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Receptor-mediated internalization of [^3H]-neurotensin in synaptosomal preparations from rat neostriatum

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Abstract

Following its binding to somatodendritic receptors, the neuropeptide neurotensin (NT) internalizes via a clathrin-mediated process. In the present study, we investigated whether NT also internalizes presynaptically using synaptosomes from rat neostriatum, a region in which NT1 receptors are virtually all presynaptic. Binding of [^3H]-NT to striatal synaptosomes in the presence of levocabastine to block NT2 receptors is specific, saturable, and has NT1 binding properties. A significant fraction of the bound radioactivity is resistant to hypertonic acid wash indicating that it is internalized. Internalization of [^3H]-NT, like that of [^{125}I]-transferrin, is blocked by sucrose and low temperature, consistent with endocytosis occurring via a clathrin-dependent pathway. However, contrary to what was reported at the somatodendritic level, neither [^3H]-NT nor [^{125}I]-transferrin internalization in synaptosomes is sensitive to the endocytosis inhibitor phenylarsine oxide. Moreover, treatment of synaptosomes with monensin, which prevents internalized receptors from recycling to the plasma membrane, reduces [^3H]-NT binding and internalization, suggesting that presynaptic NT1 receptors, in contrast to somatodendritic ones, are recycled back to the plasma membrane. Taken together, these results suggest that NT internalizes in nerve terminals via an endocytic pathway that is related to, but is mechanistically distinct from that responsible for NT internalization in nerve cell bodies. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Presynaptic terminals; Endocytosis; Neurotensin receptor; Electron microscopy

1. Introduction

Receptor-mediated endocytosis is a process by which ligands gain access into cells. The internalization of receptor-ligand complexes has been best studied for nutrient and growth factor receptors such as the transferrin and epidermal growth factor receptors, which internalize via clathrin coated pits and vesicles (Pearse and Crowther, 1982; Heuser and Anderson, 1989; Hubbard, 1989). Various G-protein-coupled receptors also internalize in a clathrin-mediated fashion upon exposure to agonist ligands (Grady et al., 1995; Lefkowitz, 1998; Ferguson et al., 1998). Although this process has mainly been documented in transfected or neoplastic cell lines, G-protein-coupled receptors were also shown to internalize in neurons from the central and peripheral nervous

systems. For example, biochemical and histochemical studies have provided evidence for ligand-induced internalization of muscarinic (Maloteaux and Hermans, 1994), dopamine (Dumartin et al., 1998), neurokinin (Mantyh et al., 1995), neurotensin (Mazella et al., 1991), opioid (Sternini et al., 1996), and somatostatin (Stroh et al., 2000) receptors in mammalian neurons. In all of these studies, receptor internalization was documented at the level of neuronal perikarya and dendrites. However, no studies have provided direct evidence for internalization of G-protein-coupled receptors at the level of axon terminals.

It has long been appreciated that the somatodendritic region of neurons differs functionally with regards to sorting and endocytic pathways as compared to axon terminals. For example, differential sorting to axonal and somatodendritic domains has been reported in neurons transfected with viral glycoproteins known to be transported via apical and basolateral segments of polarized cells, respectively (Dotti and Simons, 1990). Horseradish peroxidase and ovalbumin-gold were also shown by

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electron microscopy to be transiting through distinct endocytic pathways in cell bodies compared to terminals of hippocampal neurons in culture (Parton et al., 1992). Therefore, it seems likely that presynaptic receptors may internalize via mechanisms that differ from those of somatodendritic ones.

Neurotensin (NT) is a bioactive neuropeptide involved in a number of brain functions including regulation of locomotor activity, antinociception, temperature regulation, and regulation of pituitary secretion (for reviews, see Kitabgi and Nemeroff 1992). Most of these effects, with the possible exception of antinociception, appear to be transduced through interaction of the peptide with the NT1 receptor, a G-protein-coupled receptor linked to inositol trisphosphate and Ca^{++} signaling pathways (Vincent, 1995; Vincent et al., 1999). Autoradiographic binding and immunohistochemical studies have shown NT1 receptors to be associated with both somatodendritic and axonal domains of selective neuronal subsets, and most conspicuously with dopamine (DA) neurons of the nigro-striatal, meso-cortical, and meso-limbic pathways (Palacios and Kuhar, 1981; Quirion et al., 1985; Szigethy and Beaudet, 1989).

Using fluorescent derivatives, NT was shown to be internalized within the soma and dendrites of midbrain DA neurons, both in primary neuronal cultures and in brain slices (Faure et al., 1995; Nouel et al., 1997). This process is receptor-mediated and sensitive to disrupters of clathrin-dependent endocytosis (Faure et al., 1995; Nouel et al., 1997). Additionally, injection of iodinated or fluorophore-tagged NT into the neostriatum of adult rats in vivo was shown to result in the retrograde labeling of DA perikarya in the ipsilateral substantia nigra, suggesting that the peptide may be also internalized pre-synaptically and transported back to nigro-striatal nerve cell bodies (Castel et al., 1990; Faure et al., 1995). However, this latter experimental model does not lend itself to the characterization of the presynaptic process at the origin of the retrograde transport of the labeled ligands. The aim of the present study was therefore to develop an in vitro approach to determine whether NT indeed undergoes receptor-mediated endocytosis at presynaptic sites and to characterize the mechanisms of this internalization.

2. Materials and methods

2.1. Drugs

The selective NT2 ligand, levocabastine was a gift from Dr. A. Schotte, Janssen Pharmaceuticals, Beerse, Belgium (Schotte et al., 1986). The non-peptide NT antagonist SR48692 was kindly provided by SANOFI Recherche, Toulouse, France (Gully et al., 1993). The carboxylic ionophore monensin and the endocytosis

inhibitor phenylarsine oxide (PAO) were both purchased from Sigma (St Louis, MO).

