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

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for The degree of Master of Science

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August 31, 2000

LIST OF ABBREVIATIONS

CNS:	Central nervous system
DA:	Dopamine
NN:	Neuromedin
NT:	Neurotensin
NTS1:	Neurotensin receptor subtype 1
NTS2:	Neurotensin receptor subtype 2
NTS3:	Neurotensin receptor subtype 3
Tf:	Transferrin
TfR:	Transferrin receptor
SN:	Substantia nigra

ABSTRACT

Nothing is known about receptor-mediated internalization at the level of nerve terminals. In this study, we have attempted to biochemically characterize neurotensin (NT) receptor-mediated internalization in nerve terminals. To that aim, binding experiments were performed using [^{3}H]-NT on purified preparations of neostriatal terminals (synaptosomes) pretreated with diverse drugs or conditions known to block receptor internalization or specific intracellular pathways. Internalization of the radioligand was demonstrated by comparing [^{3}H]-NT binding before and after dissociation of surface-bound molecules by acid wash. In synaptosomal preparations, [^{3}H]-NT was found to internalize with an efficiency of 20% (acid wash-resistant / total specific binding at equilibrium). Binding is inhibited by the NTS1 antagonist SR48692 and the binding affinity (Kd =0.3nM) and capacity (B_{max} = 25 fmol/mg protein) are reminiscent of the NTS1, suggesting that this receptor accounts for the bulk of [^{3}H]-NT binding and internalization. Internalized NT accumulates inside synaptosomes. Internalization was blocked by sucrose and low temperature but not by PAO, indicating that it is a novel pathway of clathrin-dependant internalization. This pathway operates through a mechanism distinct from synaptic vesicles recycling since it was not abolished in a calcium-free medium. Following internalization, [^{3}H]-NT was found to dissociate from its receptor whereas the NTS1 recycled back to the plasma membrane. Taken together, these findings suggest that NTS1 internalizes in nerve terminals via a mechanism distinct from that responsible for NT internalization in nerve cell bodies.

RÉSUMÉ

Les mécanismes responsables de l'internalisation de la neurotensine (NT) au niveau des terminaisons nerveuses demeurent inconnu. Dans la présente étude, nous avons étudié les mécanismes biochimiques responsables de l'internalisation de la NT au niveau des terminaisons nerveuses. Pour ce faire, nous avons effectué des expériences de liaisons et d'internalisation de la NT en utilisant un ligand radioactive, le [^{3}H]-NT, sur des préparations de synaptosomes neostriataux purifiés. Nous avons soumis ces prérations aux traitements de diverses drogues et conditions connues pour leur abilité à interférer dans les mécanismes d'internalisation. L'internalisation de la NT radioactive a été démontrée en mesurant la quantité de [^{3}H]-NT restante suite au lavage des préparations en pH acid (pH = 4). L'affinité et la capacité de liaison de [^{3}H]-NT suggèrent que le ligand radioactive se lie avec le récepteur NTS1. De plus, le [^{3}H]-NT est déplacé par l'antagoniste au NTS1, le SR48692. Le [^{3}H]-NT internalise avec une efficacité de 20 % et s'accumule dans les synaptosomes. L'internalisation est bloquée lorsque les synaptomes sont incubés en présence de sucrose ou lorsque l'expérience se déroule à basse température, mais n'est pas perturbée en présence de PAO. Ces résultats indiquent que la NT internalise via a mécanisme dépendant de la clathrine, distinct des mécanismes décris précédemment. De plus, l'internalisation de la NT n'est pas associée au recyclage constitutif de la membrane, puisque le [^{3}H]-NT s'internalise toujours en absence de calcium. Nous avons également démontré que, suite à son internalistion, le [^{3}H]-NT se dissocie de son récepteur et que celui-ci est recyclé à la membrane. En somme, nos résultats suggèrent que le NTS1 internalise dans les terminaisons nerveuses via un mécanisme distinct de celui responsable de l'internalisation de la NT au niveau des corps cellulaires.

ACKNOWLEDGEMENTS

It is with immense gratitude and respect that I wish to thank the following people for their help in making this thesis become a reality.

My God and those that are not from this world anymore. Their presence in difficult moments during this project was often the only comfort.

My parents and family, who have made everything possible. Their love and constant encouragement were both inspiring and stimulating. Thanks to my sisters Tam and Anh as well as my only brother Minh Duy.

My supervisor, Dr. Alain Beaudet who has provided me with the unique opportunity to work in an exceptional environment. His constant encouragement and challenges were truly inspiring, both in the realm of science and beyond.

My friends and colleagues of the neuroanatomy laboratory; Mariette Houle, who always proved to be very helpful. Special thanks to my colleagues Dr. Catherine Cahill and Phillipe Sarret, Pierre Villeneuve, Cheryl Savdie, Mao "the Great" Cheng-Lee, Anne Morinville, Alexander "Super" Choi Jackson, James "the Kid" Esdaile, Kelly Mclellan, and last but not least, our secretary, for her kindness and help; Naomi Takeda.

Dr. Peter McPherson, for showing me how to prepare synaptosomes.

My colleagues from other laboratories in the institute and other laboratories, Ian Manns, Edmund Cape, Karen Maloney, Laurence Meadows, and Steve Frey.

Monique Lederman, who so often has bent the deadlines, to alleviate the symptoms of my pathological procrastination.

Drs. Debonnel, Hastings, and Ragsdale and for accepting to be in my thesis committee.

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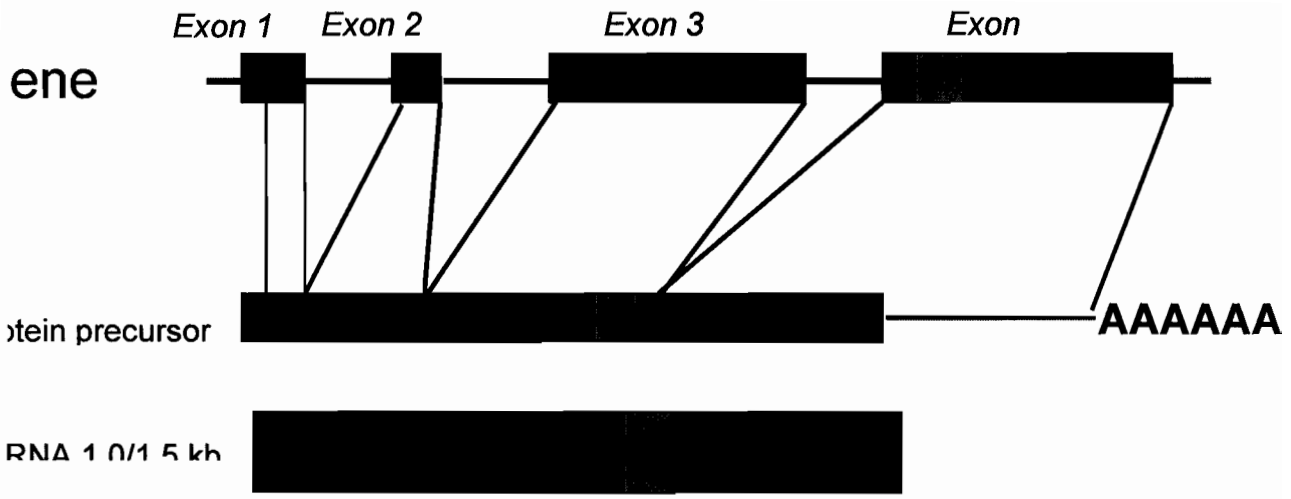
INTRODUCTION

1 NEUROTENSIN

1.1 Overview

Neurotensin (NT) is a peptide that was first isolated from bovine hypothalamus (Caraway and Leeman, 1973). It is a tridecapeptide the sequence of which was first determined by Carraway and Leeman (1975): pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg^{8}-Pro-Tyr-Ile-Leu^{13}-OH. The N-terminal fragments of the NT molecule, NT 1-6, NT 1-8, NT 1-11 are inactive whereas the C terminal residues, NT 8-13 and NT 9-13 are biologically active (Carraway et al, 1976; Kitabgi et al., 1977; Jolicoeur et al., 1984). The C-terminal hexapeptide portion of NT 8-13 contains all the structural requirements for receptor recognition and induction of biological activity (Kitabgi et al., 1980; St-Pierre et al., 1981; Kitabgi et al., 1984). Interestingly, this region (8-13) of the C-terminal is highly preserved while the N-terminal varies amongst species (Kitabgi et al., 1977; Jolicoeur et al., 1984; Elde et al., 1984).

NT and its biologically active co-derivative, neuromedin (NN) are known to be derived from a common larger precursor protein, pro neurotensin/ neuromedin (pro NT/NN) that undergoes differential cleavage (Figure 1 and 2). The rat cDNA encoding pro NT/NN was cloned (Dobner et al., 1987) and was found to consist of four exons and three introns spanning 10.2 kilobases. The NT and NN coding domains (120-169 residues) are localized to exon 4 at the C-terminal extremity of the NT precursor molecule (Dobner et al., 1987; Kislaukis et al., 1988). Exon 1 encodes for the signal peptide, while exons 2 and 3 encode amino acid 24-44 and 45-119 of the precursor, respectively (Kislaukis et al., 1988).



■ -Signal peptide

■ -Neuromedin N

■ -Neurotensin

■ -Remaining portion of the precursor

Figure 1: ***Schematic representation of the organization of the gene, mRNA and protein of prepro-NT/NN.*** The prepro-NT/NN gene is composed of 4 exons. NT and NN both arise from exon 4 and the remaining segments of the precursor from exons 1 to 3. Note that two mRNA are generated from the gene.

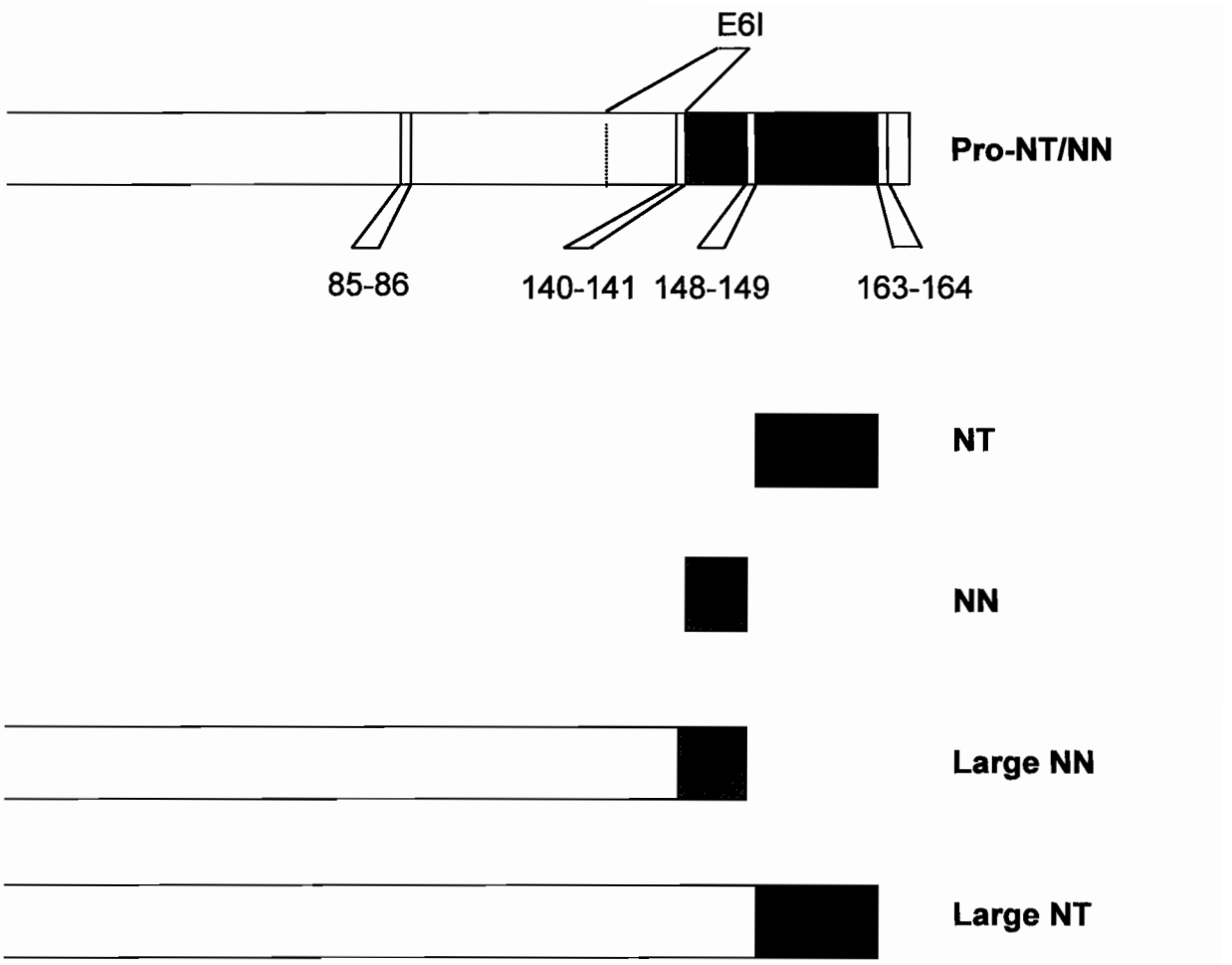


Figure 2: ***Schematic representation of pro-NT/NN and its various maturation products.*** The position of the four Lys-Arg dibasic sites are illustrated along with NT, NN, large NN, large NT and the peptide segment E6I exposed after proteolytic cleavage of the Lys^{141} - Arg^{142} dibasic site.

Comparison of the deduced amino acid sequences from the cloned pro NT/NN cDNA reveals 76% homology among species (Kislaukis et al., 1988). Indeed, NT is present in almost all classes of vertebrates including mammals, amphibians and reptiles as well as lower life forms such as bacteria (Bhatnagar et al., 1981).

In mammals, NT is predominantly expressed in the intestine (Dobner et al., 1987; Kisklauskis, 1988). It is also expressed in other peripheral tissues such as the pancreas, the heart and the adrenal medulla (for review, Reinecke M, 1985). But the highest concentration of NT, as revealed by radioimmunoassays performed on micropunched tissue samples and immunohistochemical studies on tissue sections, are found in the central nervous system (CNS) (Carraway et al., 1976 a,b; Uhl et al., 1976; Jennes et al., 1982; Goedert et al., 1984a).

1.2 Distribution of NT in the rat CNS

The development of specific anti-NT antibodies (Carraway et al., 1976a,b) allowed the use of radioimmunoassay and immunohistochemical techniques to determine regional and cellular distributions of NT, respectively. Also, the cloning of the NT precursor (Dobner et al., 1987) allowed recombinant pro NT/NN mRNA application for in situ hybridization studies of potential sites of NT synthesis.

1.2.1 Regional distribution of NT as determined by radioimmunoassay

Considerable variability exists in the content of NT detected by radioimmunoassay in analogous brain regions of several mammalian species. In adult rats, NT is distributed heterogeneously in the CNS. The highest concentrations of NT are detected in the periaqueductal gray, amygdala, nucleus

accumbens and several hypothalamic nuclei. Moderate concentrations are present in the caudate nucleus, hippocampus and globus pallidus. Only low concentrations are detected in the cerebellum (Manberg et al., 1982; Uhl et al., 1976; Kataoka et al., 1979; Jennes et al., 1982).

1.2.2 Cellular distribution of NT as determined by immunohistochemistry and in situ hybridization

In mammalian brain, NT-Immunoreactivity is exclusively found in association with neurons (Jennes et al., 1982; Woulfe et al., 1994). Most of the immunoreactive NT is concentrated in their axons and axon terminals.

Only small number of NT immunoreactive perikarya are observed in the absence of colchicine pretreatment, indicating that perikaryal levels of the peptide are usually low. Regions rich in NT-containing axons and axon terminals include the nucleus accumbens, bed nucleus of the stria terminalis, lateral septum, substantia innominata, globus pallidus, lateral and dorsal hypothalamic area, paraventricular nucleus of the hypothalamus, external zone of the median eminence, zona incerta, medial forebrain bundle, stria terminalis, mediodorsal paraventricular and paracentral thalamic nuclei, habenular nuclei, medial and central nuclei of the amygdala, cingulate, retrosplenial and perirhinal cortices, ventral tegmental area, substantia nigra, interfascicular nucleus, and raphe nuclei (Jennes et al., 1982; Woulfe et al., 1994).

In colchicine-treated rats, NT-Immunoreactive nerve cell bodies were observed in several forebrain and limbic structures including the preoptic area, hypothalamus, septum, amygdala, nucleus accumbens, caudate-putamen and pallidum (Jennes et al., 1982; Woulfe et al., 1994).

The topographic distribution of NT-immunoreactive cells bodies correlates well with that of neurons expressing pro-NT/NN mRNA as revealed by in situ hybridization histochemistry (Alexander et al., 1989). Exceptions include the subiculum and CA1 of the hippocampus, in which NT/NN mRNA is readily detectable but NT-immunoreactive cell bodies are not. This discrepancy suggests that in these precursor-expressing neurons, the NT/NN mRNA may be untranslated, poorly translated, or rapidly degraded (Alexander et al., 1989; Checler et al., 1993; Nicot et al., 1994).

1.3 Functions of NT

1.3.1 In the gastro-intestinal tract

In the gastro-intestinal tract, NT has an hormonal function. Essentially localized in type N cells, it acts in a endocrine and paracrine fashion. It is released following meal intake and stimulates gastric and pancreatic secretions and decreases contraction of the smooth muscles lining the gastro-intestinal tract (Vincent, 1995).

1.3.2 In the CNS

In CNS, NT has both neuroendocrine and neurotransmitter/ neuromodulator functions.

1.3.2.1 Neurotransmitter/neuromodulator role

NT fulfills many criteria of a neurotransmitter in the CNS including:

(1) synthesis in selective neuronal populations (see above)

(2) concentrated in synaptic vesicles within synaptosomes (Uhl et al., 1977; Kataoka et al.,1979)

(3) released upon depolarization by potassium in a calcium dependant fashion (Bean et al., 1989)

(4) is degraded by a variety of peptidases in the synaptic cleft (Checler et al., 1982, 1983).

Several peptidases are involved in the inactivation of NT. They are divided into two groups:

a) Peptidases that are responsible for primary cleavages of the parent peptide, leading to degradation products totally devoid of biological activity. These include proline endopeptidase and endopeptidase 24-11, 24-15 and 24-16 (Checler et al., 1983, 1985). Endopeptidases 24-15 and 24-16 are both widely distributed in the brain; endopeptidase 24-11 is more selectively distributed and mostly concentrated in the caudate putamen. These peptidases are inhibited by captopril and the metal chelating agent 1,10 phenanthroline (Erdos et al., 1977).

b) Enzymes that are involved in the secondary processing of NT degradation products but cannot be considered as inactivating peptidases, such as aminopeptidases and postproline dipeptidyl-aminopeptidases (Checler et al., 1988)

(5) binding to specific high affinity binding sites with pharmacological characteristics of functional receptors (Uhl et al., 1976; Young et al., 1979; Quirion et al., 1982). NT receptors will be fully discussed latter.

