three independent experiments performed in triplicates.

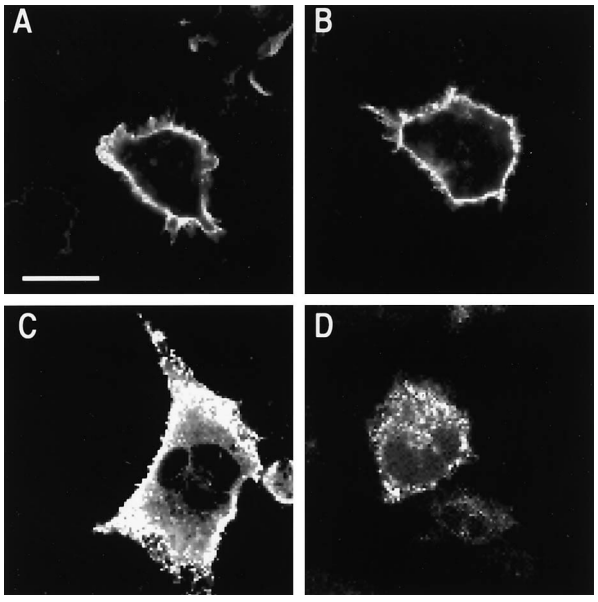

Fig. 4. Confocal microscopic imaging of COS-7 transfected with the mNTR3. The receptor was visualized by immunocytochemistry (A–C) and the ligand with Fluo-NT (D) as described in Section 2. Cells were incubated without the peptide (A and C) or with 100 nM NT (B). Immunolabeling protocol was carried out in the absence (non-permeabilized, A and B) or in the presence of Triton X-100 (permeabilized, C). In D, cells were incubated with 20 nM Fluo-NT for 20 min at 37°C, washed with the hypertonic acid buffer and air dried. Single trans-nuclear optical sections were scanned Scale bar: 10 μm.

being predominant (Fig. 3B, lanes 3 and 4). The Cyto was devoid of NTR3 protein (Fig. 3B, lanes 5 and 6) whereas the nuclear/mitochondrial fraction was enriched in both the 90 and 100 kDa forms of receptor (Fig. 3B, lanes 7 and 8). Quantification by scanning of the 100 kDa protein in each fraction revealed that the PM contained about 20% of the total cell amount of mNTR3 detected by Western blot analysis. Incubation with 0.1 μM NT increased the amount of mNTR3 to the PM (Fig. 3B, lanes 1 and 2). This effect was also observed in experiments carried out on whole COS cells on which unlabeled NT (0.1 μM) was added for various times at 37°C, extensively washed, and the binding to the cell surface was measured in the presence of the internalization blocker PheAsO. In these conditions, NT increased the amount of cell surface binding to 130% from the basal level (Fig. 3C) whereas the other NTR3 ligand RAP was ineffective.

3.5. Confocal imaging of the mNTR3 in transfected COS-7 cells

Immunolabeling of transfected cells incubated with or without unlabeled NT is shown in Fig. 4A–C. In the absence of NT, immunocytochemistry revealed that receptors were present at the cell surface (Fig. 4A) but the prominent labeling was intracellular (Fig. 4C). Following exposure to NT, NTR3 immunofluorescence was still constant at the surface of the cell (Fig. 4B) although the peptide, as visualized by labeling obtained with Fluo-NT, was intensively internalized throughout the cell (Fig. 4D), suggesting that the amount of NTR3 present at the cell surface remained constant.

4. Discussion

The major finding of this work is that the mouse NTR3 protein is expressed in part on the PM of transfected cells where it recognizes and internalizes NT. This is the first direct demonstration that the NTR3 is able to bind NT when present at the cell surface. Indeed, our previous works described the binding of NT only to CHAPS-solubilized brain or cell extracts [4,22]. Although the amount of cell surface receptors is relatively low under basal conditions, the interaction with NT appears to induce the translocation of the protein to the cell surface suggesting a dynamic regulation of the protein subcellular localization. This finding is consistent with a recent work which reported that CHO cells transfected with NTR3 expressed about 10% of receptors on the cell surface and mediated endocytosis and degradation of LpL [8].

Interestingly, under conditions which allow internalization and recycling, binding saturation was obtained only at concentrations of the NT close to 200 nM. These results suggest that in COS-7 cells, NTR3 may accumulate the peptide into the cell by a mechanism involving recruitment of intracellular receptors to the membrane, internalization of the ligand–receptor complexes and recycling of the receptor after dissociation of the ligand, thus acting as a scavenger for NT. Similar conclusions have been drawn for another type I receptor, the cation-independent mannose 6-phosphate/insulin growth factor-II (IGF II) receptor, which has been shown to internalize IGF II and may serve to clear it from the circulation [23].

The pharmacological profile of mNTR3 on whole cells is very similar to that measured on CHAPS-solubilized extracts from cells transfected with either human or mouse NTR3 cDNA. Indeed, NT and Trp^{11}NT display equivalent potencies to inhibit the binding of ^{125}I-Tyr_3-NT ($IC_{50} = 20–40$ nM) whereas levocabastine and the non-peptide antagonist SR48692 are poorly efficient ($IC_{50} > 3$ μM). The only marked difference between the human and the mouse NTR3 is observed with neuromedin N which is 10–20 fold less potent on the mouse NTR3 than on the human counterpart.

Western blot analysis performed on total cell extracts reveals the expression of two proteins with M_r of 100 and 90 kDa, respectively, the high molecular weight form corresponding to the *N*-glycosylated protein. Interestingly, only the glycosylated protein is present at the cell surface whereas the HDM/LDM fraction contains a small proportion of the non-glycosylated form. A large amount of the two NTR3 forms is found in the nuclear/mitochondrial fraction, a result suggesting a possible involvement of the NTR3 in some nuclear gene activation as suggested by the role of the receptor on the growth effects of NT in several cancer cell lines [24]. From Western blot analysis, we can conclude that the mouse NTR3 is expressed on the PM as a glycosylated protein that could be a functional receptor for NT. Photoaffinity labeling experiments confirm that only the higher molecular weight 100 kDa protein is present at the cell surface (Fig. 2). Note that in the presence of the internalization blocker PheAsO making it possible to detect the labeling of the NTR3 initially present on the cell surface, the labeling intensity is lower than in conditions where sequestration and accumulation of the ligand is allowed. These data are in agreement with the relatively low amount of the protein detected by Western blot analysis in the PM fraction (between 10 and 20%). Interestingly, although NT is efficiently internalized with NTR3, the amount of receptor present in the PM is maintained, an observation measured both by Western blot, binding experiments, and visualized by confocal imaging in the absence of permeabilization. This effect is NT-specific since RAP, another known ligand for NTR3, has no influence on the protein membrane translocation. This result suggests that NT may induce a specific activation of a signaling pathway leading to the transport of NTR3 from intracellular compartments to the cell surface. These observations are in agreement with data obtained in primary cultures of neurons naturally expressing NT binding sites [17]. In these cells, NT was shown to be efficiently internalized by a 100 kDa protein after inducing its translocation from an intracellular compartment to the PM [17]. Although the cellular mechanisms by which this regulation is mediated are not known, the mNTR3 can be actually considered a receptor for NT since the peptide triggers the growth of CHO cells expressing the protein [24].

In conclusion, we have shown that the mouse NTR3 is present on the cell surface, that the receptor mediates the binding and the internalization of NT, and that its presence on the surface membrane is regulated by the peptide.

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