2.2. Preparation of neostriatal synaptosomes

Adult male Sprague Dawley rats (220–250 g; Charles River, St. Constant, Quebec) were killed by decapitation and their brains were rapidly removed. The neostriatum was dissected from ten rats and pooled in ice-cold 0.32 M sucrose containing peptidase inhibitors (64 mM benzamidine, 10.8 μM leupeptine, 5 $\mu\text{g/ml}$ aprotinin) and 20 mM HEPES, pH 7.4. Synaptosomes were then prepared as previously described (McPherson et al., 1994). In brief, neostriatal tissue was homogenized with a hand glass homogenizer using a Teflon-coated pestle rotating at 900 rpm. The homogenate was centrifuged at 750 g for 5 min at 4 °C and the resulting supernatant recuperated and further centrifuged at 12,000 g for 15 min at 4 °C. The synaptosomal P_2 pellet was equilibrated in 0.32 M sucrose and centrifuged at 14,500 g for 15 min at 4 °C and then resuspended in modified Earles buffer (155 mM choline Cl, 5 mM KCl, 1.8 mM CaCl_2 and 0.9 mM MgCl_2) for experimental use.

2.3. Assessment of synaptosomal integrity

To assess their morphological preservation, synaptosomes were fixed with 2% acrolein-2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 10 min followed by 2% PFA in 0.1 M PB for 20 min and rinsed in 0.1 M PB. They were then post-fixed with 2% osmium tetroxide for 30 min, thoroughly rinsed with 0.1 M PB, dehydrated in graded alcohols and embedded in Epon. Ultrathin sections (80 nm) were cut on an ultramicrotome and counterstained with uranyl acetate and lead citrate prior to examination with a JEOL 100CX electron microscope (Peabody, MA).

To test the effects PAO treatment and of hypertonic acid wash on synaptosomal integrity, synaptosomes were pre-incubated, or not, for 30 min at 4 °C with 10 μM PAO in Earles buffer, followed by 15 min at 37 °C in the same buffer. They were then rinsed, or not, with a hypertonic acid solution (pH4) containing 0.5 M NaCl for 2 min at 25 °C (acid wash). Synaptosomes were then fixed and processed for electron microscopy as described above.

Viability of synaptosomes was further assessed by measuring lactate dehydrogenase activity, an index of cellular disruption (Keiding et al., 1974). In brief, synaptosomes were preincubated for 30 min in Earles buffer in the presence or absence of 10 μM PAO, incubated for 15 min at 37 °C in the same buffer at 37 °C, washed, or not, in hypertonic acid wash, and pelleted by centrifugation at 10,000 g at 4 °C. The pellet was resuspended with 0.32 M sucrose and pelleted again. Lactate dehydrogenase activity was measured spectrophotometri-

ically at 340 nm by following the oxidation of NADH (decrease in absorbance) in the presence of pyruvate (Koh and Choi, 1987).

2.4. [^3H]-NT binding and internalization assays

Prior to performing binding/internalization experiments, the protein content of synaptosomal preparations was determined using a Bio-Rad assay reagent (Bradford). In some experiments, synaptosomal preparations were pretreated at 4 °C for 30 min with Earles buffer containing either: (1) PAO (10 μM), (2) sucrose (0.32 M) or (3) monensin (25 μM) prior to ligand exposure.

For [^3H]-NT saturation binding experiments, 1 mg of synaptosomal proteins were incubated for 15 min at 37 °C with 0.2–1 nM [^3H]-NT in the presence or absence of levocabastine (10 μM) to block low affinity NT2 receptors. For determination of non-specific binding, additional samples were incubated in the presence of 1 μM non-radioactive NT. Specific binding was calculated by subtracting non-specific from total binding. Binding was terminated by two successive additions of 12 ml ice-cold Earles buffer and filtering under vacuum through GF/B filters presoaked for 1–2 hours at 4 °C in Earles buffer containing polyethylenimine (0.3% V/V). Filters were recuperated in plastic vials containing 10 ml scintillation liquid (Ecolite) and counted in a beta counter.

For competition binding experiments, synaptosomes were incubated for 15 min at 37 °C in Earles buffer containing 1 nM [^3H]-NT, 10 μM levocabastine, and increasing concentrations of the NT1 receptor antagonist SR48692 (10^{-12} to 10^{-5} M) and the binding was terminated as above.

For time course binding/internalization experiments, synaptosomes, pretreated or not with PAO, sucrose, or monensin, were incubated for 15 min at 37 °C or 4 °C in Earles buffer containing 1 nM [^3H]-NT and 10 μM levocabastine for 2, 4, 10, 15 or 45 min. To determine the proportion of internalized [^3H]-NT, synaptosomes were rinsed for 2 min with either ice-cold Earles buffer or with a hypertonic acid solution (pH 4) at 25 °C, and the bound radioactivity was counted as above.

To compare internalization of [^3H]-NT with that of [^{125}I]-transferrin ([^{125}I]-Tf) synaptosomes, pretreated or not with PAO or sucrose, were incubated at 37 °C in Earles buffer containing 8 nM [^{125}I]-Tf for 2, 4, 10, 15 or 45 min. They were then rinsed for 2 min with either ice-cold Earles buffer or with a hypertonic acid solution (pH 4) at 25 °C. Synaptosomes were filtered as above, the filters recuperated in plastic tubes, and the radioactivity was counted in a gamma counter.

Values for all binding/internalization experiments represent the mean $\pm$ standard error of the mean from three different pools of synaptosomes per condition. Apparent dissociation constant (Kd) and maximal binding capacity

(Bmax) were derived from Scatchard plot analysis of saturation binding curves.