As neurotransmitter/neuromodulator, NT has been involved in a number of brain functions including regulation of locomotor activity, antinociception, temperature regulation and regulation of cognitive behavior. Intracerebral and intraventricular injection of NT have been shown to elicit a behavioral profile characterized by hypothermia, motor hypoactivity, decreased muscle tone, hypotension and potentiation of barbiturates and ethanol-induced sedation (Bissette et al., 1976; Jolicoeur et al., 1981; Castel et al., 1989; Kalivas et al., 1985). The similarity of these effects with those induced by neuroleptic drugs suggested an interaction between NT and dopamine (Nemeroff et al., 1980; Osbahr et al., 1981). This interpretation was latter supported by various lines of electrophysiological, biochemical and anatomical evidence demonstrating interactions between NT and mesostriatal, mesocortical and mesolimbic pathways (for review see Kaschow et Nemeroff, 1991). Other non-neuroleptic effects of NT-include decreased feeding and inhibition of gastric secretions (Osumi et al., 1978; Clineshmidt et al., 1979; Luttinger et al., 1982).

1.3.2.2 Neuroendocrine Function

NT plays a neuroendocrine role, particularly in the HPA axis, where it has been shown to regulate ACTH and corticosterone secretion. It is also involved in the central regulation of the release of other pituitary hormones, including luteotrophic hormone, thyroid stimulating hormone, and growth hormone (Rostène and Alexander, 1997).

2 NT RECEPTORS

2.1 Overviews

The central and peripheral pharmacological effects of NT are mediated through the activation of specific receptors.

Three NT receptor sub-types have been cloned to date and are referred to as NTS1, NTS2 and NTS3 (Tanaka et al., 1990; Chalon et al., 1996; Mazella et al., 1998). NTS1 and NTS2 are classical G protein-coupled receptors with seven transmembrane domains, characteristic of biogenic amines and of most neuropeptide receptors. The third, NTS3, was cloned by Mazella and colleagues (Mazella et al., 1998) and was found to correspond to the protein sortilin, a single transmembrane domain receptor involved in intracellular transport (Peterson et al., 1997). Sortilin differs from the two other G-protein-coupled NT receptors and is similar to the mannose-6-phosphate receptor bearing a single transmembrane domain (Mazella et al., 1998).

Radioligand binding experiments using iodinated neurotensin [^{125}I(Tyr^{3}) or ^{125}I-(Trp^{11})-NT] on synaptic membrane preparations from whole rat brain (Mazella et al, 1983, Sadoul et al., 1984) have allowed to pharmacologically differentiate NTS1 and NTS2. NTS1 is characterized by its high affinity (Kd = 0,1-0,3 nM) and low binding capacity (B_{max} = 12-26 fmol/mg) for NT. By contrast, NTS2 has a relatively lower affinity (Kd = 4-6 nM) and higher capacity (B_{max} = 73-147 fmol/mg). These properties of NTS1 and NTS2 binding were supported by further radioligand binding experiments in intact neuronal and non-neuronal cells. Neuronal models included primary cultured neurons from mouse and rat brain (Dana et al., 1991; Mazella et al, 1991; Brouard et al., 1992; Chabry et al., 1993)

as well as rat brain synaptosomes (Awad et al., 1989). Non-neuronal cell models included cells expressing the endogenous receptors such as the HT29 human colonic adenocarcinoma cells (Turner et al., 1990), all heterologous transfection systems such as HEK293, human embryonic kidney cell line (Botto et al., 1998), Chinese hamster ovary CHO cells (Hermans et al., 1994) or simian kidney epithelial COS7 cells transfected with cDNA encoding either NTS1 or NTS2 (Mazella et al., 1996). The affinities of NTS1 and NTS2 for NT in these intact cell models correspond to values obtained in membrane preparations. Binding capacity of NTS2 was consistently higher than that of NTS1 but varied in absolute value from one cell model to another and even from one brain region to an other in the case of synaptosomal preparations (Awad et al., 1989).

Levocabastine, a known histamine H1 antagonist has been shown to inhibit NT binding to low affinity binding sites (NTS2) without affecting binding to the high affinity site (NTS1) in rat brain (Kitabgi et al., 1987). Discrimination between these two subtypes has also been possible by the development non-peptide antagonists such as SR48692. SR48692 recognizes preferentially high affinity binding sites (NTS1). In rat brain homogenates, ^{125}I (Tyr^3)-NT binding is displaced may SR48692 with an IC_{50} value of 82 nM. This value drops to 5 nM when low binding sites are blocked by levocabastine (Gully et al., 1993) (Figure 3). Little is known of the binding properties of the NTS3/sortilin in the mammalian brain. However, ^{125}I (Tyr^3)-NT was found to bind to CHAPS-solubilized extracts of COS7 cells transfected with human cDNA coding for NTS3 in a saturable and reversible fashion with an affinity of 10-15 nM (Mazella et al., 1998). Affinity labeling and binding experiments suggest that the NTS3 can be partly cleaved by

the prohormone convertase furin into a higher affinity receptor (Kd = 0,3 nM) (Mazella et al., 1998). The latter would correspond to the mature form of the receptor (Mazella et al., 1998). Studies in primary culture neurons from embryonic mice suggest that the targeting of NTS3 is markedly enhanced following stimulation and internalization of NTS1 and/or NTS3 present on the cell surface (Chabry et al., 1993).

	NTS1	NTS2	NTS3	
Structure	7 transmembrane domain, G-protein coupled receptor	7 transmembrane domain, G-protein coupled receptor	Single transmembrane domain, Sortilin	
Regional distribution	Highly selective	Widespread	Widespread	
Cellular distribution	Neurons exclusively	Glial cells & neurons	Glial and Neuronal cells	
Affinity, Kd (nM)	High (0,1 - 0,3)	Low (4 – 6)	Very low or High (10 –15)	(0,3-0,7)
Capacity, B_{max}, (fmol/mg protein)	Low (12 – 26)	High (73-147)	Very low (4)	Very high (320)
Antagonized by Levocabastine	NO	YES	NO	**?**
Affinity for SR48692 (nM)	High (5)	Low (82)	Very Low (3000)	**?**

Figure 3: ***Summary of pharmacological properties and central distribution of the three NT receptor subtypes: NTS, NTS2 and NTS3.***

The respective cDNAs coding for NT receptors have been cloned in rat, mice and human for NTS1 and NTS2 (Tanaka et al., 1990; Chalon et al., 1996) and in mice and human for NTS3 (Mazella et al., 1998). In rat brain, the two amino acid sequences deduced for NTS1 and NTS2 are 424 and 416 amino acids in length respectively, and show 43% homology with one another. Amino

acids critical for the binding of NT to NTS1 include the Arg327 as well as residues located in the first (Asp139) and the third extracellular loop (Tyr347) and the N-terminal extracellular segment (Labbé-Jullié et al., 1995). Thr-422 and Tyr-424 residues located in the carboxyl terminal cytoplasmic tail of the NTS1 were found to be critical for internalization of the receptor (Chabry et al., 1995). Recently, a comparative study has revealed a difference of only one amino acid between the second transmembrane domains of NTS1 and NTS2 (Martin et al., 1999). The Asparagine 113 in NTS1 was replaced by an alanine in NTS2. Substitution of the alanine in NTS2 by an asparagine has been shown to endow NTS2 with a sodium-dependant reduction in affinity for NT (Martin et al., 1999). Human cDNA of NTS3 codes for a 833 residues containing a signal peptide, a putative cleavage site of furin and a single transmembrane domain (Mazella et al., 1998).

2.2 Localization of NT receptors in the CNS

The existence and distribution of NT receptors was first revealed by radioligand binding experiments on brain homogenate or brain sections using tritiated (Quirion et al., 1982) and iodinated NT (Kitabgi et al., 1987, Moyse et al, 1987). In latter experiments, NTS1 and NTS2-associated radiolabeling were differentiated based on their sensitivity to levocabastine (Kitabgi et al.,1987). Further central distribution of NT receptors was documented by immunohistochemical technique which was made possible by development of a specific antibody against specific sequences of cloned NT receptor subtypes: antibody against NTS1 (Boudin et al., 1996) has allowed the mapping of this

subtype in rat brain whereas studies have yet to be performed for NTS3 for which an antibody has been recently developed (Petersen et al., 1997). A specific antibody for NTS2 has yet to be developed. In rat brain, NTS1 and NT2 exhibit marked differences in both their regional and cellular distribution whereas very little is known of the central distribution of NTS3 in mammalian brain.

2.2.1 Regional distribution of NT receptor subtypes in rat brain

NTS1 was found to be heterogeneously distributed in rat brain and was concentrated in the following regions in decreasing order of densities: the subtantia nigra, ventral tegmental area, cingulate cortex, dentate gyrus, olfactory tubercle, rhinal sulcus, caudate nucleus, nucleus accumbens, superior colliculus, hypothalamus and dorsal raphe nucleus (Kitabgi et al., 1987; Moyse et al., 1987; Boudin et al., 1996). By contrast, NTS2 displayed a more homogeneous and diffuse distribution in most of brain regions, particularly, the cortex, dorsal hippocampus and thalamus (Schotte et al., 1986; Kitabgi et al., 1987). Concerning NTS3, very little is known about its central localization. A recent in situ hybridization study revealed an accumulation of NTS3 mRNA in the piriform cortex, cerebral cortex and hippocampal formation in adult mice (Hermans et al., 1999).

2.2.2 Cellular distribution of NT receptor subtypes in rat brain

Light (Moyse et al., 1987; Kessler et al., 1987) and electron microscopic (Dana et al., 1989; Szigethy et al., 1990) radioligand binding studies in rat brain demonstrated the selective localization of high affinity NT binding sites (NTS1)

within neurons as opposed to glial cells in various regions of the CNS. In these studies, radiolabeling was observed over cell bodies, dendrites and axon terminals of neurons and over both the plasma membrane and intracellular organelles. The concept of an extensive association of NTS1 with neurons is supported by in situ hybridization studies which revealed the presence of strong hybridization signal for NTS1 mRNA exclusively in neurons (Elde et al., 1990; Nicot et al., 1994; Alexander and Leeman, 1998).

By contrast, several lines of evidences suggest that NTS2 might be predominantly associated with glial cells. On the basis of ontogenic and lesion studies, Schotte and Laduron (1987) were the first to suggest that low affinity NT receptors (NTS2) were associated with glial cells. Thus, the late appearance of NTS2 in developing rat brain (at day 30 after birth) corresponds to the development of glial cells and raises the possibility of NTS2 expression in these cell types rather than neurons (Kitabgi et al., 1987; Schotte and Landuron, 1987). Further evidence was demonstrated by kainic acid lesion in the neostriatum leading to a transitory reduction followed by a massive increase in low affinity NT binding which correlates with astrocyte proliferation (Schotte et al., 1988). In culture, this type of glial cell was shown, using a fluorescent analogue of NT, to be endowed with low affinity, levocabastine-sensitive NT binding sites (Nouel et al., 1997). However, in rodent brain slices, NTS2 mRNA were found to be predominantly concentrated in neurons as indicated by in situ hybridization studies (Sarret et al., 1998; Walker et al., 1998), suggesting that, in vivo, NTS2 is mainly expressed in neurons. A recent study combining immunohistochemical labeling of astrocyte marker proteins and in situ hybridization of NTS2 mRNA

performed on sections of normal and stab wounded rat brain was conducted by Nouel and colleagues (1999). In normal brain sections, hybridization signal for NTS2 mRNA was faint and correlated very weakly with glial immunolabeling. However, following lesion, both the number of hybridizing glial cells and the intensities of hybridization signal over individual glial cells were markedly increased around the wound, suggesting that in vivo, physical stress triggers the expression of NTS2 mRNA in astrocytes, the level of which is very low under normal conditions. To determine which NT receptor subtypes were expressed by cortical astrocytes in culture, transcription-polymerase chain reaction of their mRNA was performed (Nouel et al., 1999). As expected, cortical astrocytes in culture were devoid of NTS1 but expressed NTS2. Interestingly, NTS3 was also found to be expressed by this type glial cells.

2.3 Mediation of NT action in the CNS

In general, pharmacological evidence points to a participation of NTS1 in the mediation of neuroleptic-like effects of NT resulting from its interaction with mesocortical dopaminergic system (Labbé-Jullié et al.1994). For instance, SR48692, which preferentially antagonizes NTS1, has been shown to attenuate the hypomotility induced by intracerebroventricular injection of NT (Dubuc et al.,1994). However, SR48692 was unable to block a series of NT effects including central analgesia and hypothermia, suggesting that these effects did not involve activation of NTS1 and could therefore be mediated either by NTS2, by the newly cloned NTS3, or by a yet undiscovered subtype of NT receptors (Dubuc et al., 1994). Recently, in vivo antisense strategies to selectively block

central expression of NTS2 in mice, demonstrated that NTS2 was essential for the mediation of naloxone-insensitive analgesic effects of NT (Dubuc et al., 1999). Hypothermia was not affected by this treatment, implying that this function might be subserved by NTS3 or another as yet unknown NT receptor subtype.

2.4 Molecular mechanisms of NT receptor action

2.4.1 Second messenger system

Several studies have shown that NT receptors are coupled to a variety of second messenger cascade systems. Variations in the level of different molecules implicated in intracellular signaling have been linked to the activation different receptor subtypes following NT binding.

It is well established NTS1 activation induces variations in the level of different molecules implicated in intracellular signaling (for review see Hermans and Maloteaux, 1998). For instance, NT induces inositol triphosphate (IP3) production in the majority of primary and cell line cultures in which rat NTS1 are expressed (cultured neurons, neuroblastomas, human colon carcinomas, NG108-15 cells, bovine chromaffin cells). In cultured neurons and in cell lines, this IP3 production was further correlated with an increase in the concentration of intracellular calcium. This increase in intracellular calcium concentration was reported to trigger an accumulation of cyclic GMP in mouse neuroblastoma cell lines (N1E115) expressing NTS1 (Gilbert et al., 1984). NTS1 activation was also found to differently affect intracellular levels of cyclic AMP depending on cell types. NT induces a decrease in cAMP level in N1E115 cells whereas it has the opposite effect in CHO cells transfected with NTS1. In the latter cell type, NT

was recently found to activate transcription of early genes such as Krox-24 following activation of MAP kinases cascade by protein kinase C (Poinot-chazel et al., 1996; Portier et al., 1998).

Evidences for NTS2 association to intracellular signaling pathways are not as numerous as for NTS1. To date, transfected CHO cells and xenope oocytes expressing mouse and rat NTS2, respectively, were among the few models implicating NTS2 in the mediation of NT induction of intracellular signaling. Accordingly, in transfected CHO cells, NT stimulates mobilization of intracellular calcium (Mazella et al., 1996) while in transfected xenope oocytes, NT induces a calcium-dependent increase in chloride currents (Yamada et al., 1998).

Concerning NTS3, the intracellular signaling cascade has yet to be determined.

2.4.2 Control mechanism of receptor sensitivity

Chronic exposure of neurons to neuropeptide agonists acting on cell surface receptors results in a loss of neuronal responsiveness to the transmitter, a phenomenon known as desensitization.

Desensitization may be achieved either by uncoupling the receptor from its associated G protein and/or effector, or by decreasing the density of cell surface receptors. At short-term intervals (5-45 minutes) exposure to ligand, the loss of specific cell surface receptors usually reflects their sequestration into the cell interior, a process called internalization (Maloteaux et al., 1983a). Internalized receptors may be recycled back to the cell surface or degraded (Maloteaux et al., 1983b; Vanisberg et al., 1991). At longer time intervals

exposure to the ligand (> 45 minutes), the loss of cell surface receptors may be due to decreased synthesis, a process referred to as down regulation (Gilbert et al., 1988).

In rat embryonic neuronal cell, 60% of high affinity NT receptors (NTS1) disappear from the surface following 10 minutes exposure to NT (Vanisberg et al., 1991). This corresponds to a reduction of the B_{max} value without change in the binding affinity.

3 INTERNALIZATION OF RECEPTORS

3.1 Overview

Receptor endocytosis is a general process by which after exposure to its ligand, the ligand-receptor complex is taken up with high specificity and affinity into cells and are enclosed in membrane-bound vesicles and taken into the cell (Dautry-Varsat et al., 1984). It is a rapid and energy-dependent process.

The initial event consists in the binding of the ligand to specific receptors on the cell surface. Then, ligand-receptor complexes move to specialized regions of the membrane where they form clusters. These specialized regions have been referred to as bristle-coated pits (Roth et al., 1964; Pearse et al., 1975) and are composed of three clathrin heavy chains (180 kDa protein), three clathrin light chains (33-36 kDa protein) (Brodsky et al., 1988) and a protein complex composed of 100, 50, and 16 kDa components termed accessory proteins or adaptors (AP-2) (Ahle et al., 1988; Keen et al., 1990) which interact with the cytoplasmic domains of various receptors (Glickman et al., 1989). However, recent studies have suggested that internalization can occur along other

pathways than the clathrin-mediated one. The caveolin-dependant pathway is one of such pathway (Anderson, 1998; Okamoto et al., 1998). Both clathrin and caveolin-dependant pathways were reported to be associated with dynamin, a GTPase important for the scission of endocytosed compartment from the plasma membrane (Urrutia et al., 1997; Oh et al., 1998; Henley et al., 1998)

Following clathrin-mediated internalization, the ligand-receptor complexes are then internalized into the cell along the following classical itinerary: clathrin-coated (or non-coated) vesicles first bud from the plasma membrane and fuse together to give rise to early endosomes of 150 to 500 nm in diameter (Pearse et al., 1990; Rodman et al., 1990). Endosomal compartments contain an ATP-dependant proton pump (Galloway et al., 1983; Brown et al., 1987) and the receptor-ligand complexes encounter a progressively lower pH as they move from coated/or non-coated vesicles through the endocytotic pathway (Tycko et al., 1982; Yamashiro et al., 1987; Fuchs et al., 1989). Within this acidic milieu, most ligands dissociate from their receptors, which in some cases return to the cell surface (receptor recycling) (Dautry-Varsat et al., 1984; Mellman et al., 1983). Some endocytosed material is then transferred from early endosomes to late endosomes or pre-lysosomes (Schmid et al., 1988; Stoorvogel et al., 1991) which have been described as multivesicular compartments containing receptors, ligands, and other solutes destined for degradation (Griffiths et al., 1988). Lysosomes provide the terminal compartment of the endocytotic pathway in which proteins and other macromolecules are degraded by acid hydrolases.

3.2 Internalization of receptors

3.2.1 In peripheral cell models

Most of our knowledge concerning receptor internalization and trafficking is derived from studies of single transmembrane domain receptors in transfected peripheral cell models. For instance transferrin, epidermal growth factor (EGF) and low density lipoprotein (LDL) receptors, like most receptors studied to date, are known to internalize via clathrin-dependant mechanisms (Pearse et al, 1982; Larkin et al., 1983; Heuser and Anderson, 1989). These receptors, however, differ not only in internalization requirements but also in the subsequent intracellular route taken by both ligand and receptor. Transferrin and LDL receptors are internalized constitutively i.e. independently from their ligand occupancy, whereas EGF receptor internalization is ligand-induced. Both transferrin receptors and LDL receptors are recycled back to the membrane, however transferrin remains bound to its receptor following the release of its iron content in acidic early endosomes. By contrast, in these compartments, LDL dissociates from its receptor and is targeted to late endosomes and lysosomes for degradation (Goldstein et al., 1985). This fate is met by both EGF and its receptors following internalization in human fibroblasts (Carpenter et Cohen., 1976; Stoscheck et Carpenter, 1994). However, internalized EGF receptors were also shown to recycle back to the membrane in rat hepatic cells (Kato et al., 1992).