3. Results

3.1. Integrity of synaptosomes

Synaptosomes were prepared from rat neostriatum, a region in which most if not all NT1 receptors are associated with axon terminals (Quirion et al., 1985; Masuo et al., 1990; Boudin et al., 1996; Fassio et al., 2000). By electron microscopy, synaptosomes corresponded for the most part to sealed axon terminals filled with clear synaptic and large dense cored vesicles and occasionally showing an attached postsynaptic membrane segment (Fig. 1A). Clathrin-coated pits and clathrin-coated vesicles were also occasionally observed (Fig. 1A). In addition to axon terminals, a small number of glial and dendritic remnants were also present amongst terminal profiles (Fig. 1A).

3.2. [^3H]-NT binding to striatal synaptosomes

Neostriatal synaptosomes were incubated with increasing concentrations of [^3H]-NT for 15 min at 37 °C in the presence or absence of levocabastine to inhibit NT binding to low affinity NT2 receptors (Fig. 2A). In the presence of levocabastine, specific [^3H]-NT binding was specific and saturable (Fig. 2A). Scatchard plot analysis revealed a binding affinity (Kd) of 0.3 nM and a maximal binding capacity (Bmax) of 25 fmol/mg protein (Fig. 2A, insert). Non-specific binding, as determined by the addition of 1 μM unlabeled NT, accounted for approximately 20% of total binding at saturation (data not shown). In the absence of levocabastine, [^3H]-NT binding capacity was higher than that measured in the presence of the NT2 receptor ligand and did not reach saturation at the concentration of radioactive ligand used (Fig. 2A).

To confirm that the specific [^3H]-NT binding observed in the presence of levocabastine represented binding to the NT1 receptor, competition experiments were performed in the presence of both levocabastine and increasing concentrations of the selective NT1 antagonist, SR48692. SR48692 competed for specific [^3H]-NT binding with an IC₅₀ of 3.8 nM (Fig. 2B).

3.3. Internalization of [^3H]-NT in striatal synaptosomes

To determine whether specifically bound [^3H]-NT was internalized within neostriatal synaptosomes, synaptosomal preparations were incubated for 2–45 min at 37 °C with 1 nM [^3H]-NT in the presence of levocabastine and the proportion of internalized [^3H]-NT was assessed

Fig. 1. Electron microscopic assessment of striatal synaptosomes. A. Control. Two well preserved axon terminals showing numerous clear synaptic vesicles as well as several clathrin-coated vesicles (arrowheads) are visible in this field. The terminal on the right makes an asymmetric synaptic contact with a preserved dendritic spine (arrow). B. Following hypertonic acid wash, morphological preservation of synaptosomes is comparable to that of controls. Note the asymmetric synaptic contact with the remnants of a post-synaptic element (arrow). C. Synaptosomes treated with PAO also show a fine structure comparable to that of controls. Note the good preservation of plasma membranes and synaptic specialization (arrow). Scale bar=0.4 μ m.

Fig. 2. Pharmacological profile of [3 H]-NT binding to rat neostriatal synaptosomes. A. Saturation of specific [3 H]-NT binding at 37 °C in the absence or presence of levocabastine (10 μ M) at 15 min. Specific binding was obtained by subtracting non-specific from total binding, where non-specific binding was determined in the presence of an excess of unlabeled NT (1 μ M). Insert: Scatchard plot of [3 H]-NT binding in the presence of levocabastine. B. Displacement of specific [3 H]-NT binding by the non-peptide NT1 antagonist SR48692. Specific binding was determined by incubation with 1 nM [3 H]-NT in the presence of 10 μ M levocabastine and increasing concentrations of SR48692 for 15 min at 37 °C.

after dissociating surface-bound ligand by hypertonic acid wash. Specific [3 H]-NT binding saturated at 2 min, reaching a plateau of approximately 20 fmol/mg protein (Fig. 3). Acid wash-resistant [3 H]-NT binding reached a plateau at 15 min with a value of approximately 4.0 fmol/mg protein (Fig. 3). This corresponds to an internalization efficiency (acid wash-resistant/total specific binding at equilibrium) of about 20%.

To determine whether the internalization process was temperature-dependent, saturation experiments were repeated at 4 °C and the results compared with those obtained at 37 °C (Fig. 3). Low temperature reduced the affinity of [3 H]-NT binding as evidenced by the fact that no saturation was achieved at up to 4 nM of [3 H]-NT (data not shown). At this temperature, radioligand binding of 1 nM [3 H]-NT reached a plateau within approximately 4 min to a value of 5.5 fmol/mg protein (Fig. 3). Virtually all [3 H]-NT specifically bound at 4 °C was washed out with hypertonic acid buffer, indicating that there was no ligand internalization at this temperature (Fig. 3).

Fig. 3. Effects of low temperature (4 °C) on the binding and internalization of [3 H]-NT in rat neostriatal synaptosomes. Kinetics of [3 H]-NT binding and internalization at 37 °C versus 4 °C, in the presence of 10 μ M levocabastine. Specific binding was obtained by subtracting non-specific from total binding, non-specific binding being determined in the presence of an excess of unlabeled NT (1 μ M). Hypertonic acid wash, pH 4, was used to dissociate membrane-bound radioligand, thereby providing a measure of internalized ligand.

To determine whether [^3H]-NT internalization was clathrin-dependent, time course experiments were performed at permissive temperature (37 °C) on synaptosomes pretreated with PAO or sucrose, two compounds documented to inhibit clathrin-coated pit formation (Hertel et al., 1985; Heuser and Anderson, 1989). Synaptosomes pretreated with PAO displayed a [^3H]-NT binding profile similar to that of untreated ones: [^3H]-NT binding saturated within 2 min to reach a value of 20 fmol/mg protein (Fig. 4A). By contrast, pretreatment with sucrose decreased both the rate (plateau reached in 4 vs 2 min) and plateau value (13.0 fmol/mg protein) of specific [^3H]-NT binding compared to untreated synaptosomes (Fig. 4B).

Synaptosomes pretreated with PAO displayed an internalization profile virtually identical to that of untreated ones (Fig. 4), indicating that this drug had no effect on [^3H]-NT internalization. By contrast, pretreatment with sucrose significantly reduced the amount of acid wash-resistant [^3H]-NT binding (Fig. 4B), indicating that sucrose efficiently inhibited [^3H]-NT internalization.

To ensure that the PAO-resistant decrease in [^3H]-NT binding observed after hypertonic acid wash was not due to a loss of synaptosomal integrity, we tested the effects of hypertonic acid wash and of treatment with PAO on

synaptosomal fine structure. Following either procedure, synaptosomes displayed the same morphological features as controls and, in particular, equally well preserved plasma membranes (Fig. 1B,C). To confirm that the plasma membranes of acid-washed and/or PAO-treated synaptosomes were not only intact morphologically but were also physiologically viable, we tested the resistance of the plasma membrane to leakage of the cytoplasmic marker, lactate dehydrogenase (Clark and Nicklas, 1970). Lactate dehydrogenase assays showed no leakage of the enzyme, whether synaptosomes had been subjected to hypertonic acid wash or to PAO treatment (not shown), indicating that the synaptosomes were still intact following each of these treatments.

To determine whether the differential effect of PAO and sucrose on [^3H]-NT internalization was specific for NT or a more general characteristic of presynaptic endocytosis, we examined the effects of these two treatments on the receptor-mediated internalization of [^{125}I]-diferric human transferrin ([^{125}I]-Tf). Transferrin receptors are known to internalize constitutively via a clathrin-dependent mechanism (Prekeris et al., 1999) and to be presynaptically associated with dopamine axon terminals in rat neostriatum (Mash et al., 1991). Specific [^{125}I]-Tf binding was specific and saturable (Fig. 4C, D). Non-specific binding accounted for less than 25% of total binding at

Fig. 4. Kinetics of [^3H]-NT and [^{125}I]-Tf binding and internalization following sucrose or phenylarsine oxide (PAO) treatments. Synaptosomes were prepared in Earles buffer containing or not 10 μM PAO (A, C) or 0.32 M sucrose (B, D). A, B. Total [^3H]-NT binding was obtained by incubation with 1 nM [^3H]-NT in the presence of 10 μM levocabastine for increasing time intervals at 37 °C and non-specific binding was determined in the presence of an excess of unlabeled NT (1 μM). Specific binding was obtained by subtracting non-specific from total binding. Internalized ligand defined as radioactivity resistant to hypertonic acid wash (pH 4). C, D. Total [^{125}I]-Tf binding was obtained by incubation with 8 nM [^{125}I]-Tf for increasing time intervals at 37 °C and non-specific binding was determined in the presence of an excess of unlabeled Tf (5 μM). Again, the proportion of internalized ligand was determined by hypertonic acid wash.

saturation. Approximately 53% of this specific binding was acid wash-resistant, i.e. corresponded to internalized ligand (Fig. 4C, D). Neither PAO nor sucrose treatment affected the kinetics of [125 I]-Tf binding, however, there was a 17% decrease in [125 I]-Tf maximal binding capacity in the presence of sucrose (Fig. 4C, D). Following treatment with PAO, the proportion of acid wash-resistant binding remained as high as 65% of total at saturation (Fig. 4C), indicating that the drug had no effect on [125 I]-Tf internalization. By contrast, in the presence of sucrose, the proportion of acid wash-resistant binding was reduced to 28.6% of specific [125 I]-Tf binding at saturation (Fig. 4D), indicating that this treatment did not completely prevent, but greatly reduced [125 I]-Tf internalization.

To determine whether internalization of [3 H]-NT was followed by recycling of internalized receptors to the plasma membrane, internalization assays were repeated in the presence of the ionophore monensin, a drug documented to prevent endosome acidification and to thereby impair ligand–receptor dissociation, an event essential for receptor recycling (Pressman, 1976; Tartakoff, 1983). As can be seen in Fig. 5, treatment with monensin did not significantly alter [3 H]-NT binding kinetics. However, it did decrease the binding plateau by approximately 25% as compared to untreated conditions (Fig. 5). Furthermore, monensin treatment greatly reduced the proportion of hypertonic acid-wash resistant [3 H]-NT binding (Fig. 5), indicating that receptor recycling was essential for efficient ligand internalization.

4. Discussion

The present study provides the first direct evidence for receptor-mediated internalization of a neuropeptide in axon terminals and suggests that mechanisms of receptor internalization in axons may differ from those

previously documented at the level of perikarya and dendrites.

4.1. Validation of synaptosomes as a model system

Synaptosomes are a well-established model for studying nerve terminal function and have been used extensively to examine exocytosis and clathrin-mediated endocytosis. For example, Ca $^{2+}$ -induced glutamate release from synaptosomes, like in intact neurons, is sensitive to clostridial neurotoxins, which cleave components of the exocytic machinery including synaptobrevin, syntaxin, and SNAP-25 (McMahon et al., 1992; Blasi et al., 1994). FM dyes have been used to reveal that clathrin-mediated endocytosis in synaptosomes is dependent on dynamin and amphiphysin (Marks and McMahon, 1998) as is the case for many forms of clathrin-mediated endocytosis in multiple cell types (McPherson et al., 2001). Finally, many proteins of the endocytic regulatory machinery, such as dynamin, synaptojanin, and amphiphysin, undergo depolarization-dependent dephosphorylation as a means to control their association and endocytic function (Cousin and Robinson, 2001). In most cases, these phenomena were originally observed in synaptosomes and were later confirmed in other cell systems (Cousin and Robinson, 2001). Thus, synaptosomes appear to reproduce faithfully most aspects of nerve terminal exo/endocytosis.