Very little is known concerning the mechanisms of internalization and intracellular targeting of seven-transmembrane receptors, and even less concerning the fate of their ligand in peripheral cell models. Most of these

receptors were found to recycle back to the plasma membrane following internalization. These include β2-adrenergic receptors, human thrombin receptors, neurokinin 1 receptors, opioid receptors, gastrin-releasing peptide receptors and the A-type cholecystokinin receptors (Von Zastrow et al., 1992; Hoxie et al., 1993; Brass et al., 1994, Grady et al., 1995a,b; Tarasova et al., 1997).

3.2.2 In brain cells

Biochemical and/or autoradiographic studies have provided evidence for receptor-mediated internalization of muscarinic (Maloteaux et al., 1994), β-adrenergic (Goldstein et al., 1985) and a variety of other G protein-coupled receptors for neuropeptides in central neurons (Laduron et al., 1992; Maloteaux et al., 1994). Central mechanisms of internalization however, have been most extensively documented for tyrosine kinase receptors including epidermal growth factor (Chabot et al., 1986); nerve growth factor (Stoeckel et al., 1975), and basic fibroblast growth factor receptors (Walicke et al., 1991; Eckenstein et al., 1994). However, for many of these receptors, and particularly for G protein-coupled receptors, little is known of the sequence of events triggered by their internalization or of the subcellular compartment that subserves it.

3.3 Internalization of NT receptor subtypes

3.3.1 In peripheral cell models

All three cloned NT receptors have been reported to internalize upon interaction with agonist ligands in a temperature and time-dependant fashion.

This internalization was sensitive to low temperature (4°C) as well as to endocytosis blockers such as hyperosmolar sucrose and phenylarsine oxide (PAO), strongly suggesting that all 3 receptors internalize via a clathrin-dependant mechanism.

In HT29 (Turner et al., 1990), fibroblasts deficient in thymidine kinase LTK (Chabry et al., 1994), COS7 (Chabry et al., 1995), and CHO cell lines (Hermans et al., 1994), NTS1 internalized with an efficiency of 60 to 90% and saturated within 20 to 30 minutes of exposure to NT. Furthermore, in COS7 cells, NTS1 was found to internalize in a calcium-independent fashion and was subsequently targeted to lysosomes for degradation (Vandenbulcke et al., 2000), consistent with biochemical evidence in LTK cell suggesting that internalized NTS1 does not recycle to the plasma membrane (Botto et al., 1998). Concerning NT, it was found to be recruited to the Trans-Golgi Network (Vandenbulcke et al., 2000).

By contrast, in HEK293 cells, NTS2 was found to undergo recycling following internalization, with properties similar to NTS1 (60% efficiency, 20 minutes for saturation) (Botto et al., 1998). A recent study by Mazella and colleagues (1998) reported that NTS3 also internalizes following NT exposure in COS7 cells transfected with this NT receptor subtype.

3.3.2 In brain cells

Several studies suggest that, in neurons, the interaction of NTS1 with its receptor is also followed by ligand-induced internalization of ligand-receptor complexes. The internalization of NT receptors was demonstrated in embryonic brain cultures and in SN17 cells (hybridoma of embryonic mouse septal cells and murine neuroblastoma) (Vanisberg et al., 1991; Mazella et al., 1991, Chabry et

al., 1993, Faure et al., 1995, Hermans et al., 1997). These experiments showed that radiolabeled-NT induced a rapid decrease in the number of NT receptors from the cell surface, which resulted from an internalization process. Internalization in neuronal cells, much like in non-neuronal cells, was found to be time- ($t_{1/2}$ = 15 minutes) and temperature-dependent (non-permissive T° = 4°C) and was sensitive to PAO. Studies using fluorescein-tagged NT in brain slices and in cultured neurons indicated that the internalization process was endocytotic in nature and was mediated via small intracytoplasmic particles the size and maturation of which corresponded to that of endosomes (Faure et al., 1995a).

Studies in mesencephalic dopaminergic cultured neurons, in which NT - NTS1 complex is prevented from dissociation by photoaffinity cross-linking suggest that following their internalization, ligand and receptor dissociate (Nouel et al., 1997). In neurons, much like in non-neuronal cell, following it internalization, NTS1 is degraded rather than being recycled. This is suggested by the insensibility of NTS1 internalization to monensin, a blocker of receptor recycling (Chabry et al., 1993) and its sensibility to lysomotropic drugs chloroquine and methylamine (Hermans et al., 1997). On the other hand, the internalized ligand is mobilized from neuronal processes to perikarya, and, within perikarya, from the periphery to the center of the cells, to end up clustered in a juxtanuclear region that may correspond to the Trans-Golgi Network i.e. to the compartment towards which NTS1 is targeted in non-neuronal cells.

As opposed to what was observed in transfected cell models, in cortical astrocytes in culture, no ligand-induced internalization of NTS1 is observed

following exposure to fluorescent NT (Nouel et al., 1997). Concerning NTS3, it is still not known whether it internalizes or not in brain cells.

4 THE NIGROSTRIATAL DOPAMINERGIC NEURON AS A MODEL TO STUDY NTS1 INTERNALIZATION

4.1 Connectivity of nigrostriatal dopaminergic neurons

The substantia nigra (SN) is localized in the mesencephalon and is divided into a pars reticulata and a pars compacta. The latter contains dopaminergic (DA) neurons (A9) that project topographically to different parts of the neostriatum. Intermingled clusters of cells in the SN project ipsilaterally either to the caudate or the putamen. Conversely, striatal neurons also project fibers to the SN (striatonigral projection). Striatonigral and nigrostriatal fibers appear to form a closed feedback loop in which striatonigral fibers form the afferent limb and nigrostriatal fibers constitute the efferent limb. This reciprocal arrangement is evident in that horseradish peroxidase injected into the neostriatum is transported retrogradely from axon terminals to cells of the pars compacta (von Krosigk et al., 1992) and anterogradely via neostriatal neurons to terminals in the pars reticulata of the SN (Druga, 1992). Clinically, the importance of the nigrostriatal dopaminergic pathway is underscored by the fact that degeneration of this system is a hallmark feature in Parkinson's Disease.

4.2 Anatomical evidence for NT innervation of nigrostriatal neurons

Neuroanatomically, NT containing axon terminals are closely associated with A9 DA neurons at the level of both their cell bodies in the SN and their

terminal field in the neostriatum (Levant, 1988). In fact, NT fibers were seen by fluorescent histochemistry to approximate dopaminergic perikarya in the SN (Hokfelt et al., 1984). Furthermore, studies using double labeling immunohistochemistry at the electron microscopic level reported that NT terminals form synaptic contacts with dopamine perikarya and dendrites (Woulfe et al., 1989). Using radiolabeling and immunohistochemistry, NT terminals have also been reported to innervate the neostriatum (Quirion et al., 1982; Jennes et al., 1982).

4.3 Evidence of association of NTS1 with nigrostriatal neurons in culture

High affinity NT binding sites (NTS1) were detected in mesencephalic cells in primary cultures by receptor autoradiography (Dana et al., 1991) and fluorescence study (Nouel et al., 1999). The combination of receptor autoradiography and immunohistochemistry demonstrate that in these mesencephalic cultures, NTS1-associated NT labeling was localized in dopaminergic neurons at both the level of their perikarya and nerve terminals (Brouard et al., 1992; Nouel et al., 1999).

4.3.1 NTS1 in dopaminergic cell body in the substantia nigra

The association of NT receptors with DA neurons in the SN was first suspected by autoradiographic receptor binding studies in rat, monkey and human brain (Palacios and Kuhar, 1981; Quirion et al., 1982; Rostene et al., 1992). A later investigation combining [^{125}I]-NT autoradiography and tyrosine hydroxylase (TH) immunohistochemistry in rat brain sections demonstrated that

[^{125}I]-NT binding in the SN was associated with levocabastine-insensitive high affinity binding sites (NTS1) and was mainly localized to 95-100% of [^{125}I]-NT-labeled cells to TH immunopositive-neurons (DA neurons) (Szigethy and Beaudet, 1989). Recently, light and electron microscopy (EM) studies using a newly developed specific anti-NTS1 antibody as well as in situ hybridization studies using NTS1 complementary probes confirmed the presence of high affinity NT binding sites in the SN (Boudin et al., 1996, 1998; Elde et al., 1990; Nicot et al., 1994; Alexander et al., 1999). Combined autoradiographic and immunohistochemical studies revealed that within DA perikarya and dendrite, functional NTS1 were associated with both the plasma membrane and intracellular organelles (Boudin et al., 1998).

4.3.2 NTS1 in dopaminergic nerve terminals in the neostriatum

The first evidence of NT receptor association with striatal dopaminergic nerve terminals comes from a lesions studies in which 6-hydroxydopamine neurotoxin locally injected into the neostriatum was found to result into a large depletion of NT binding sites (Palacios and Kuhar, 1981). Further lesion studies of the neostriatum in the presence of levocabastine indicated that these NT binding sites belong to the high affinity component (NTS1) and that they were all located on presynaptic dopaminergic nerve endings (Schotte et al., 1989). More recently, light microscopic immunohistochemical studies using a specific anti-NTS1 antibody also reported the presence of NTS1 in nerve terminals in the neostriatum (Boudin et al., 1996). A biochemical study using purified preparations of nerve terminals (synaptosomes) from the neostriatum has

characterized NT binding sited in these structures (Awad et al., 1989). NT binding properties in the neostriatum were found to correspond to those of high affinity NT binding sites (NTS1). Interestingly, a low affinity component (NTS2) was also detected but its localization was not assessed. However, a more detailed subcellular analysis performed on cytoplasmic extracts containing intact synaptosomes suggested that in the neostriatum NTS1 was located in dopaminergic terminals whereas NTS2 was associated with glial elements (Schotte et al., 1988).

Nigrostriatal neurons have been extensively used as a model to explore the neurotransmitter/neuromodulator role of NT through the activation of specific high affinity receptors (NTS1) at both somatodendritic and terminal level. Accordingly, injection of NT in the SN was shown to increase electrical activity of DA neurons (Pinnock et al., 1992; for review, see Shi et al., 1992) and to stimulate the release of DA both locally in the SN (Faggin et al., 1990; Meyers et al., 1983) and distally in the neostriatum. Similarly, application of NT to the neostriatum was found to locally augment the release of DA by nigrostriatal terminals (Kalivas et al., 1982; Shi et al., 1992; Widerlov et al., 1982). Ligand-induced internalization of NTS1 was also among the modulatory process studied and was shown to occur at the level of both perikarya in the SN and terminals in the neostriatum

4.4 Internalization of NTS1 in A9 dopaminergic neurons at the level of perikarya in the SN and nerve terminals in the neostriatum

Cell bodies and nerve terminals of nigrostriatal dopaminergic neurons are endowed with the capacity to internalize fluorescent or radiolabled NT and to

transport it to the perinuclear region as demonstrated by retrograde labeling studies as well as studies on cultured neurons and mesencephalic brain slices.

Evidences for internalization of NT in somatodendritic domain of dopaminergic nigrostriatal neurons come from studies in cultures cell and in brain slices in which fluorescent derivative of NT was found to be transported inside the cell in a temperature and time-dependant manner (Faure et al., 1995; Nouel et al., 1997). This process was prevented by preincubation in PAO, strongly suggesting that it corresponds to clathrin-mediated internalization. Performing immunolabeling EM studies using a specific anti-NTS1 antibody, Boudin and colleagues (1998) observed immunoreactivity in endosomes of nigral neurons, suggesting that internalization in these cells was subserved by NTS1 and was vesicular in nature.

NT internalization in dopaminergic nerve terminals in the neostriatum was documented by autoradiographic study in which injection of tritiated NT to the rat neostriatum was followed by an accumulation of radioactivity in the ipsilateral SN two hours later (Catel et al, 1990). The appearance of this labeling was prevented by pretreatment with colchicine, or 6-hydroxydopamine as well as by injection of an excess of unlabelled NT or of NT 8-13, an NT fragment that contains all the structural requirements for receptor recognition (Kitabgi et al., 1980; St-Pierre et al., 1981; Kitabgi et al., 1984). However, NT 1-8, which has no affinity for NT receptors had no effected. It was suggested that NT internalized through a receptor dependant fashion and was retrogradely transported to the perikarya in the SN through a process that was microtubule-dependant. Further retrograde labeling studies using confocal microscopy to follow in vivo injection of

fluo NT in the neostriatum supported this first finding (Beaudet et al., 1994). Data from these latter investigations suggested that the compartments implicated in ligand-induced NT receptor internalization correspond to endosomal and lysosomal elements (Beaudet et al., 1994). HPLC experiments demonstrated that the radioactivity appearing in the SN following injection of radiolabeled NT in the neostriatum was composed of both intact NT and degradation products of this peptide (Castel et al., 1992).

Comparison between intracellular distribution of retrogradely transported NT from neostriatal nerve terminals with that observed following internalization of the peptide at the somatodendritic level in the SN was done by Faure and colleagues (1995). To document somatodendritic internalization, brain slices from rat ventral midbrain were incubated with a fluorescent derivative of NT. To document retrograde transport, rats were injected with the same compound into the neostriatum. At short time intervals (5-15 min), labeling was found to be distributed throughout the cytoplasm of perikarya from the SN and took the form of small intense fluorescent particles suggesting that the endocytotic compartment subserving NT receptor internalization was vesicular in nature. A comparable labeling was also detected following intrastriatal injection with fluo NT. It was also found that intracellular distribution of the fluorescent ligand reorganizes with time. After 30 minutes to an hour, the intensity and size of granules increase in the cell bodies but diminish in the neuropil and cluster around the nucleus. Taken together, these experiments suggest that in the mesocortical DA neuron, internalization of NT occurs both at somatodendritic and axon terminal plasma membrane through unknown endosomal-like

compartments, the number of which diminishes with time while they increase in size and clustering near the cell nucleus.

OBJECTIVES

Neuronal cells differ from non-neuronal cells in that it is highly polarized into somatodendritic and terminal domains (Dotti et al., 1990). These two regions are physically separated by a functional barrier at the level of the axon Hillock (Kobayashi et al., 1992). The dendritic and axonal processes differ from each other in morphology (Bartlett et al., 1984), in their capacity for protein synthesis (Davis et al., 1992), in some of the molecular constituents of their cytoskeleton (Careres et al., 1984; Shaw et al., 1985) and in the composition of their plasma membrane (Lindse et al., 1985; Banker et al., 1988). Somatodendritic and terminal domains were also found to differ functionally, with regards to sorting and endocytotic pathways. Differential sorting to the axonal and somatodendritic domains have been detected in neurons transfected with viral glycoproteins known to be transported to the apical and basolateral plasma membrane domains, respectively (Dotti et al., 1990). Cell bodies and terminals were shown to display differential endocytotic pathways in hippocampal neurons culture at the EM level (Parton et al., 1992). Late endosomes (possessing rab7 protein) and lysosomes were shown to be predominantly located in the cell body and in the proximal segments of the dendrites. Multivesicular bodies appear to be the major structures mediating transport of endocytosis markers between the nerve terminal and the cell body (Parton et al., 1992).

Given that the membrane domains of dendrites and terminals exhibit these differential properties, the question arises whether NT receptor activation in dendrites and axons gives rise to the same pattern of internalization. There has been so far in the litterature no direct experimental evidence for the

internalization of any G protein-coupled receptor. There is however, evidence for in vivo retrograde transport of labeled NT from axon terminals in the neostriatum to cell bodies in the subtantia nigra following injection of high concentrations of the radioactive or fluorescent ligand. It has been hypothesized that this retrograde transport was triggered by receptor mediated internalization of the labeled ligand, but the mechanisms of such internalization has never been addressed. In fact, NT binding sites in neostriatal terminals have themselves been only poorly characterized at biochemical level (Faure et al., 1995).

To both characterize the pharmacological properties of radioalabeled NT binding to presynaptic receptors, as well as to document the existence and establish the kinetics of NT internalization in the presynaptic compartment, we resorted to the use of purified preparation of terminals referred to as synaptosomes. Synaptosomes present double advantages consisting of a closed system in which the phenomenon of internalization is not only isolated (from events in the cell body) but is also amplified due to purification. They also allow the use of lower more physiological concentrations of radioligand, thereby affording a better characterization of NT binding sites and their internalization.

As extensively reviewed above, in the rat neostriatum, high affinity NT receptors (NTS1) are localized to dopamine-releasing nerve terminals which originate from the substantia nigra in the mesencephalon. Consequently, synaptosomal preparations from the neostriatum appeared to constitute an excellent model:

1- To confirm the presence of high affinity binding sites in neostriatal terminals.

2- To determine whether there is NTS1 mediated internalization of NT in neostriatal dopaminergic terminals.

3- Characterize the kinetics and define the pathway (s) of NT internalization in the neostriatum.

4- To bring a better understanding of the fate of NTS1 following internalization in neostriatal terminals.

METHOD PROPOSED

To determine the identity of NT receptor subtypes expressed in synaptosomes from the neostriatum, binding experiments using [^{3}H]-NT were performed in the presence levocabastine to inhibit low affinity NT binding sites (NTS2). To determine whether specifically bound [^{3}H]-NT was internalized, hypertonic acid wash, pH 4, was used to dissociate surface-bound ligand molecules (Botto et al., 1998; Nouel et al., 1997).

To gain insight into the process underlying NT receptor internalization, treatments with endocytosis inhibitors (PAO and sucrose) or conditions such as low temperature were applied.

To gain insight into the events that followed internalization of [^{3}H]-NT-NT receptor complexes, inhibition of receptor recycling was performed using monensin, a drug documented to inhibit endosome acidification and thereby, dissociation of receptor-ligand complexes.

1 SYNAPTOSOMAL PREPARATIONS FROM THE RAT NEOSTRIATUM

Ten adult male Sprague-Dawley rats (220-240 g) were killed by stunning and decapitated. The brains were rapidly removed and the neostriatum or the cerebellum dissected out and pooled in 10 volumes (ml) of cold 0,32 M sucrose medium (4°C) containing peptidases inhibitors (see List of Major Solutions at the end of this section). Synaptosomes were isolated using either one of the three following methods, all of which were performed at 4°C to prevent protein degradation.

1.1 Method I

Method I as used by McPherson and colleagues (1994) yields crude synaptosomal preparations. Neostriatum extracts were homogenized by 9 slow up and down strokes of hand glass homogenizer using a Teflon—coated pestle rotating at 900 rpm. The homogenate was centrifuged in a *Sorvall RC 5C Plus* centrifuge at 750 g (2500 rpm) for 5 minutes using a *SS-34* rotor. The resulting supernatant (S1) was centrifuged again at 12 000 g (10 000 rpm) for 15 minutes. The pellet (P2) was resuspended in 10 volumes (ml) of 0,32 M sucrose medium and centrifuged at 14,500 g (11 000 rpm) for 15 minutes. The resulting pellet was recuperated and resuspended in modified Earle's buffer.

1.2 Method II

Method II gives purified synaptosomal preparations, developed by Dodd and colleagues (1981). Neostriatum extracts were homogenized by 12 up and down trokes of hand glass homogenizer using a Teflon—coated pestle rotating at 800 rpm. The homogenate was centrifuged in a *Sorvall Discovery 90* ultracentrifuge at 1850 g (4500 rpm) for 10 minutes using a Swing-out *SW 50,2 Ti* rotor. The resulting supernatant (S1) was layered on top of 1,2 M Sucrose medium (4 ml) and centrifuged again at 18,500 g (45 000 rpm) for 15 minutes. Two ml of the gradient interface was collected, diluted with 5 ml of 0,32 M Sucrose medium, layered on top of 0,8 M Sucrose medium (4 ml), and centrifuged for an other 15 minutes at 18 500 g (45 000 rpm). The resulting pellet in which purified synaptosomes had collected was recuperated and resuspended in modified Earle's buffer.

1.3 Method III

Like Method II, Method III gives purified synaptosomal preparations. It is a method developed by Nagy and colleagues (1984). Neostriatum extracts were homogenized by 10 up and down trokes of hand glass homogenizer using a Teflon—coated pestle rotating at 800 rpm. The homogenate was centrifuged in a *Sorvall RC 5C Plus* centrifuge at 1000 g (2900 rpm) for 10 minutes with a *SS-34* rotor. The resulting supernatant (S1) was centrifuged again at 12 000 g (10 000 rpm) for 20 minutes. The pellet (P2) was recuperated in about 3 volumes of 0,32 M Sucrose medium (to a protein concentration of 6-8 mg/ml) and diluted in 8,5% of Percoll/sucrose medium (8:1) to a final concentration of 7,5%. This preparation

was layered on top of a discontinuous Percoll/sucrose gradient consisting of two phases (from bottom to top, 16% and 10%) and centrifuged at 15 000 g (11 200 rpm) for another 15 minutes. The gradient interface between 16% and 10% Percoll/sucrose solutions which is documented to contain the synaptosomes (**Na**gy et al., 1989) was collected and was washed 3 times to remove the Percoll silica balls by consecutive dilution in 10 ml of 0,32 M Sucrose medium and centrifugation at 15 000 g (11 200 rpm). Synaptosomal preparations were resuspended in modified Earle's buffer.

2 ELECTRON MICROSCOPY

To compare the integrity and enrichment of synaptosomal preparations using Method I, II and III, as well as to determine the effects of our internalization assays on synaptosomes prepared according to Method I.

Synaptosomal preparations were then processed for EM microscopy. For this purpose, they were fixed with prefiltered 2% Acrolein-2% Paraformaldehyde in 0,1M phosphate buffer (PB) for 10 minutes followed by 2% PFA in 0,1M phosphate buffer for 20 minutes and rinsed in 0,1 M PB. In experiments designed to test the effects of our labeling conditions on synaptosomal integrity, synaptosomes were incubated for 30 minutes in Modified Earle's buffer in the presence or absence of PAO (10mM), incubated in Earle's at 37°C and acid washed or not as described below and then fixed.

All preparations were then post-fixed with 2% osmium tetroxide for 30 minutes and rinsed 3 times with 0,1 M SPB for 3 minutes. The fixed synaptosomes were dehydrated through successive 10 minute incubations in

increasing concentrations of ethanol (50, 70, 80, 90, 95 et 100 %) and embedded in Epon 812 through successive 30 minute incubations in increasing concentrations of Epon 813/Propylene oxide (1:1 and 3:1), followed by 100% epon. The synaptosomes were pelleted in a plastic mold using a table centrifuge and incubated for 4 days at 60°C. Ultrathin sections (80 nm) were cut from each resulting Epon block, laid on grids and counterstained with uranyl acetate for 20 minutes and lead citrate for 2,5 minutes and examined with a *JOEL 100CX* electron microscope. Photographs were acquired at 27000, 40000 and 50000 X magnifications.

3 BINDING STUDIES

Synaptosomal preparations were assayed for protein concentration using a *Bio-Rad* assay reagent, with bovine serum albumin as a standard. In some experiments, synaptosomal preparations were pretreated at 4°C for 30 minutes with phenylarsine oxide (PAO)(10µM), Sucrose buffer (modified Earle's buffer containing 0,32 M sucrose) or monensin (25 µM) prior to ligand exposure. Unless otherwise mentioned, all experiments were performed in the presence of levocabastine (1mM). Incubation was performed at 4 or 37°C depending on the conditions being tested. Incubation was performed a series of plastic tubes containing 1000 µg of synaptosomal protein in 200µl of Sucrose buffer, Regular or Modified Earle's buffer, 25 µl of radiolabeled ligand [[^{3}H]-Neurotensin ([^{3}H]-NT) or [^{125}I]-diferric human transferrin ([^{125}I]-Tf)] and one of the two following: 25 µl of unlabeled ligand (Neurotensin, 1µM or human apotransferrin, 5µM) for determination of non-specific binding or 25 µl of the same buffer used to

resuspend the synaptosomal preparations for determination of total binding. Specific binding was obtained by subtracting non-specific from total binding. All of the ligands (radiolabeled or unlabeled) and drugs (sucrose, PAO and monensin) were prepared in the same buffer used to resuspend the synaptosomal preparations.

Incubations were terminated by two successive addition of 12 ml of ice-cold buffer and filtration under vacuum trough GF/B filters presoaked for 1-2 hours at 4°C in modified Earle's buffer containing polyethylenimine (0,3% V/V). For [^{3}H]-NT binding studies, Filters were recuperated in plastic vials with 10 ml scintillation liquid, *Ecolite (+)* and counted in a gama counter. For [^{125}I]-Tf binding study, filters were recuperated in plastic tubes and counted in a beta counter.

To determine internalized [^{3}H]-NT following incubation, 1ml of ice cold (4°C) hypertonic acid wash solution (Regular Earle's buffer, pH 3,75) was added on top of the synaptosomal incubation medium for a final pH of 4. The cumulative solution, thus obtained was incubated at room temperature (25°C) for 2 minutes for time course studies and in increasing time intervals (2, 4 and 6 minutes) to test acid wash efficiency.

3.1 Saturation studies

For saturation studies at 37°C, non-pretreated synaptosomal preparations, were resuspended in ice cold (4°C) modified or regular Earle's buffer or Sucrose buffer for [^{3}H]-NT studies and in modified Earle's buffer for [^{125}I]-Tf studies. Tubes containing synaptosomal preparations were then incubated in a heated water

bath (37°C) in increasing concentrations of [^{3}H]-NT (from 0,2 to 4nM) or [^{125}I]-human Tf (from 0,1 to 15nM) for 15 and 30 minutes, respectively.

For [^{3}H]-NT saturation studies at 4°C, 30 minutes incubation was done with increasing concentrations of [^{3}H]-NT ranging from 0,1 to 4 nM.

3.2 Time course studies

For time course experiments, pretreated (sucrose or PAO) and non-pretreated synaptosomal preparations were resuspended in ice cold (4°C) modified Earle's buffer without or with levocabastine (1mM) and incubated in 1nM of [^{3}H]-NT at 37°C (heated water bath) or 4°C (on ice) for increasing time intervals of 2, 4, 10, 15 and 45 minutes. For [^{125}I]-Tf studies, incubation was performed at 37°C in 8nM of [^{125}I]-Tf for increasing time intervals of 4, 15 and 45 minutes.

3.3 Competition studies

Synaptosomal preparations were resuspended in ice cold (4°C) modified Earle's buffer and incubated for 15 minutes in 1nM of [^{3}H]-NT at 37°C with increasing concentrations of SR48692, from 10^{-12} to 10^{-5} M at regular intervals.

3.4 Product specifications

Phenylarsine oxide (PAO): PAO is a trivalent arsenic compound that is though to form stable rings between vicinal sulfhydryl groups. This was associated with the inhibition of clathrin-mediated internalization (Hertel et al., 1985).

Sucrose: Sucrose treatment (0,45 M) has been shown under EM microscopy to prevent the formation of functional clathrin-coated pits (Heusser et al., 1989).

Both Phenylarsine oxide and sucrose are well documented to block clathrin mediated internalization for many receptors. For instance, PAO inhibits clathrin-mediated internalization of epidermal growth factor receptors (Hertel et al., 1985; Kato et al., 1992), transferrin receptor (Sturrock et al., 1990), insulin receptors (Devaskar et al., 1985) and high affinity neurotensin receptors (NTS1) (Chabry et al., 1995, Botto et al., 1998; Beaudet et al., 1998). Sucrose was shown to block clathrin-mediated internalization of LDL receptors (Heusser et al., 1989), EGF receptors (Moss et al., 1991), human growth hormone receptors (Ilondo et al., 1991), alpha1B-adrenoreceptor (Hirasawa et al., 1998) and of NTS1 (Vandenbulck et al., 2000).

Levocabastine: Levocabastine is an H1 antihistamine that is shown to specifically inhibit low affinity neurotensin binding sites (NTS2) without affecting high affinity component (NTS1) (Schotte et al., 1986; Kitabgi et al., 1987).

Monensin: Monensin is a carboxylic ionophore that is well known to prevent recycling to the plasma membrane of many receptors, including LDL receptors (Sandip et al., 1981), atrial-natriuretic factor B and C receptors (Rathinavelu et al., 1991) and insulin receptors (Devaskar et al., 1985)

SR48692: SR48692 is a non-peptide antagonist developed by SANOFI Recherche (France) that specifically recognizes high affinity neurotensin receptors (NTS1).

3.5 Statistical analysis

Values for all binding experiment represent means ± standard error of the mean for n=3 per condition. The scatchard plot calculated for each specific binding curve represents the ratio of bound radioligand to free radioligand (B/F) relative to bound ligand in fmol/mg protein (B, fmol/mg protein). The Bmax was determined as the value at X-intercept (fmol/mg protein) and the Kd corresponded to the inverted value of the plot slope (slope=1/Kd).

All the graphs and data manipulations were done by the Microsoft Excel.

List of major solutions:

-*Acetate Uranyl:* Acetate uranyl (5%) in Ethanol 40%.

-*Calcium-free Modified Earle's buffer*: Modified Earle's Buffer without $CaCl_2$ but with EDTA (20mM)

-*Hypertonic Acid Wash solution:* Modified Earle's buffer with NaCl (400mM), adjusted to pH 3,75 with glacial acetic acid.

-*Lead citrate:* N_20_6Pb (0,15 M), $C_6H_5Na_3O_7.2H_2O$ (0,15 M), pH with 4% NaOH

-*Modified Earle's buffer:* choline chloride (155 mM), KCl (5 mM), $CaCl_2$ (1,8 mM), $MgCl_2$ (0.9 mM), HEPES (25 mM), BSA (30 mM) glucose (5 mM) and phenanthroline (1 mM) to prevent NT degradation.

-*Osmium tetroxide(2%):* 2% osmium tetroxide, Sorensin's phosphate buffer (0,1M)

-*Regular Earle's buffer:* same as modified Earle's buffer but choline chloride is replaced by NaCl (140 mM)

-*Sucrose medium for Method I:* Sucrose (320mM), HEPES (20 mM), Benzamidine (64 mM), Leupeptine (5 µg/ml) and Aprotinin (5 µg/ml).

-*Sucrose medium for Method II and III:* Sucrose (320, 800 or 1200 mM), HEPES (5 mM), EDTA (0,1mM).

-*Sorensin's phosphate buffer (0,1M):* Na_2PO_4 (150 mM), $NaH_2PO_4.H_2O$ (45 mM) (pH not adjusted)

RESULTS

1 OPTIMIZING SYNAPTOSOME PREPARATION FROM THE RAT NEOSTRIATUM

Three methods were tested to isolate synaptosomes from the neostriatum (Methods I, II and III). Method I is the shortest (about 1,5 hr) as it uses only 3 centrifugation steps to yield a crude preparation of synaptosomes. The two other techniques take longer (about 3,5 hrs) and essentially add a purification step with the use of discontinuous gradients. A sucrose gradient was used in Method II whereas in Method III, a Percoll/sucrose gradient was used (for details see Materials and Methods). Methods II and III both yielded a purified preparation of synaptosomes, however, the purification steps in these latter two methods reduced the final amount of protein recuperated in the synaptosomal fraction by a factor of 10 compared with that obtained in Method I (0,9 +/- 0,3 µg protein/µl vs 8,8 +/- 0,4 µg protein/µl per 10 rats).

1.1 Synaptosomal integrity as assessed at the EM level

The morphological integrity of synaptosomes isolated from Methods I, II and III was assessed by electron microscopy (EM) level.

1.1.1 Synaptosomal preparation from Method I

Synaptosomes prepared according to Method I contained a large number of contaminants (Figure 4 A, B, C and D). The largest number of these contaminants was glial membrane remnants, which often took the appearance of multiple parallel dark bands closely packed together. These structures probably corresponded to disrupted myelin sheaths, which are well known to display this

appearance under EM. However, most of the contaminants were mitochondria and unidentified polymorphic profiles. Mitochondria were easily recognized by their dark cylindrical-shape and their inner parallel cisternae (Figure 4B, D). By contrast, unidentified profiles were usually irregular in shape and contained a variety of cellular structures including irregular and sometimes elongated vesicular compartments as well as mitochondria (Peters et al., 1976). Synaptosomes were most easily identified by the presence of a small portion of the postsynaptic membrane that sometimes remained attached to the pre-synaptic element. These specialized regions were characterized by the classical accumulation of dense material on both pre and post-synaptic domains (Peters et al., 1976). Synaptosomes were filled with clear spherical vesicles that were often clustered next to the synaptic density. Interestingly, synaptosomes occasionally exhibit vesicles about twice the size of synaptic vesicles with dense short spikes regularly distributed around its periphery (Figure 4A). These structures are reminiscent of clathrin-coated vesicles (Peters et al., 1976). Furthermore, at the level of plasma membrane, clathrin-coated pits could also be observed (Figure 4C). Synaptosomes also contained mitochondria as well as larger irregular vesicular compartments, which were usually located away from the synapse and may correspond to endosomes (Figure 4A). Most synaptosomes lacked a synaptic specialization but could be identified by their high content in closely packed synaptic vesicles. Synaptosomes isolated using Method I had an average diameter of 0,93 μm, however, some of them measured up to 2 μm in diameter.

Synaptosomes isolated using Method I displayed an undisrupted plasma membrane indicating that they had been successfully sealed following

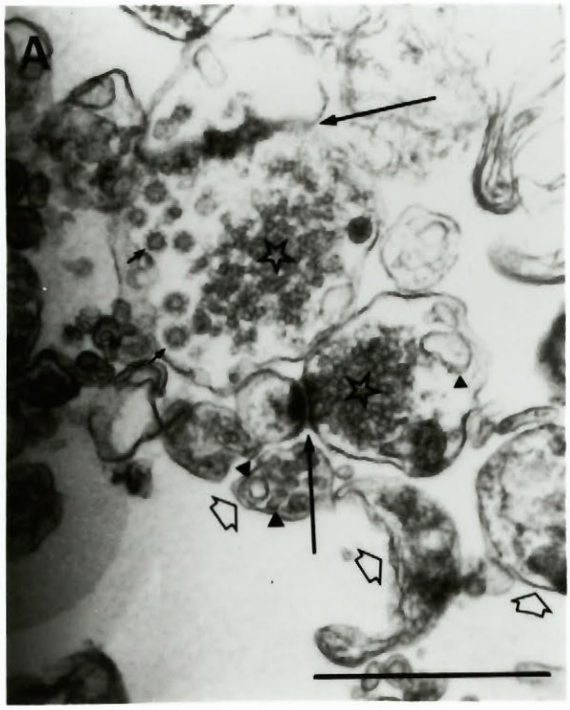
homogenization. Furthermore, they displayed a morphology characteristic of intact nerve terminals, indicating that the procedure to isolate them had not been too damaging.

Figure 4: ***Electron micrographs of synaptosomes isolated using Method I (A, B, C and D)*** Synaptosomal preparations in Modified Earle's Buffer at 37°C were fixed, stained and studied by electron microscopy as described in Materials and Methods. A, Synaptosomes are recognizable by the presence of synaptic vesicle clusters in their cytoplasm (stars). Note the presence of clathrin-coated vesicles (small arrow). Synaptic densities associated with post-synaptic remnant can also be seen (long arrows). Both synaptosomes and unidentified vesicles (empty arrow) contain irregular vesicular compartments (arrowhead). B, Mitochondria are also observed in synaptosomes (white asterisk). Oligodendrocyte debris (black dot) are abundant in the preparations. C, Note the presence of clathrin-coated pits in a synaptosome. Panel D exhibits a large synaptosome (2µm) and most of the previously described structures. Scale Bar = 1µm.

1.1.2 Synaptosomal preparations from Method II

As opposed to Method I, Method II yields a synaptosomal preparation with less contamination than in the crude synaptosomal preparation obtained by Method I. The number of intact synaptosomes was much higher with Method II compared to Method I. Much like synaptosomes from crude preparations (Method I), the synaptosomes obtained using Method II display morphological characteristics of nerve terminals (Figure 5A, B). They contain synaptic vesicle clusters and often show synaptic membrane specialization characterized by a post-synaptic density and by the occasional association of post-synaptic membrane remnants (see Figure 5B). A large intracellular space was also observed, and the plasma membrane was generally well preserved. Compared to synaptosomes isolated from Method I, the synaptosomes from Method II were smaller in size, averaging 0,75 μm. Clathrin-coated vesicles are also less readily identifiable and were fewer in number. As mentioned earlier, although Method II yields synaptosomes of high purity and preserved morphology, it does not yield high protein concentration.