In the present study, we verified the viability of our synaptosomal preparations by physical and biochemical approaches. Electron microscopic examination demonstrated that isolated synaptosomes were well-preserved and often exhibited clathrin-coated pits and vesicles, indicating that they were endowed with clathrin-dependent internalization machinery (Maycox et al., 1992). Additionally, the lack of lactate dehydrogenase leakage suggested that synaptosomal plasma membranes were intact.

4.2. [3 H]-NT binding to striatal synaptosomes

Binding experiments confirmed that [3 H]-NT binds specifically and in a saturable manner to synaptosomes prepared from rat neostriatum (Awad et al., 1989). As previously reported for brain membrane preparations (Mazella et al., 1983; Schotte et al., 1986), NT binding in synaptosomes comprised both a high affinity component, insensitive to the antihistamine levocabastine, and low affinity component, displaceable by this drug. In the presence of levocabastine, the affinity (K_d) and capacity (B_{max}) of [3 H]-NT binding closely approximated those reported for the high affinity NT binding component in rat brain membranes (Mazella et al., 1983). It also corresponded to the affinity of NT for the NT1 receptor subtype, as determined in transfected cell systems (Vincent, 1995; Vincent et al., 1999). Accordingly, [3 H]-NT bind-

Fig. 5. Kinetics of [3 H]-NT binding and internalization in the presence of monensin (25 μ M). Synaptosomes were prepared in Earles buffer containing 25 μ M monensin. Total [3 H]-NT binding was obtained by incubation with 1 nM [3 H]-NT in the presence of 10 μ M levocabastine for increasing time intervals at 37 °C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1 μ M) and specific binding determined by subtracting non-specific from total binding. Hypertonic acid wash, pH 4, was used to remove membrane-bound radioligands without extracting internalized ones.

ing was competed for by the NT antagonist SR48692 with an affinity comparable to that of the drug at the NT1 site (Gully et al., 1993). The fact that in the presence of levocabastine [^3H]-NT binding was fully competed for by SR48692 indicates that none of the levocabastine-resistant binding was due to the NT3 receptor, as this receptor was shown to be insensitive to SR48692 (Navarro et al., 2001). The present demonstration of an association of NT1 receptors with axon terminals in rat neostriatum conforms to the results of immunohistochemical (Boudin et al., 1996; Fassio et al., 2000) and lesion studies which have shown NT receptors to be selectively associated with the terminal arborizations of NT1-expressing nigro-striatal neurons in rat caudate putamen (Palacios and Kuhar, 1981; Quirion et al., 1985; Goedert et al., 1984; Masuo et al., 1990; Nicot et al., 1994; Alexander and Leeman, 1998).

Because of the observed glial profiles in our synaptosomal preparations, the possibility that a proportion of the labeled NT1 receptors might have been associated with glial processes rather than with axon terminals cannot be formally excluded. Nonetheless, this possibility is remote, given that neither NT1 receptor mRNA, nor NT1 receptor protein was found in association with glial cells in the neostriatum using *in situ* hybridization or immunohistochemistry, respectively (Nicot et al., 1994; Alexander and Leeman, 1998; Boudin et al., 1996; Fassio et al., 2000).

4.3. [^3H]-NT internalization in striatal synaptosomes

Following incubation of striatal synaptosomes with [^3H]-NT at 37 °C, approximately 20% of specifically bound ligand was resistant to hypertonic acid wash, suggesting that it had been internalized. The loss of [^3H]-NT binding observed following hypertonic acid wash could not be attributed to leaky synaptosomal membranes, since both electron microscopy and lactate dehydrogenase assays showed good preservation of plasma membrane integrity following this dissociation procedure. Two lines of evidence further supports the view that the acid wash-resistant fraction corresponds to truly internalized, as opposed to artifactually trapped, radioactivity. First, no residual radioactivity was detected in synaptosomes incubated at 4 °C, i.e. at a temperature previously documented to abolish receptor-mediated neuropeptide internalization (Mazella et al., 1991; Vanisberg et al., 1991; Chabry et al., 1993). Second, [^3H]-NT binding to synaptosomes prepared from rat cerebellum, which contain NT2, but not NT1 receptors, was totally abolished following hypertonic acid wash demonstrating that the internalization of [^3H]-NT observed here is receptor-specific (Sarret et al., submitted).

The internalization of [^3H]-NT in striatal synaptosomes was totally inhibited by sucrose, suggesting that

the internalization of presynaptic NT1 receptors is clathrin-dependent. Surprisingly, however, internalization in synaptosomes was entirely resistant to treatment with the endocytosis inhibitor PAO, a drug documented to block clathrin-mediated internalization (Hertel et al., 1985) and shown to prevent NT internalization at the level of perikarya and dendrites in central neurons (Chabry et al., 1993; Nouel et al., 1997; Faure et al., 1995). The lack of PAO blocking effects cannot be attributed to artifactual disruption of synaptosomal integrity as electron microscopy showed good morphological preservation of synaptosomes following treatment with this drug. Our results therefore indicate that the mechanisms of presynaptic NT internalization differ from those at play in the somatodendritic domain.