Figure 5: ***Electron micrographs of synaptosomes isolated using Method II (A, B) and III (C, D)*** Synaptosomal preparations in Modified Earle's Buffer at 37°C were fixed, stained and studied by electron microscopy as described in Materials and Methods. As seen in panels A and B, the synaptosomal preparation contain the same elements as those described in Method I. Thus, we detect synaptic vesicle clusters in synaptosomes (stars), synaptic density (long arrow), mitochondria (white asterisk), unidentified vesicles (empty arrow) and irregular vesicular compartments (arrow head). In panel C and D, note the absence of intracytoplasmic space in the synaptosomes. Synaptic vesicles, synaptic density, mitochondria and oligodendrocytes remnants are observed. Scale bar = 1μm.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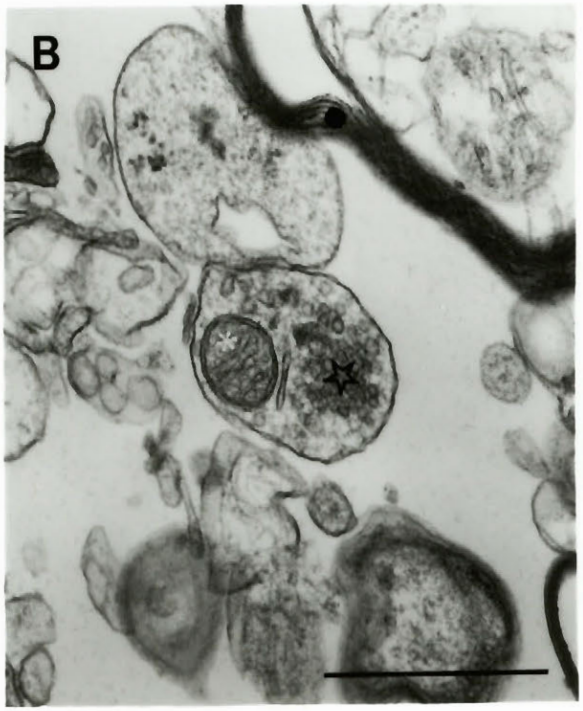
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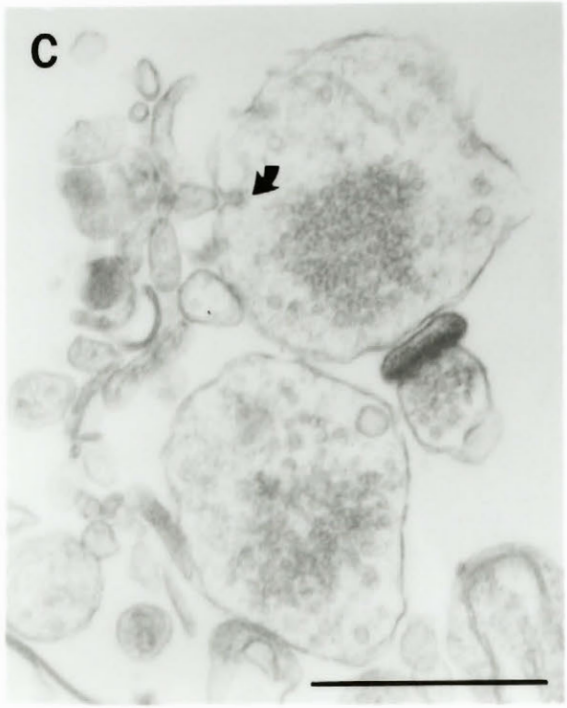
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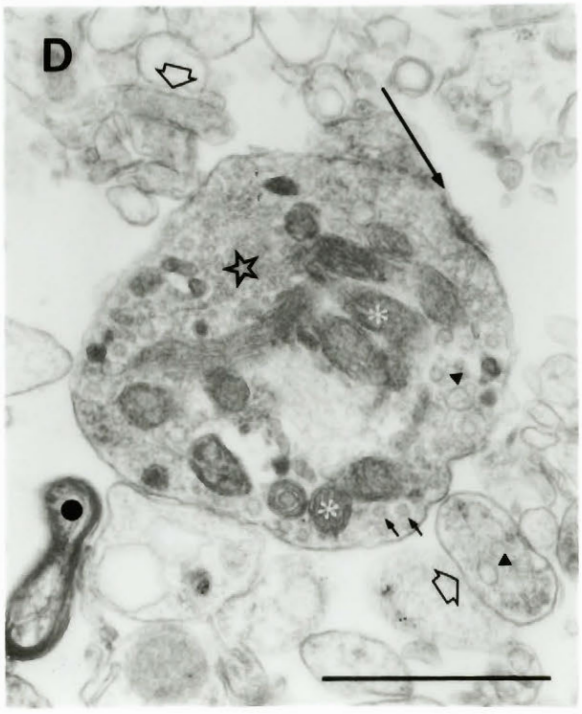
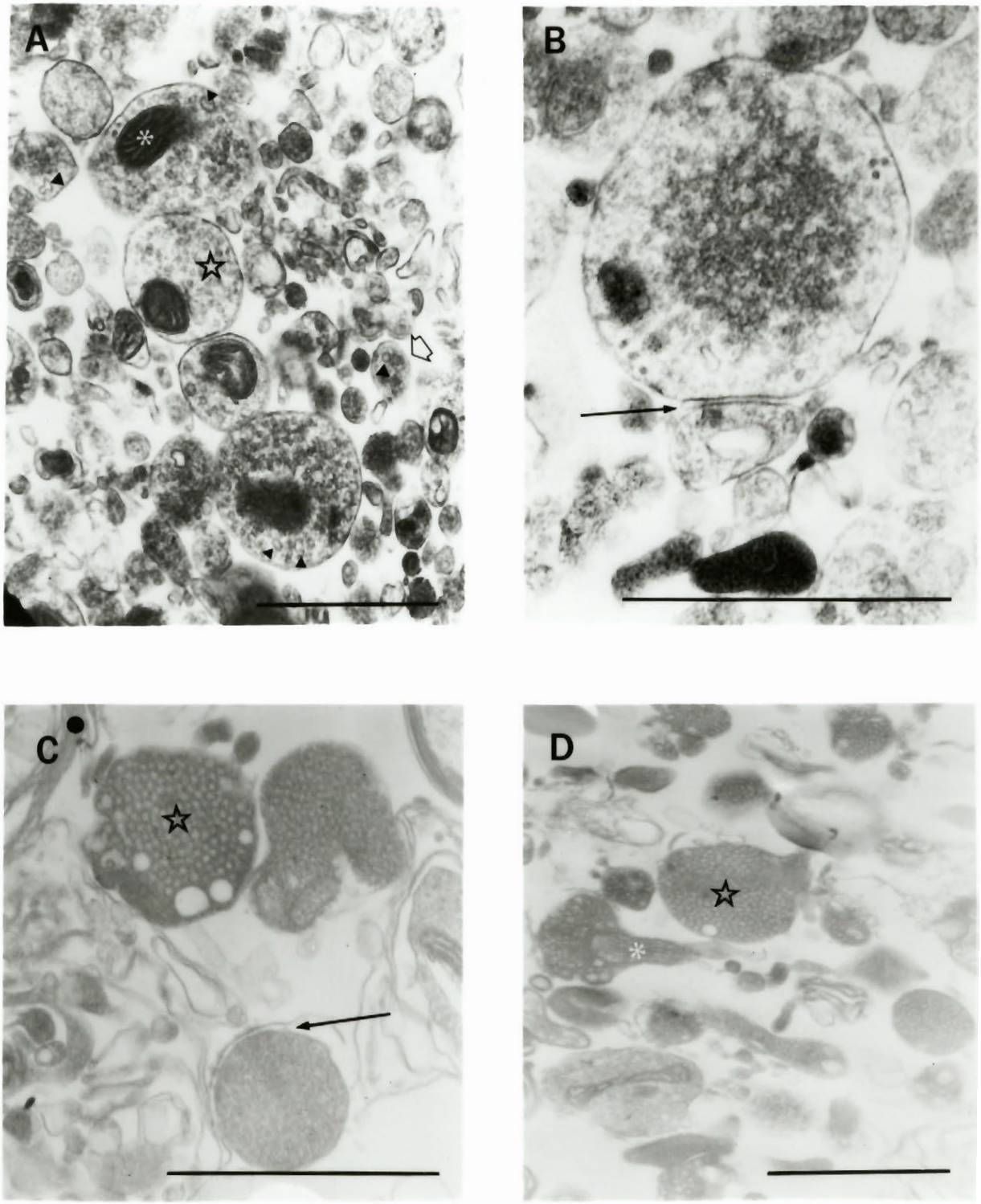

D

1.1.3 Synaptosomal preparations from Method III

The last method tested also yields purified synaptosomes, however, EM studies of synaptosomes from this preparation reveal a different morphology (Figure 5C, D). Much like synaptosomes purified by Method II, the synaptosomes obtained by Method III contain closely packed spherical structures resembling synaptic vesicles, which were often seen to closely surround mitochondria. However, synaptosomes isolated from Method III display a rather irregular contour and clathrin-coated vesicles were not observed. The most striking difference was the absence of intracellular space, as the plasma membrane was almost indistinguishable from the periphery of synaptic vesicle clusters. This gives the synaptosomes a shrunken appearance congruent with their being smaller in size (average diameter 0,44 μm) than the ones prepared from Method II or Method I.

In summary, Methods I and II yield comparably well-preserved synaptosomes. However, Method II yields a protein concentration that is insufficient for binding studies, making crude synaptosomal preparations from Method I the most appropriate model for our studies. Method I has been shown to produce morphologically well preserved synaptosomes, the protein concentration of which is sufficient for biochemical analysis. Furthermore, Method I offers the added advantage of being more expeditive than the other two methods.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2 [^{3}H]-NT BINDING TO NEOSTRIATAL SYNAPTOSOMES PREPARED USING METHOD I

2.1 Effects of different incubation media on [^{3}H]-NT association properties at 37°C

2.1.1 Sucrose and Modified Earle's buffer containing choline chloride

Neostriatal synaptosomes were kept in the sucrose medium (Medium I) they were isolated in and were incubated with increasing concentrations of [^{3}H]-NT up to 1 nM for 15 minutes at 37°C (Figure 6). Non-specific binding was determined by adding an excess (1µM) of unlabeled NT to the incubation (Figure 6). Experiments were done in the presence of levocabastine to inhibit the binding of [^{3}H]-NT to low affinity NT binding sites (NTS2).

Figure 6 indicates that specific [^{3}H]-NT binding to these synaptosomal preparations was saturable. Scatchard plot analysis yields a binding affinity (Kd) for specific [^{3}H]-NT binding at 0,20 nM and a maximal binding capacity (B_{max}) of 25,3 fmol/mg protein. Non-specific binding was very low (about 20% of total binding at saturation) and increased linearly with radioligand concentration.

Because we were concerned that the high concentration of sucrose (0,32 M) present in this incubation buffer might inhibit receptor-mediated endocytosis (Heuser and Andersen, 1989), we also tested [^{3}H]-NT binding using modified Earle's as a binding buffer. Regular Earle's buffer contains sodium chloride that has been reported to reduce the affinity of NTS1 to its ligand (Uhl et al., 1977; Martin et al., 1999), therefore we used a solution in which sodium chloride was replaced by choline chloride (modified Earle's buffer). To determine the efficiency of this medium to detect [^{3}H]-NT binding, the same saturation experiments, as described above, were performed using the modified Earle's buffer (Figure 7).

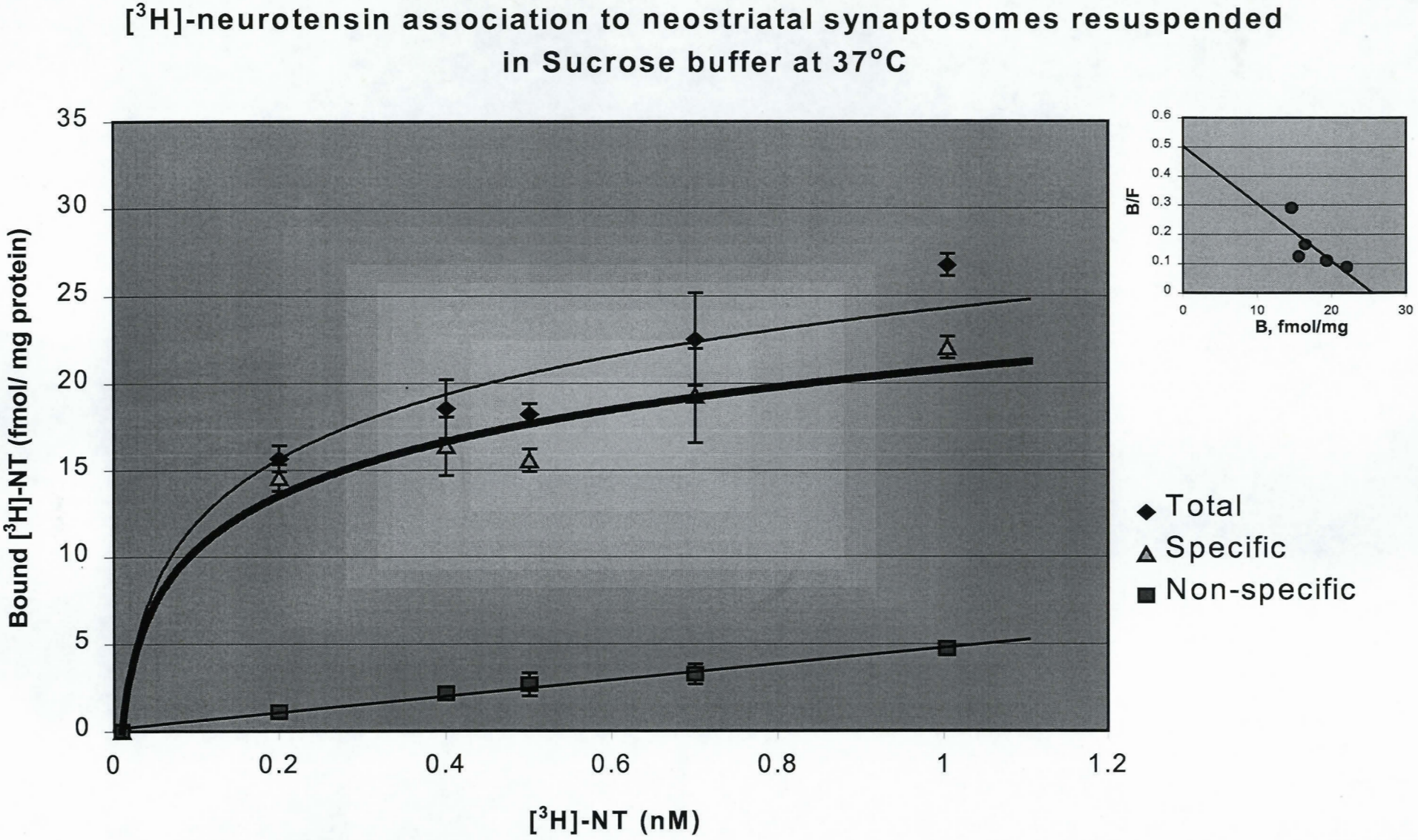


Figure 6: ***Saturation binding of [^{3}H]-NT in Sucrose Buffer (0,32 M) at 37°C.*** Synaptosomal preparations were resuspended in Sucrose Buffer (0,32 M). Total binding was obtained by incubation in increasing concentrations of [^{3}H]-NT for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

As with the buffer containing sucrose, modified Earle's buffer yielded specific saturable [^{3}H]-NT binding (Figure 7). The B_{max} and Kd calculated from the Scatchard plot of these data yielded values that were comparable to those obtained using synaptosomes resuspended in medium containing sucrose. There was however, a slight reduction in both the capacity (22,76 fmol/mg protein) and affinity (0,42 nM) for [^{3}H]-NT binding as compared to values obtained in synaptosomes in sucrose. Non-specific binding was also slightly higher than in experiments using sucrose buffer reaching up to 50% of total binding at saturation (Figure 7).

2.1.2 Regular Earle's buffer containing sodium

The last set of saturation experiments was performed on neostriatal synaptosomes resuspended in regular (non-modified) Earle's buffer to assess the sensitivity of [^{3}H]-NT binding sites to sodium (140 mM) (Figure 8).

In regular Earle's buffer, the binding capacity of neostriatal synaptosomes was significantly decreased (8,3 fmol/mg protein) as compared to values obtained when the experiments were performed in modified Earle's buffer. However, [^{3}H]-NT binding affinity was determined to be 0,3 nM, a value similar to that obtained in modified Earle's buffer.

2.2 Effect of low temperature (4°C) on [^{3}H]-NT association properties

Low temperature slows metabolic activity and intracellular movement of molecules. In various models studied to date, low temperature has been found to alter properties of [^{3}H]-NT binding (Chabry et al., 1993; Mazella et al., 1993). To

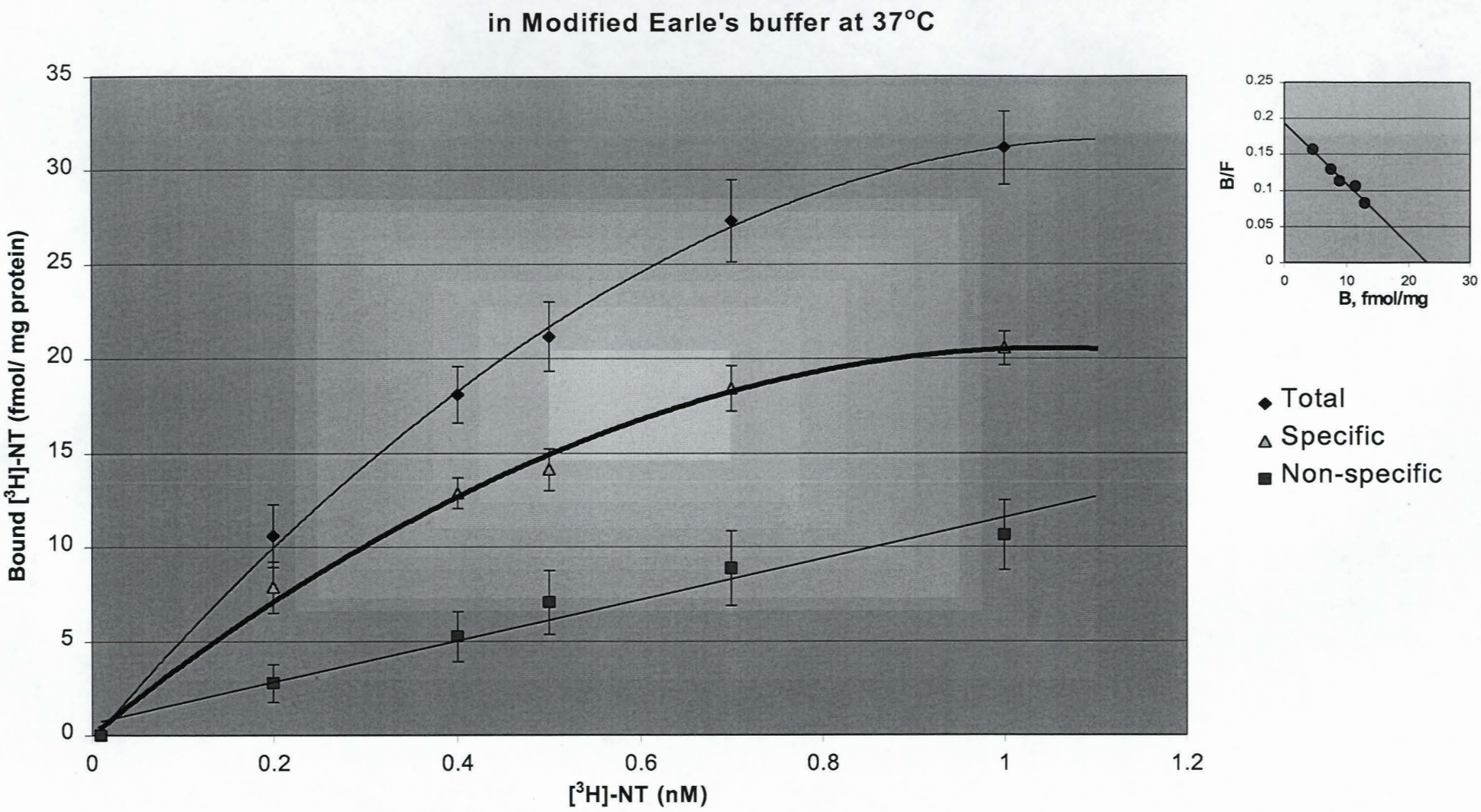


Figure 7: ***Saturation binding of [^{3}H]-NT in Modified Earle's Buffer at 37°C.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in increasing concentration of [^{3}H]-NT for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