To determine whether the internalization of other presynaptic receptors might be similarly resistant to PAO, the effect of the drug was tested on the internalization of transferrin (Tf) receptors, which has been extensively documented to be clathrin-dependent in both non-neuronal (Sturrock et al., 1990; Martyris et al., 1995; Warren et al., 1997) and neuronal cells (Prekeris et al., 1999). Although Tf receptors were reported to be absent from axon terminals in hippocampal neurons in culture (Cameron et al., 1991; Parton et al., 1992), they were shown to be associated with nigrostriatal dopaminergic axon terminals in both human and rodent brain (Mash et al., 1991). Accordingly, in the present study, [^{125}I]-Tf was found to bind specifically and in a saturable fashion to neostriatal synaptosomes. The affinity and maximal capacity of this binding were comparable to those previously reported for Tf binding to membrane preparations from rat and human brain (Mash et al., 1991; Roskams and Connor, 1992). Fifty-five percent of [^{125}I]-Tf bound at saturation was acid wash resistant, i.e. was trapped inside synaptosomes. Similar to NT1 receptor-mediated internalization, Tf receptor internalization was sensitive to sucrose but insensitive to PAO. Yet, Tf receptor internalization had been reported to be PAO-sensitive both in non-neuronal cells (Sturrock et al., 1990) and at the level of cell bodies and dendrites in neurons (Takeuchi et al., 1992). The reason for the lack of PAO sensitivity of clathrin-dependent internalization in synaptosomes is unclear, but this finding is not unprecedented since a component of epidermal growth factor receptor internalization in rat hepatocytes, which is well documented to be clathrin-mediated (Hanover et al., 1985), was also reported to be PAO-insensitive (Kato et al., 1992). In any event, the present results suggest that clathrin-mediated internalization operates through different mechanisms in axon terminals and somatodendritic arbors.

Treatment with the ionotropic agent monensin, which prevents endosome acidification and thereby dissociation of receptor-ligand complexes (Rathinavelu and Isom, 1991; Devaskar and Karycki, 1985), was found to reduce

both the capacity and the saturation time of [^3H]-NT internalization in neostriatal synaptosomes. This finding suggests that following ligand dissociation, NT1 receptors are normally recycled back to the plasma membrane, making them available for further binding and internalization of [^3H]-NT. Again, this property would differ from that reported in transfected epithelial cells (Botto et al., 1998; Vandenbulcke et al., 2000) or at the somatodendritic level in neurons (Chabry et al., 1993), where dissociated NT1 receptors were reported to recycle very poorly, but rather to be targeted to lysosomes for degradation.

The percentage of radiolabeled NT internalized in striatal synaptosomes was considerably lower than that reported in neuronal perikarya and dendrites where it amounted up to 80% of the total bound radioactivity (Mazella et al., 1991; Vanisberg et al., 1991; Chabry et al., 1993). Several factors could account for this difference. First, synaptosomes are cut from their supply of newly synthesized receptors and can therefore only rely on recycling of pre-existing receptors for peptide uptake. Second, endocytosis may be less active in synaptosomes than in whole cells because of their more limited energy supply. Third, the proportion of internalized ligand might appear lower than it is in reality due to the fact that our preparations also contained disrupted synaptosomes that might possess [^3H]-NT binding sites but cannot accumulate the ligand. Finally, the efficiency of the internalization process may differ between somatodendritic and axonal compartments due to the inability of axon terminals to internalize via PAO-sensitive mechanisms.

4.4. Conclusions

In summary, the present results suggest that NT is internalized in striatal axon terminals via a mechanism which differs in its efficiency and PAO-sensitivity from that responsible for its internalization through the same receptor at the level of neuronal perikarya and dendrites. Furthermore, our data indicates that presynaptically internalized NT1 receptors may recycle, whereas somatodendritic receptors do not. These observations are congruent with reported differences in endocytic machinery between somatodendritic and axonal domains (Parton et al., 1992) and suggest that receptor internalization may fulfil a different role at the level of axonal and somatodendritic compartments.

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References

- Alexander, M.J., Leeman, S.E., 1998. Widespread expression in adult rat forebrain of mRNA encoding high-affinity neurotensin receptor. *Journal of Comparative Neurology* 402, 475–500.
- Awad, E.W., Nassar, C.F., Tabbara, M.S., Abou-Alfa, G.K., Saade, N.E., Jabbur, S.J., 1989. Characteristics and displaceability of neurotensin binding sites in the rat cerebral cortex and corpus striatum. *General Pharmacology* 20, 725–729.
- Blasi, J., Binz, T., Yamasaki, S., Link, E., Niemann, H., Jahn, R., 1994. Inhibition of neurotransmitter release by clostridial neurotoxins correlates with specific proteolysis of synaptosomal proteins. *Journal of Physiology (Paris)* 88, 235–241.
- Botto, J.M., Chabry, J., Sarret, P., Vincent, J.P., Mazella, J., 1998. Stable expression of the mouse levocabastine-sensitive neurotensin receptor in HEK 293 cell line: binding properties, photoaffinity labelling, and internalization mechanism. *Biochemical Biophysical Research Communications* 243, 585–590.
- Boudin, H., Pelaprat, D., Rostene, W., Beaudet, A., 1996. Cellular distribution of neurotensin receptors in rat brain: immunohistochemical study using an antipeptide antibody against the cloned high affinity receptor. *Journal of Comparative Neurology* 373, 76–89.
- Cameron, P.L., Sudhof, T.C., Jahn, R., De Camilli, P., 1991. Colocalization of synaptophysin with Tf receptors: implications for synaptic vesicle biogenesis. *Journal of Cell Biology* 115, 151–164.
- Castel, M.N., Malgouris, C., Blanchard, J.C., Laduron, P.M., 1990. Retrograde axonal transport of neurotensin in the dopaminergic nigrostriatal pathway in the rat. *Neuroscience* 36, 425–430.
- Chabry, J., Gaudriault, G., Vincent, J.P., Mazella, J., 1993. Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. *Journal of Biological Chemistry* 268, 17138–17144.
- Clark, J.B., Nicklas, W.J., 1970. The metabolism of rat brain mitochondria. Preparation and characterization. *Journal of Biological Chemistry* 245, 4724–4731.
- Cousin, M.A., Robinson, P.J., 2001. The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends in Neurosciences* 24, 659–665.
- Devaskar, S.U., Karycki, L., 1985. Internalization of the neonatal brain insulin receptor. *Biochemistry Biophysics Research Communications* 133, 670–679.
- Dotti, C.G., Simons, K., 1990. Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* 62, 63–72.
- Dumartin, B., Caille, I., Gonon, F., Bloch, B., 1998. Internalization of D1 dopamine receptor in striatal neurons in vivo as evidence of activation by dopamine agonists. *Journal of Neuroscience* 18, 1650–1661.
- Fassio, A., Evans, G., Grishammer, R., Bolam, J.P., Mimmack, M., Emson, P.C., 2000. Distribution of the neurotensin receptor NTS1 in the rat CNS studied using an amino-terminal directed antibody. *Neuropharmacology* 39, 1430–1442.
- Ferguson, S.S.G., Zhang, J., Barak, L.S., Caron, M.G., 1998. Role of β -arrestins in the intracellular trafficking of G-protein-coupled receptors. *Advanced Pharmacology* 42, 420–424.
- Faure, M.P., Nouel, D., Beaudet, A., 1995. Axonal and dendritic transport of internalized neurotensin in rat mesostriatal dopaminergic neurons. *Neuroscience* 68, 519–529.
- Goedert, M., Pittaway, K., Williams, B.J., Emson, P.C., 1984. Specific binding of tritiated neurotensin to rat brain membranes: characterization and regional distribution. *Brain Research* 304, 71–81.