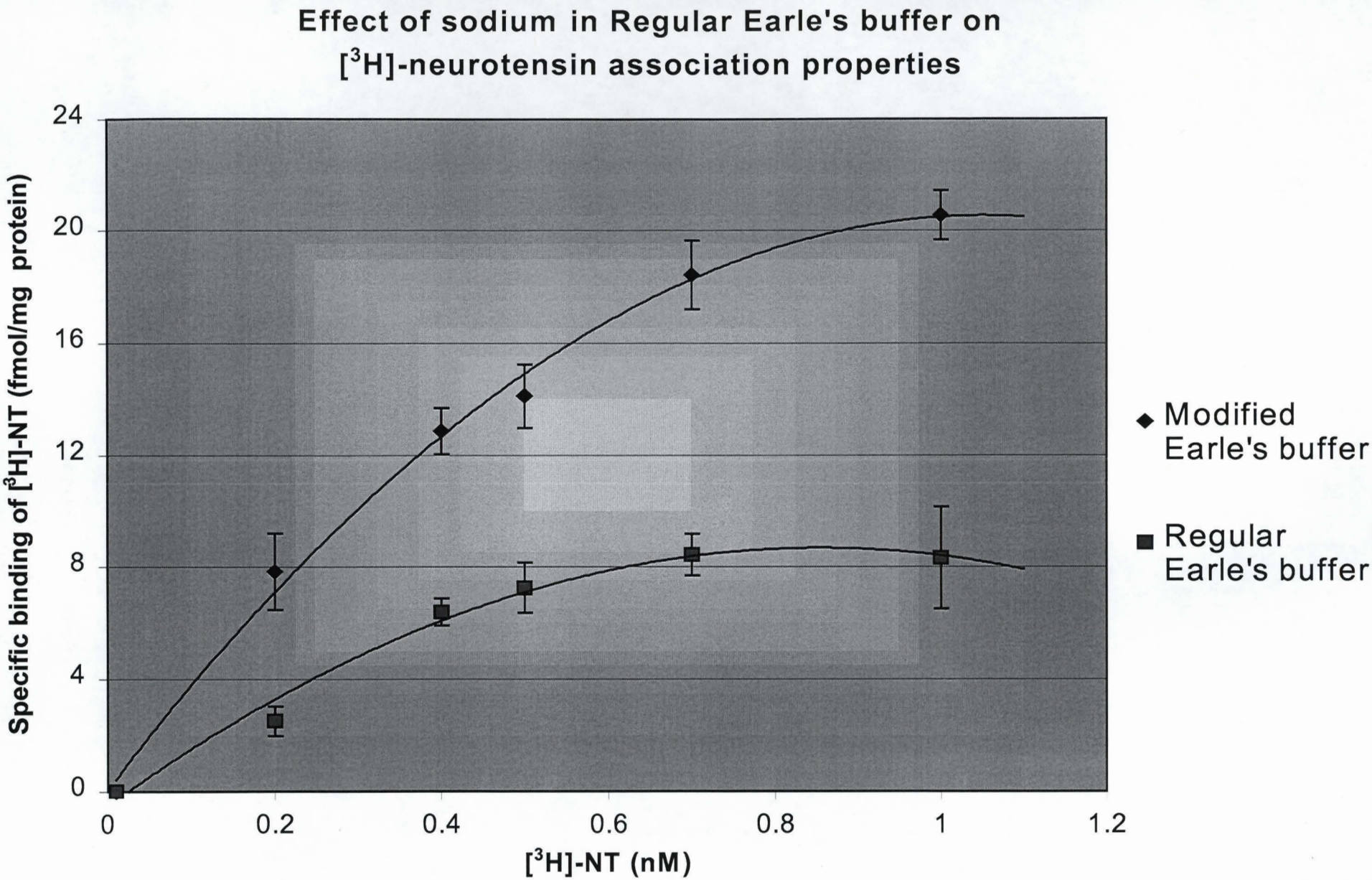


Figure 8: ***Saturation binding of [^{3}H]-NT in Regular Earle's Buffer at 37°C.*** Synaptosomal preparations were resuspended in Regular Earle's Buffer. Total binding was obtained by incubation in increasing concentration of [^{3}H]-NT for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

investigate whether temperature affects [^{3}H]-NT binding to neostriatal synaptosomes, saturation experiments were performed at 4°C and the results compared with those obtained at 37°C (Figure 9).

Low temperature reduced the affinity of [^{3}H]-NT to its binding sites as evidenced by the fact that no saturation was achieved at up to 4 nM of [^{3}H]-NT. Also, at this radioligand concentration, only 11,9 fmol/mg protein was specifically bound, indicating that low temperature reduces [^{3}H]-NT binding capacity (Figure 9).

2.3 Displacement of [^{3}H]-NT binding sites by SR48692, an antagonist of NTS1

To determine whether [^{3}H]-NT binding, observed in neostriatal synaptosomes in the presence of an excess of the NTS2 antagonist levocabastine, corresponded to binding of the NTS1, competition experiments were performed in the presence of the selective NTS1 antagonist SR48692. Levocabastine was also present. As observed in Figure 10, synaptosomes incubated in 1 nM of [^{3}H]-NT in the presence of increasing concentrations of SR48692 showed a sigmoidal displacement of SR48692 by the cold NTS1 antagonist. Half of the specifically bound [^{3}H]-NT was displaced by 3,76nM of SR48692. However, micromolar concentrations of SR48692 were unable to displace a remaining 15% of initially bound [^{3}H]-NT. This corresponds to a value of 3,2 fmol/mg protein (Figure 10).

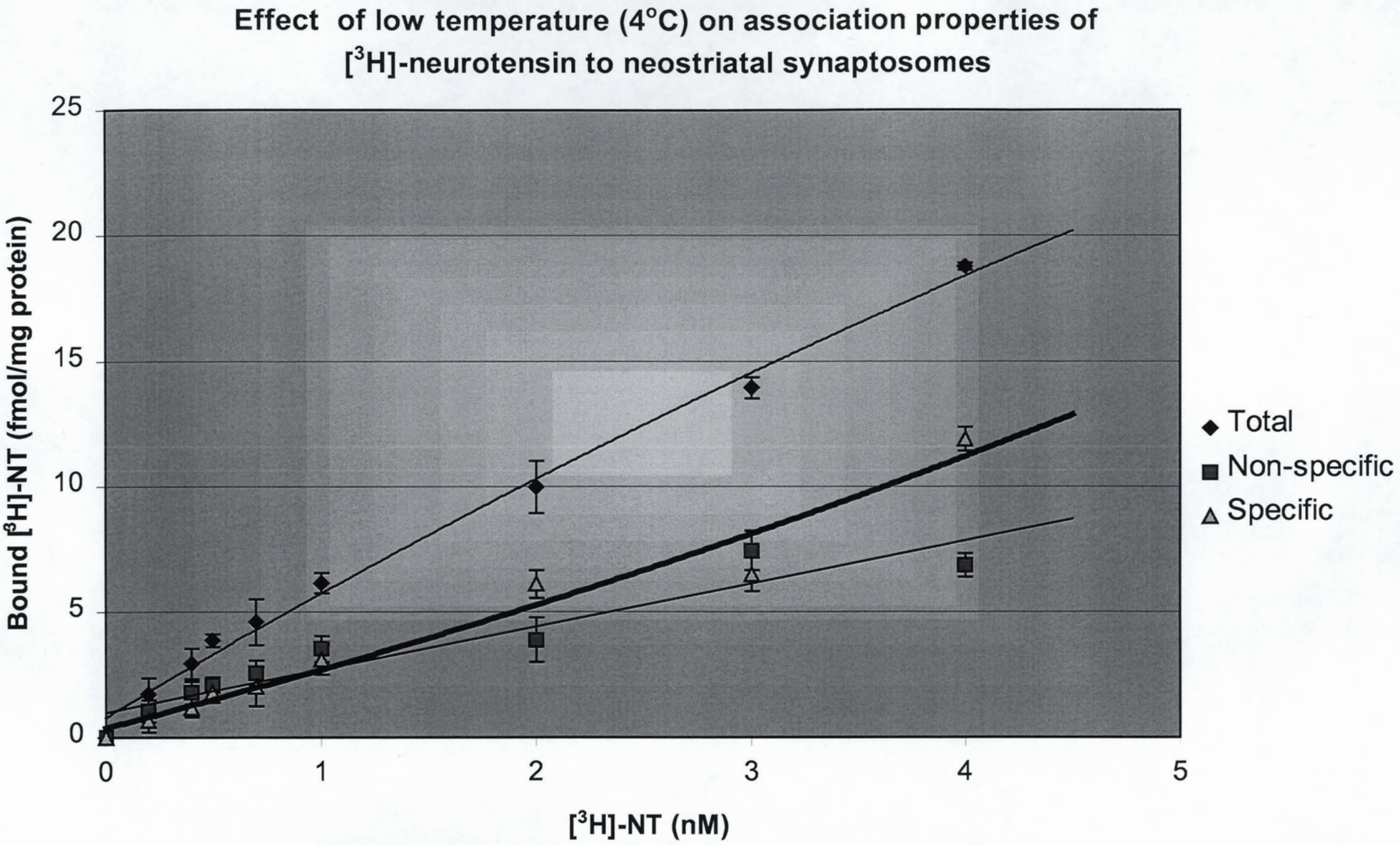


Figure 9: ***Saturation binding of [^{3}H]-NT at 4°C in Modified Earle's buffer.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in increasing concentration of [^{3}H]-NT for 15 minutes at 4°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

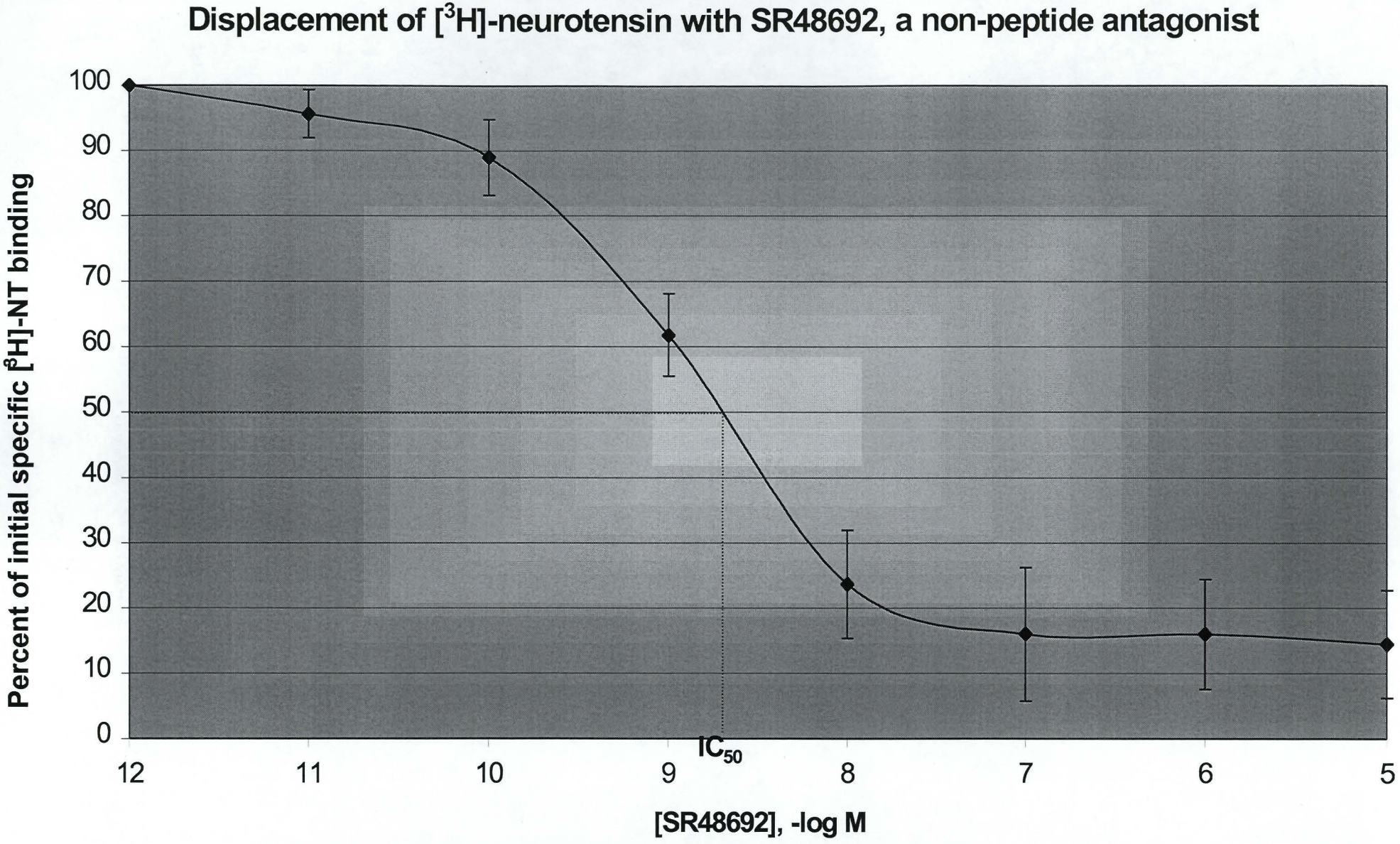


Figure 10: ***Displacement of [^{3}H]-NT by SR48692.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in [^{3}H]-NT (1nM) with increasing concentrations of SR48692 for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1µM). Specific binding was obtained by subtracting non-specific from total binding.

2.4 [^{3}H]-NT binding sites in neostriatal and cerebellar synaptosomes: the relative contribution of high (NTS1) and low (NTS2) affinity NT receptors

Saturation experiments were performed in the presence or absence of levocabastine in synaptosomes from both the neostriatum (Figure 11) and the cerebellum (Figure 12). The cerebellum was used as a control since it has been documented to express a massive concentration of NTS2 (Schotte et al., 1986; Kitabgi et al., 1987) and only low concentrations of NTS1 (Kitabgi et al., 1987; Moyse et al., 1987; Boudin et al., 1996).

In the absence of levocabastine, [^{3}H]-NT binding to synaptosomal preparations from either brain region does not completely saturate and was characterized by an increased binding capacity as compared to that observed in the presence of the NTS2 ligand. Thus, binding capacities of 45,2 and 49,5 fmol/mg protein were observed in synaptosomes from the neostriatum and the cerebellum, respectively (Figures 11 & 12). Synaptosomes from these brain regions also displayed comparable affinity, the estimated value of which was in the nanomolar range. Scatchard plot analysis of specific NT binding to neostriatal synaptosomes yielded a binding capacity (B_{max}) of 106,0 fmol/mg protein and an apparent affinity (Kd) of 1,3 nM, respectively (Figure 11).

In the presence of levocabastine, synaptosomes from these two brain regions displayed different [^{3}H]-NT binding properties (Figures 11& 12). In the neostriatum, as seen previously, [^{3}H]-NT binding sites were saturable, and characterized by a maximum binding capacity (B_{max}) of 21 fmol/mg protein and an apparent affinity (Kd) of 0,2 nM (Figure 11). By contrast, in synaptosomes from the cerebellum, [^{3}H]-NT binding increased linearly with radioligand

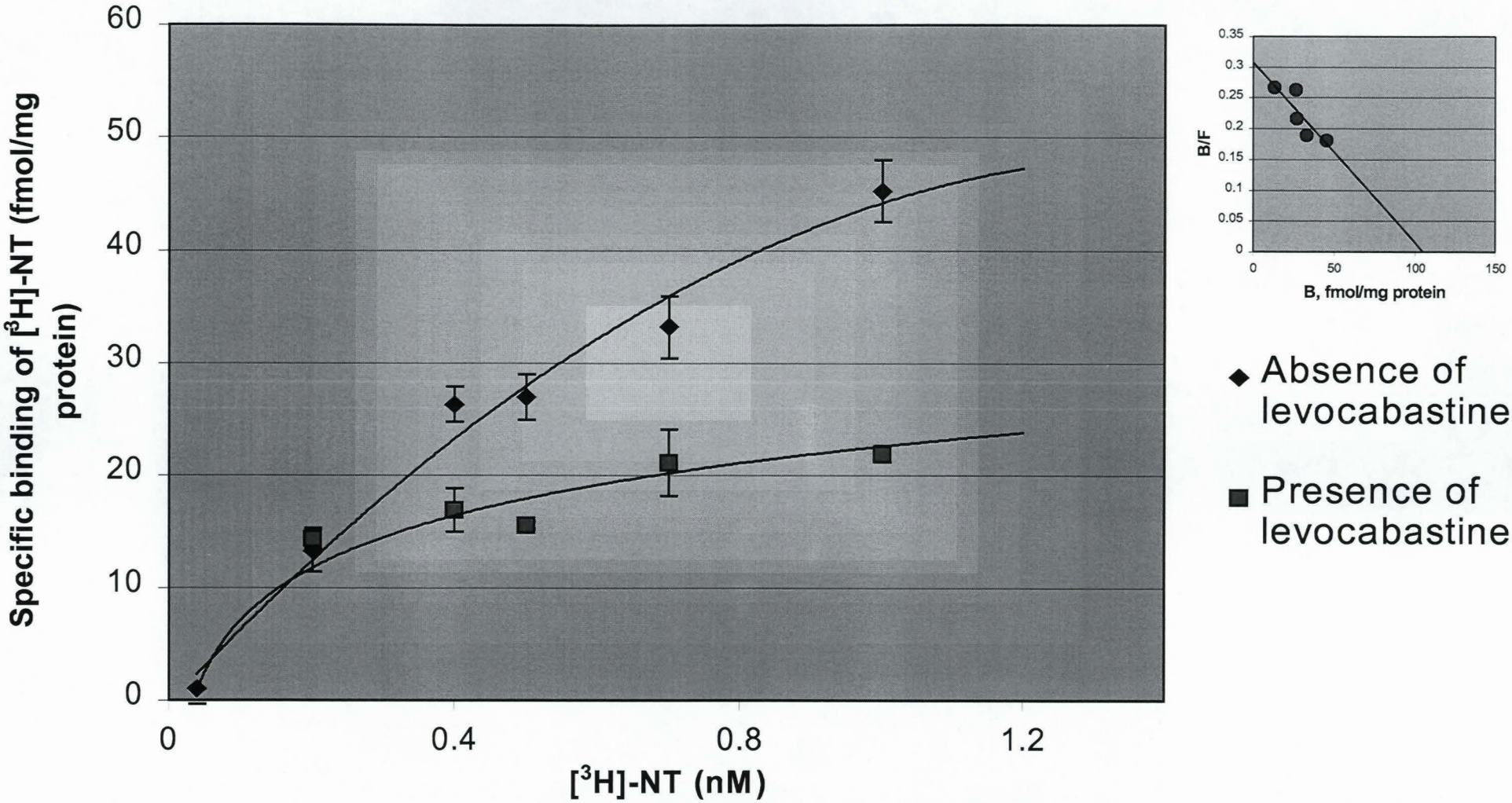
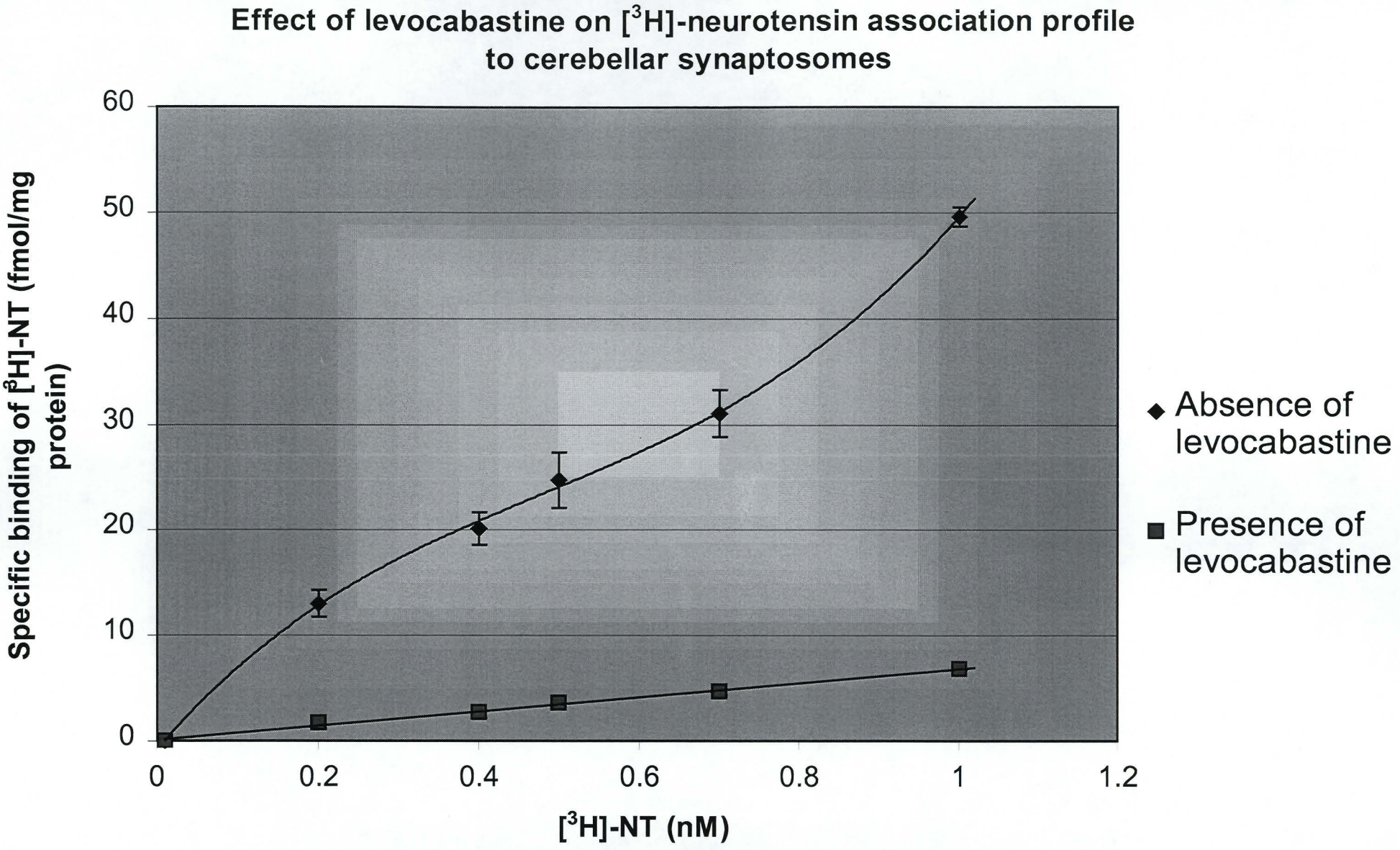