- Grady, E.F., Slice, L.W., Brant, W.O., Walsh, J.H., Payan, D.G., Bunnett, N.W., 1995. Direct observation of endocytosis of gastrin releasing peptide and its receptor. *Journal of Biological Chemistry* 270, 4603–4611.
- Gully, D., Canton, M., Boigegrain, R., Jeanjean, F., Molimard, J.C., Poncelet, M., Gueudet, C., Heaulme, M., Leyris, R., Brouard, A., 1993. Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. *Proceedings of the National Academy of Sciences USA* 90, 65–69.
- Hanover, J.A., Beguinot, L., Willingham, M.C., Pastan, I.H., 1985. Transit of receptors for epidermal growth factor and transferrin through clathrin-coated pits. Analysis of the kinetics of receptor entry. *Journal of Biological Chemistry* 260, 15938–15945.
- Hertel, C., Coulter, S.J., Perkins, J.P., 1985. A comparison of catecholamine-induced internalization of beta-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. Inhibition by phenylarsine oxide. *Journal of Biological Chemistry* 260, 12547–12553.
- Heuser, J.E., Anderson, R.G., 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *Journal of Cell Biology* 108, 389–400.
- Hubbard, A.L., 1989. Endocytosis. *Current Opinion in Cell Biology* 1, 675–683.
- Kato, Y., Sato, H., Ichikawa, M., Suzuki, H., Sawada, Y., Hanano, M., Fuwa, T., Sugiyama, Y., 1992. Existence of two pathways for the endocytosis of epidermal growth factor by rat liver: phenylarsine oxide-sensitive and -insensitive pathways. *Proceedings of the National Academy of Sciences USA* 89, 8507–8511.
- Keiding, R., Horeder, M., Gerhardt, W., Pitkanen, E., Tenhunen, R., Strömme, J.H., Theorderson, L., Waldenström, J., Tryding, N., Westlund, L., 1974. Recommended methods for the determination of four enzymes in the blood. *Scandinavian Journal of Clinical Laboratory Investigation* 33, 291–306.
- Kitabgi, P., Nemerof, C.B. (Eds.), 1992. *Proceedings of the 2nd International Conference on Neurotensin*. Annals of the New York Academy of Science, 668.
- Koh, J.Y., Choi, D.W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *Journal of Neuroscience Methods* 20, 83–90.
- Lefkowitz, R.J., 1998. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *Journal of Biological Chemistry* 273, 18677–18680.
- Maloteaux, J.M., Hermans, E., 1994. Agonist-induced muscarinic cholinergic receptor internalization, recycling and degradation in cultured neuronal cells. Cellular mechanisms and role in desensitization. *Biochemical Pharmacology* 47, 77–88.
- Mantyh, P.W., DeMaster, E., Malhotra, A., Ghilardi, J.R., Rogers, S.D., Mantyh, C.R., Liu, H., Basbaum, A.I., Vigna, S.R., Maggio, J.E., 1995. Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* 268, 1629–1632.
- Marks, B., McMahon, H.T., 1998. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. *Current Biology* 8, 740–749.
- Martys, J.L., Shevell, T., McGraw, T.E., 1995. Studies of transferrin recycling reconstituted in streptolysin O permeabilized Chinese hamster ovary cells. *Journal of Biological Chemistry* 270, 25976–25984.
- Mash, D.C., Pablo, J., Buck, B.E., Sanchez-Ramos, J., Weiner, W.J., 1991. Distribution and number of transferrin receptors in Parkinson's disease and in MPTP-treated mice. *Experimental Neurology* 114, 73–81.
- Masuo, Y., Montagne, M.N., Pelaprat, D., Scherman, D., Rostene, W., 1990. Regulation of neurotensin-containing neurons in the rat striatum. Effects of unilateral striatal lesions with quinolinic acid and ibotenic acid on neurotensin content and its binding site density. *Brain Research* 520, 6–13.
- Maycox, P.R., Link, E., Reetz, A., Morris, S.A., Jahn, R., 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *Journal of Cell Biology* 118, 1379–1388.
- Mazella, J., Leonard, K., Chabry, J., Kitabgi, P., Vincent, J.P., Beaudet, A., 1991. Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. *Brain Research* 564, 249–255.
- Mazella, J., Poustis, C., Labbe, C., Checler, F., Kitabgi, P., Granier, C., van Rietschoten, J., Vincent, J.P., 1983. Monoiodo-[Trp¹¹]neurotensin, a highly radioactive ligand of neurotensin receptors. Preparation, biological activity, and binding properties to rat brain synaptic membranes. *Journal of Biological Chemistry* 258, 3476–3481.
- McMahon, H.T., Foran, P., Dolly, J.O., Verhage, M., Wiegant, V.M., Nicholls, D.G., 1992. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *Journal of Biological Chemistry* 267, 21338–21343.
- McPherson, P.S., Kay, B.K., Hussain, N.K., 2001. Signaling on the endocytic pathway. *Traffic* 2, 375–384.
- McPherson, P.S., Takei, K., Schmid, S.L., De Camilli, P., 1994. p145, a major Grb2-binding protein in brain, is co-localized with dynamin in nerve terminals where it undergoes activity-dependent dephosphorylation. *Journal of Biological Chemistry* 269, 30132–30139.
- Navarro, V., Martin, S., Sarret, P., Nielsen, M.S., Petersen, C.M., Vincent, J., Mazella, J., 2001. Pharmacological properties of the mouse neurotensin receptor 3. Maintenance of cell surface receptor during internalization of neurotensin. *FEBS Letters* 495, 100–105.
- Nicot, A., Rostene, W., Berod, A., 1994. Neurotensin receptor expression in the rat forebrain and midbrain: a combined analysis by in situ hybridization and receptor autoradiography. *Journal of Comparative Neurology* 341, 407–419.
- Nouel, D., Faure, M.P., St Pierre, J.A., Alonso, R., Quirion, R., Beaudet, A., 1997. Differential binding profile and internalization process of neurotensin via neuronal and glial receptors. *Journal of Neuroscience* 17, 1795–1803.
- Palacios, J.M., Kuhar, M.J., 1981. Neurotensin receptors are located on dopamine-containing neurones in rat midbrain. *Nature* 294, 587–589.
- Parton, R.G., Simons, K., Dotti, C.G., 1992. Axonal and dendritic endocytic pathways in cultured neurons. *Journal of Cell Biology* 119, 123–137.
- Pearse, B.M., Crowther, R.A., 1982. Packing of clathrin into coats. *Cold Spring Harbour Symposium Quantitative Biology* 46, 703–706.
- Prekeris, R., Foletti, D.L., Scheller, R.H., 1999. Dynamics of tubulovesicular recycling endosomes in hippocampal neurons. *Journal of Neuroscience* 19, 10324–10337.
- Pressman, B.C., 1976. Biological applications of ionophores. *Annual Review of Biochemistry* 45, 501–530.
- Quirion, R., Chiueh, C.C., Everist, H.D., Pert, A., 1985. Comparative localization of neurotensin receptors on nigrostriatal and mesolimbic dopaminergic terminals. *Brain Research* 327, 385–389.
- Rathinavelu, A., Isom, G.E., 1991. Regulation of ANF receptor internalization: involvement of extracellular calcium. *Biochemistry Biophysics Research Communications* 175, 1017–1022.
- Roskams, A.J., Connor, J.R., 1992. Transferrin receptor expression in myelin deficient (md) rats. *Journal of Neuroscience Research* 31, 421–427.
- Sarret, P., Gendron, L., Kilian, P., Nguyen, H.M.K., Gallo-Payet, N., Payet, M.-D., Beaudet, A., 2002. Pharmacology and functional properties of NTS2 neurotensin receptors in cerebellar granule cells, submitted.
- Schotte, A., Lysen, J.E., Laduron, P.M., 1986. Evidence for a displaceable non-specific [3H]neurotensin binding site in rat brain. *Naunyn-Schmiedeberg's Archives of Pharmacology* 333, 400–405.
- Sternini, C., Spann, M., Anton, B., Keith, D.E. Jr., Bunnett, N.W., von Zastrow, M., Evans, C., Brecha, N.C., 1996. Agonist-selective