Figure 11*: Neostriatal synaptosomes. Saturation Binding of [^{3}H]-NT in the presence and absence of levocabastine.* Synaptosomal preparations were resuspended in Modified Earle's Buffer in the presence of the absence of levocabastine (1mM). Total binding was obtained by incubation in increasing concentration of [^{3}H]-NT for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.



Figue 12: ***Cerebellar synaptosmes. Saturation Binding of [^{3}H]-NT in the presence and absence of levocabastine.*** Synaptosomal preparations from the cerebellum were resuspended in Modified Earle's Buffer in the presence of the absence of levocabastine (1mM). Total binding was obtained by incubation in increasing concentration of [^{3}H]-NT for 15 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

concentration and was not saturated by up to 1 nM of [^{3}H]-NT (Figure 12). At this concentration, only 6,8 fmol/mg protein of [^{3}H]-NT was specifically bound. This value represents about 1/7 of the value obtained in the absence of levocabastine. By contrast, in neostriatal synaptosomes, the Bmax in the presence of levocabastine was of 21,9 fmol/mg protein as compared to 45,2 fmol/mg protein in the absence of levocabastine (Figure 11).

In the rat brain, levocabastine inhibits NT binding to NTS2 without affecting binding to NTS1. When levocabastine is absent, [^{3}H]-NT binding sites include both NTS1 and NTS2. The difference between specific [^{3}H]-NT binding in the absence of levocabastine and in its presence gives the value of radioligand binding that is contributed by the NTS2. At 1 nM of radioligand, this difference corresponded to a value of 23,84 and 42,8 fmol/mg protein for synaptosomes from the neostriatum and cerebellum, respectively.

3. KINETICS OF [^{3}H]-NT ASSOCIATION AND INTERNALIZATION AT PERMISSIVE TEMPERATURE (37^{o}C) IN NEOSTRIATAL SYNAPTOSOMES

To determine whether binding of [^{3}H]-NT to NTS1 sites resulted in receptor-mediated internalization, neostriatal synaptosomes were incubated for 2-45 minutes at 37^{o}C with a saturating concentration of [^{3}H]-NT (1nM) in the presence of levocabastine to block NTS2 sites (Figure 13). To assess the proportion of internalized [^{3}H]-NT, synaptosomal preparations were washed with hypertonic acid at the end of the incubation to dissociate surface-bound ligand (all of these experiments were performed in modified Earle's buffer).

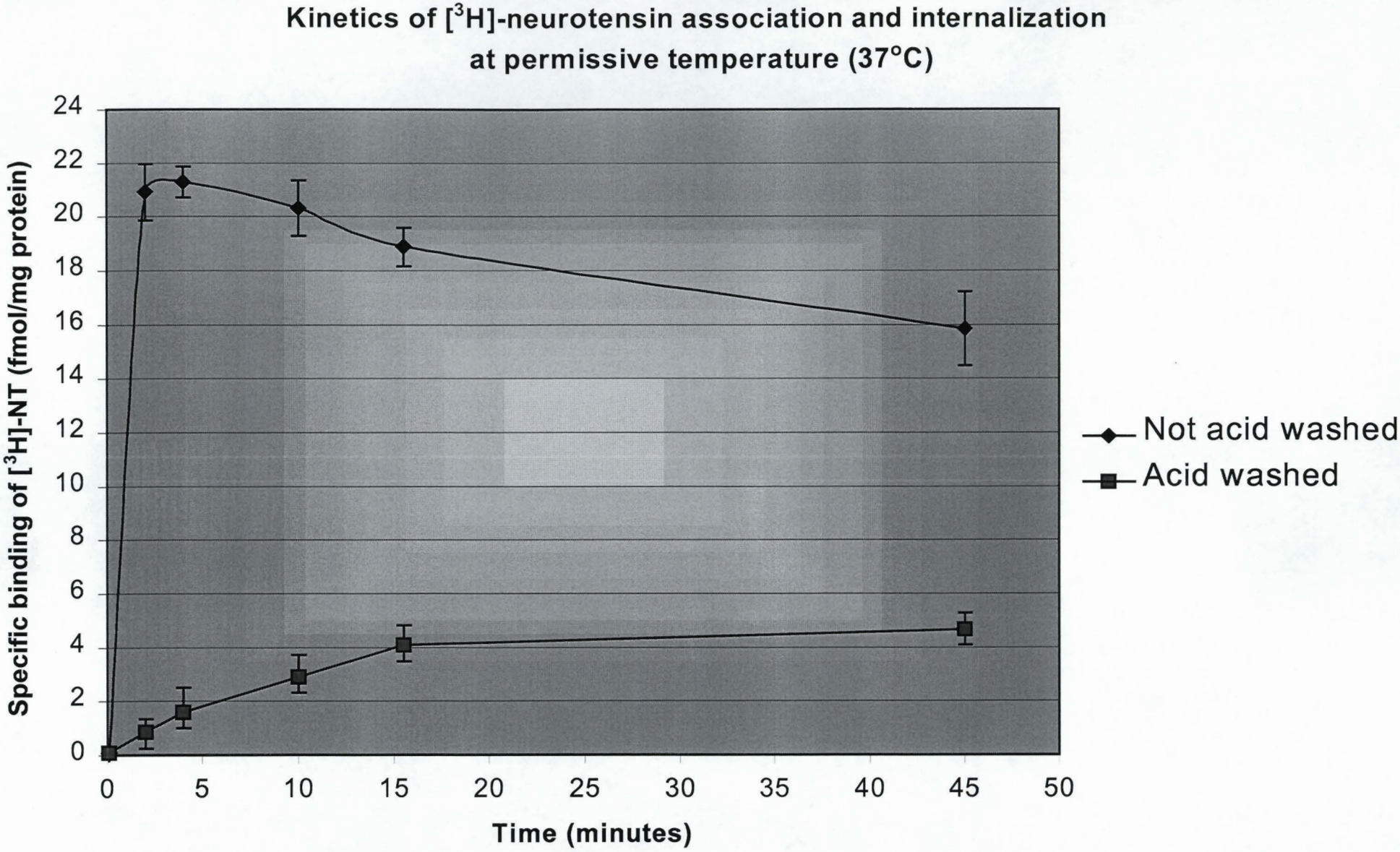


Figure 13: ***Kinetics of [^{3}H]-NT association and internalization at 37°C.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in [^{3}H]-NT (1nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 efficiently removes membrane-bound radioligands without extracting internalized ones.

As can be seen in Figure 13, total specific [^{3}H]-NT binding saturates within 2 minutes or less to a value of 20,9 fmol/mg protein whereas acid wash-resistant association (i.e. internalization) was slower and takes about 15 minutes to reach a plateau value of approximately 4,0 fmol/mg protein. This corresponds to an internalization efficiency (acid wash-resistant / total specific binding at equilibrium) of about 20,0%. As can be seen in Figure 13, [^{3}H]-NT internalization proceeded in 2 phases. A first phase (2-4 min) during which the internalization was rapid and a second phase (4-15 minutes) during which the internalization was slower. On average, the internalization was calculated at 0,27 fmol/mg per minute.

Only a few minutes after saturation, the [^{3}H]-NT binding curve slowly starts to drop and reaches 74 % of its original value after 45 minutes of incubation. This corresponded to a value of 15,8 fmol/mg protein of [^{3}H]-NT. By contrast, the internalization curve of [^{3}H]-NT remains relatively constant up to 45 minutes of incubation (Figure 13).

4 ENDOCYTOTIC MACHINERY INVOLVED IN THE INTERNALIZATION OF [^{3}H]-NT VIA NTS1 IN NEOSTRIATAL SYNAPTOSOMES

4.1 Effect of inhibitors of endocytosis on the kinetics of [^{3}H]-NT association and internalization

4.1.1 Low temperature

Incubation at low temperature (4°C) prevents NT internalization in many cell models including transfected COS cells (Herman et al., 1994) and cultured neurons (Vanisberg et al.,1991). To determine the effect of low temperature on [^{3}H]-NT internalization in neostriatal synaptosomes, time course binding

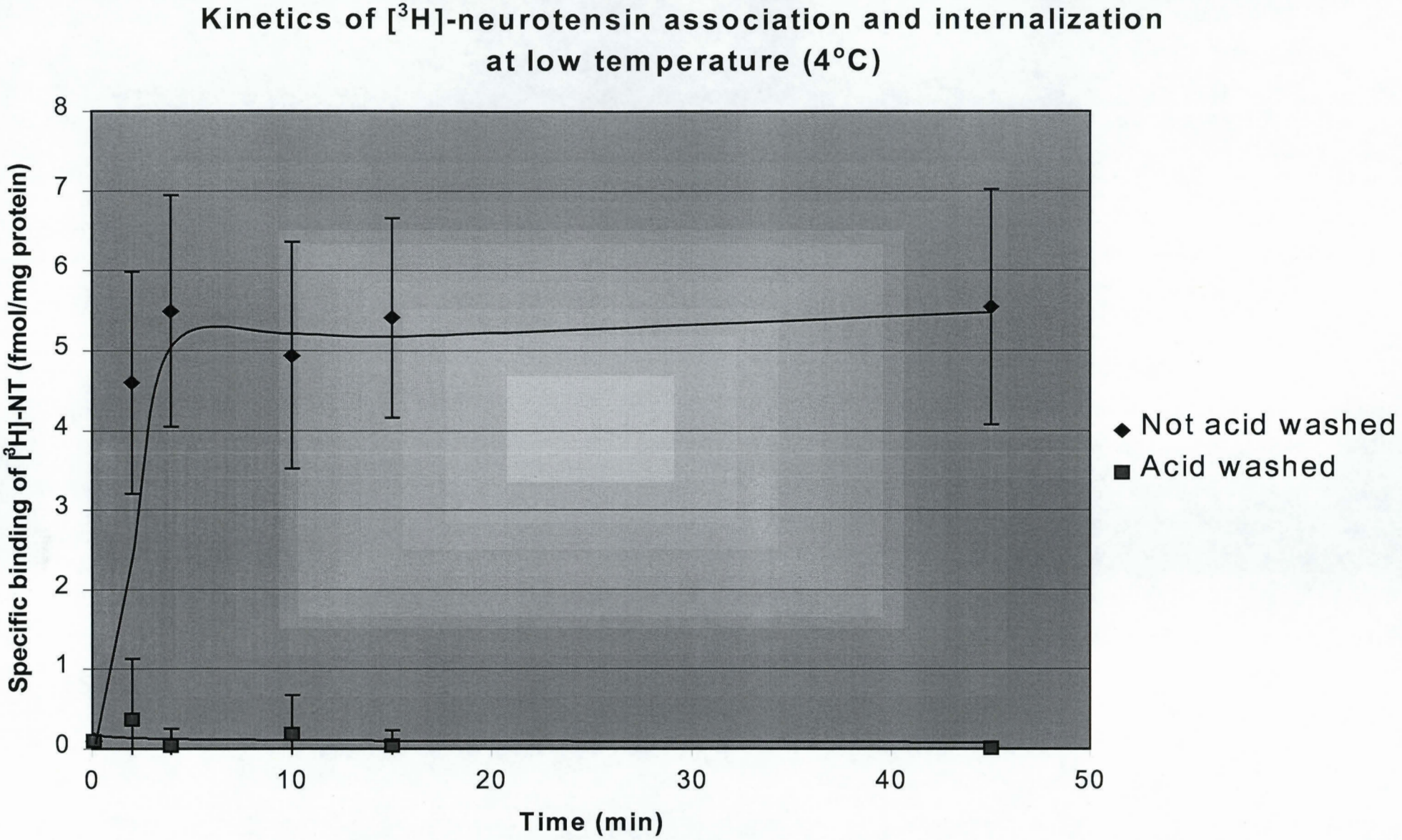


Figure 14: ***Kinetics of [^{3}H]-NT association and internalization at 4°C.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in [^{3}H]-NT (1nM) for increasing time intervals at 4°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 efficiently removes membrane-bound radioligands without extracting internalized ones.

experiments were performed at 4°C. Other parameters were kept the same as in previous time course experiments.

Lowering the temperature from 37 °C (Figure 13) to 4°C (Figure 14) reduced [^{3}H]-NT binding capacity and kinetics. At 4°C, [^{3}H]-NT binding saturates within about 4 minutes to a value of 5,5 fmol/mg protein (Figure 14). In contrast to the descending curve previously obtained for experiments at 37°C, this plateau was maintained for up to 45 minutes of incubation (Figure 14).

Following hypertonic acid wash, only insignificant amounts of specifically bound [^{3}H]-NT remained associated with the synaptosomal preparation, indicating that at 4°C, the total [^{3}H]-NT binding detected was on the plasma membrane rather than intracellular.

4.1.2 Sucrose and PAO

Time course binding experiments were repeated but at a permissive temperature (37°C) using synaptosomes pretreated with compounds known to inhibit receptor endocytosis such as sucrose and phenylarsine oxide (PAO) (Figure 15). All experiments were performed in modified Earle's buffer, in the presence of levocabastine.

Synaptosomes pretreated with PAO displayed a [^{3}H]-NT binding profile very similar to untreated ones (compare Figure 15 and Figure 13). [^{3}H]-NT binding saturated within 2 minutes or less to reach a value of 20,1 fmol/mg protein. Like untreated synaptosomes, synaptosomes pretreated with PAO display a progressive loss of [^{3}H]-NT binding over time to reach 74% of its initial value after 45 minutes of incubation.

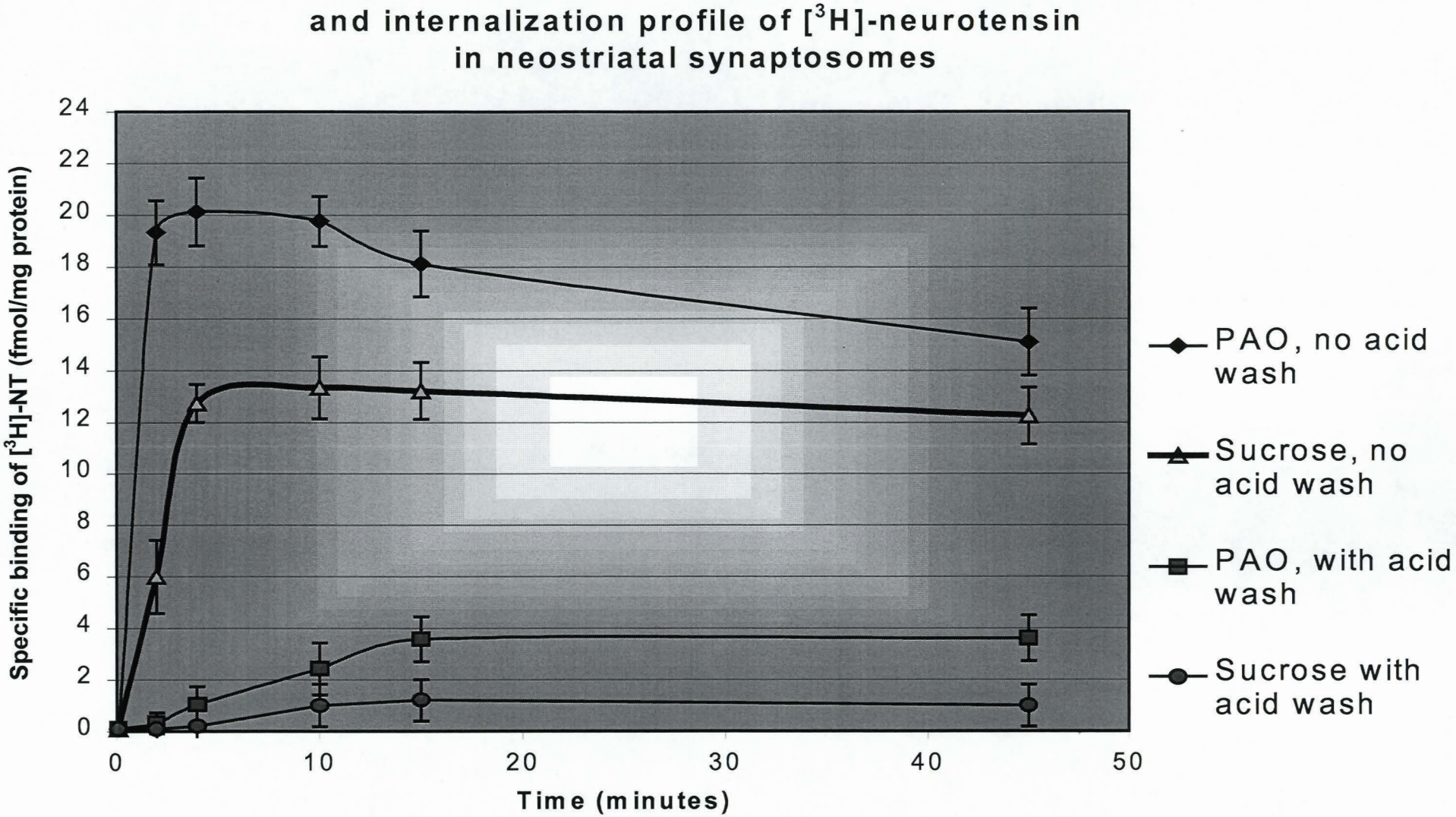


Figure 15: ***Kinetics of [^{3}H]-NT association and internalization in Sucrose and Phenylarsine oxide (PAO).*** Synaptosomal preparations were resuspended in Modified Earle's Buffer containing 0,32M sucrose (Sucrose Buffer) or 10mM PAO. Total binding was obtained by incubation in [^{3}H]-NT (1nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 efficiently removes membrane-bound radioligands without extracting internalized ones.