- endocytosis of mu opioid receptor by neurons in vivo. *Proceedings of the National Academy of Sciences USA* 93, 9241–9246.
- Stroh, T., Jackson, A.C., Sarret, P., Dal Farra, C., Schonbrunn, A., Vincent, J.P., Beaudet, A., 2000. Receptor-mediated internalization of somatostatin in rat cortical and hippocampal neurons. *Synapse* 38, 177–186.
- Sturrock, A., Alexander, J., Lamb, J., Craven, C.M., Kaplan, J., 1990. Characterization of a transferrin-independent uptake system for iron in HeLa cells. *Journal of Biological Chemistry* 265, 3139–3145.
- Szigethy, E., Beaudet, A., 1989. Correspondence between high affinity ¹²⁵I-neurotensin binding sites and dopaminergic neurons in the rat substantia nigra and ventral tegmental area: a combined radioautographic and immunohistochemical light microscopic study. *Journal of Comparative Neurology* 279, 128–137.
- Takeuchi, Y., Yanagishita, M., Hascall, V.C., 1992. Recycling of transferrin receptors and heparan sulfate proteoglycans in a rat parathyroid cell line. *Journal of Biological Chemistry* 267, 14685–14690.
- Tartakoff, A.M., 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* 32, 1026–1028.
- Vandenbulcke, F., Nouel, D., Vincent, J.P., Mazella, J., Beaudet, A., 2000. Ligand-induced internalization of neurotensin in transfected COS-7 cells: differential intracellular trafficking of ligand and receptor. *Journal of Cell Science* 113, 2963–2975.
- Vanisberg, M.A., Maloteaux, J.M., Octave, J.N., Laduron, P.M., 1991. Rapid agonist-induced decrease of neurotensin receptors from the cell surface in rat cultured neurons. *Biochemical Pharmacology* 42, 2265–2274.
- Vincent, J.P., 1995. Neurotensin receptors: binding properties, transduction pathways, and structure. *Cell Molecular Neurobiology* 15, 501–512.
- Vincent, J.P., Mazella, J., Kitabgi, P., 1999. Neurotensin and neurotensin receptors. *Trends in Pharmacological Sciences* 20, 302–309.
- Warren, R.A., Green, F.A., Enns, C.A., 1997. Saturation of the endocytic pathway for the transferrin receptor does not affect the endocytosis of the epidermal growth factor receptor. *Journal of Biological Chemistry* 272, 2116–2121.