By contrast, synaptosomes pretreated with sucrose displayed a [^{3}H]-NT binding profile that differed in several aspects from untreated ones. [^{3}H]-NT binding rate was slightly reduced since it reached a plateau in 4 minutes rather than 2 minutes (Figure 15). The value of this plateau was of 13,0 fmol/mg protein, i.e. was only 65% of specific binding in untreated synaptosomes (Figure 15). Furthermore, this plateau value did not decrease over time and remained constant for up to 45 minutes of incubation.

PAO and sucrose pretreatment also had very different effects on the kinetics of [^{3}H]-NT internalization. Synaptosomes pretreated with PAO displayed an internalization profile virtually identical to that of untreated ones (Figure 15), indicating that this drug had no effect on [^{3}H]-NT internalization. Acid wash-resistant [^{3}H]-NT accumulation proceeded at a constant rate for 15 minutes until it reached a plateau of 3,61 fmol/mg protein (Figure 15). By contrast, the amount of [^{3}H]-NT in synaptosomes treated with sucrose, significantly reduced following acid wash compared to untreated synaptosomes (Figure 15), indicating that sucrose efficiently inhibited [^{3}H]-NT internalization.

4.2 Synaptosomal integrity following treatment with PAO and acid wash

Low temperature, sucrose and PAO have all been reported to block NT internalization in every model developed to date. However, in neostriatal synaptosomes, while low temperature and sucrose efficiently blocked [^{3}H]-NT internalization, PAO was without effect. To rule out the possibility that the lack of effect of PAO could have been due to artifactual intra-synaptosomal diffusion of the radioligand through a partially disrupted plasma membrane, the effects of

PAO and/or acid wash treatments on synaptosomal integrity were tested both biochemically and by EM.

4.2.1 Biochemical Evidence for preservation of synaptosomal integrity

Binding experiments in which PAO-treated synaptosomes were subjected to hypertonic acid wash of increasing duration is shown in Figure 16. When synaptosomal preparations were not acid washed (time = 0), the maximal binding capacity was of 20,6 fmol/mg protein. This fits with values of [^{3}H]-NT binding capacity from previous experiments. Two minutes of hypertonic acid wash was sufficient to remove the majority of specifically bound [^{3}H]-NT (Figure 16). Following 2 to 6 minutes of acid wash, the value of non-washable [^{3}H]-NT remained constant at 14.6% of the non-acid wash value (Figure 16). Additional biochemical experiments were performed to determine the extend of plasma membrane destruction using the leakage of cytoplasmic markers (lactate dehydrogenase) into the extracellular space (Clark and Nicklas, 1970). These experiments demonstrated that the plasma membrane of synaptosomes treated with PAO and acid wash was still intact (Results not shown).

4.2.2 EM evidence for preservation of synaptosomal integrity

The integrity of synaptosomes treated with PAO alone (Figure 17A, B) or with PAO followed by 2 minutes of hypertonic acid wash, was assessed by electron microscopy (Figure 17C, D). Untreated synaptosomes subjected to acid wash alone were also included for purposes of comparison (Figure 18A, B).

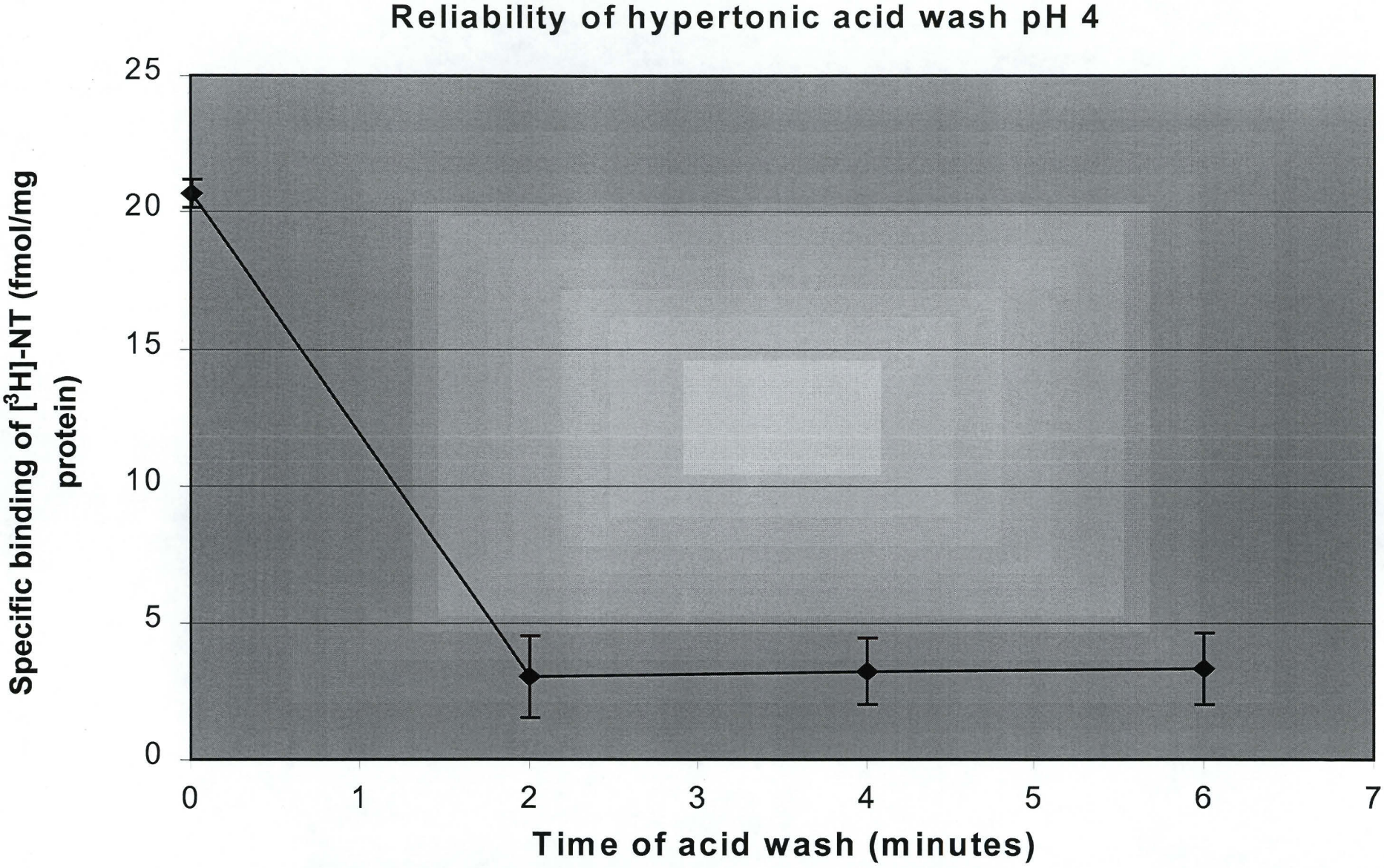


Figure 16: ***Time course for hypertonic acid wash pH 4.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Synaptosomal preparations where incubated in 1nM [^{3}H]-NT followed by an increasing time intervals of hypertonic acid wash pH 4. Non-specific binding was determined in the presence of an excess of unlabeled NT (1μM). Specific binding was obtained by subtracting non-specific from total binding.

Figure 17: ***Electron micrographs of synaptosomes isolated using Method I in the presence of PAO with acid wash (C, D) or without (A, B).*** Panel A and B show synaptosomes with a morphology comparable to that previously presented for Method I. Upon acid wash, a reduction in the intracytoplasmic space is evident (C and D). Scale bar = 1µm.

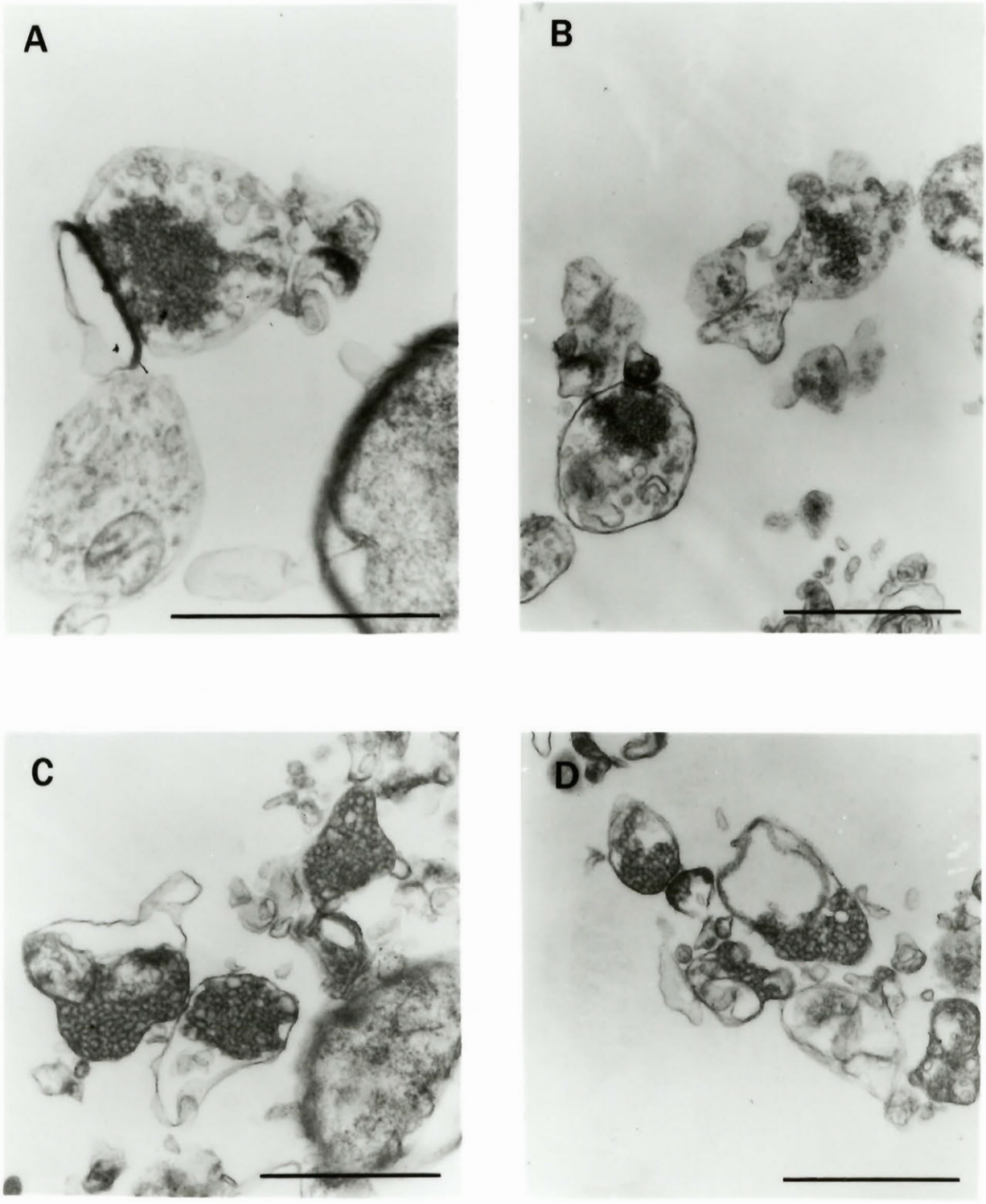
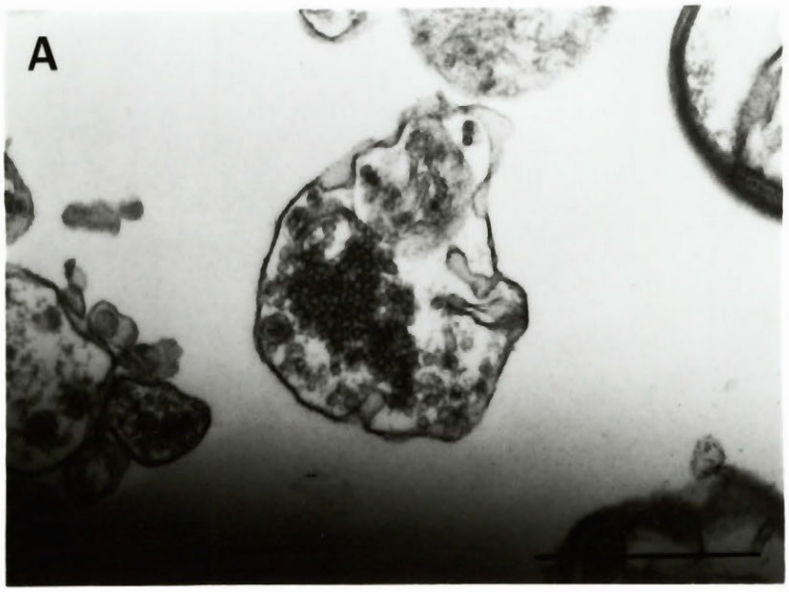
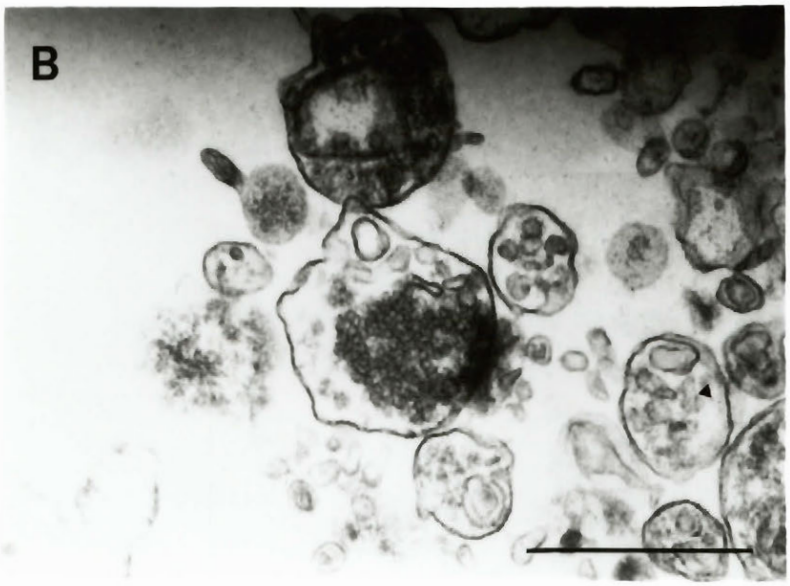

A
B
C
D

Figure 18: ***Electron micrographs of untreated synaptosomes isolated using Method I following acid wash (A, B).*** Acid wash does not significantly alter the morphology of untreated synaptosomes. Scale bar = 1µm.


A


B

As previously described, for untreated synaptosomes, PAO-treated synaptosomes displayed typical morphological features of nerve terminals and showed well-preserved plasma membranes (Figure 17A, B). They often showed synaptic specializations to which post-synaptic membranes were still attached. They also contained clusters of synaptic vesicles and a relatively large intracellular space. Clathrin-coated vesicles could also be seen inside some of them.

After hypertonic acid wash, PAO-treated synaptosomes showed a marked reduction of their intracellular space (Figures 17C, D). Interestingly, acid wash did not produce this feature in untreated synaptosomes (Figures 18A, B), which were indistinguishable from the ones that had not been subjected to acid washed (Figures 4A, B, C, D). Apart from these differences, acid-washed, PAO-treated synaptosomes displayed the features usually observed in untreated synaptosomes (Figure 4) and, in particular, showed good preservation of the plasma membrane (Figure 4). Clathrin-coated vesicles, however, were not observed.

4.3 Effects of PAO and sucrose on the kinetics of [^{125}I] diferric human transferrin association and internalization in neostriatal synaptosomes

PAO and acid wash treatments did not alter synaptosomal integrity, as evidenced by both biochemical and EM experiments. This indicates that the inability of PAO to block receptor internalization in neostriatal synaptosomes was a real effect, and not due to artifactual entry of the radioligand into disrupted synaptosomes. To determine whether this lack of effect of PAO was unique for NT or was also true for other receptor-mediated, clathrin dependent

internalization processes occurring at the level of axon terminals, we tested the effect of PAO on the internalization of [^{125}I]-diferric human transferrin ([^{125}I]-Tf) in the same synaptosomal preparations.

To determine the binding properties of [^{125}I]-Tf to neostriatal synaptosomes, a saturation experiment was performed at 37°C. As can be seen in Figure 19, specific [^{125}I]-Tf binding was saturable. Scatchard analysis of the data yielded a maximal binding capacity (B_{max}) and an apparent affinity (Kd) of 166,7 fmol/mg of protein and 5.1 nM, respectively. Non-specific binding was very low initially (about 25% of total binding at saturation) and increased linearly with radioligand concentration (Figure 19).

To determine the kinetics of [^{125}I]-Tf association and internalization in neostriatal synaptosomes, time course binding experiments at 37°C followed by hypertonic wash were performed (Figure 20). [^{125}I]- Tf binding to synaptosomal preparations from the neostriatum saturated later and with a greater capacity than [^{3}H]-NT (Figure 20). In fact, [^{125}I]- Tf saturated within about 15 minutes to a plateau value averaging 84 fmol/mg protein (Figure 20). Unlike [^{3}H]-NT binding, [^{125}I]- Tf binding does not decrease over time and remains stable for up to 45 minutes. [^{125}I]-Tf internalization closely parallels its binding profile, saturating at the same time, to about 46 fmol/mg of [^{125}I]-Tf. This represents an internalization efficiency of about 55% at saturation (Figure 20). Furthermore, the [^{3}H]-Tf internalization plateau was constant and did not decrease with time (Figure 20).

The next set of experiments was performed to determine the effect of sucrose (Figure 21) and PAO treatment (Figure 22) on [^{125}I]-Tf internalization in neostriatal synaptosomes.

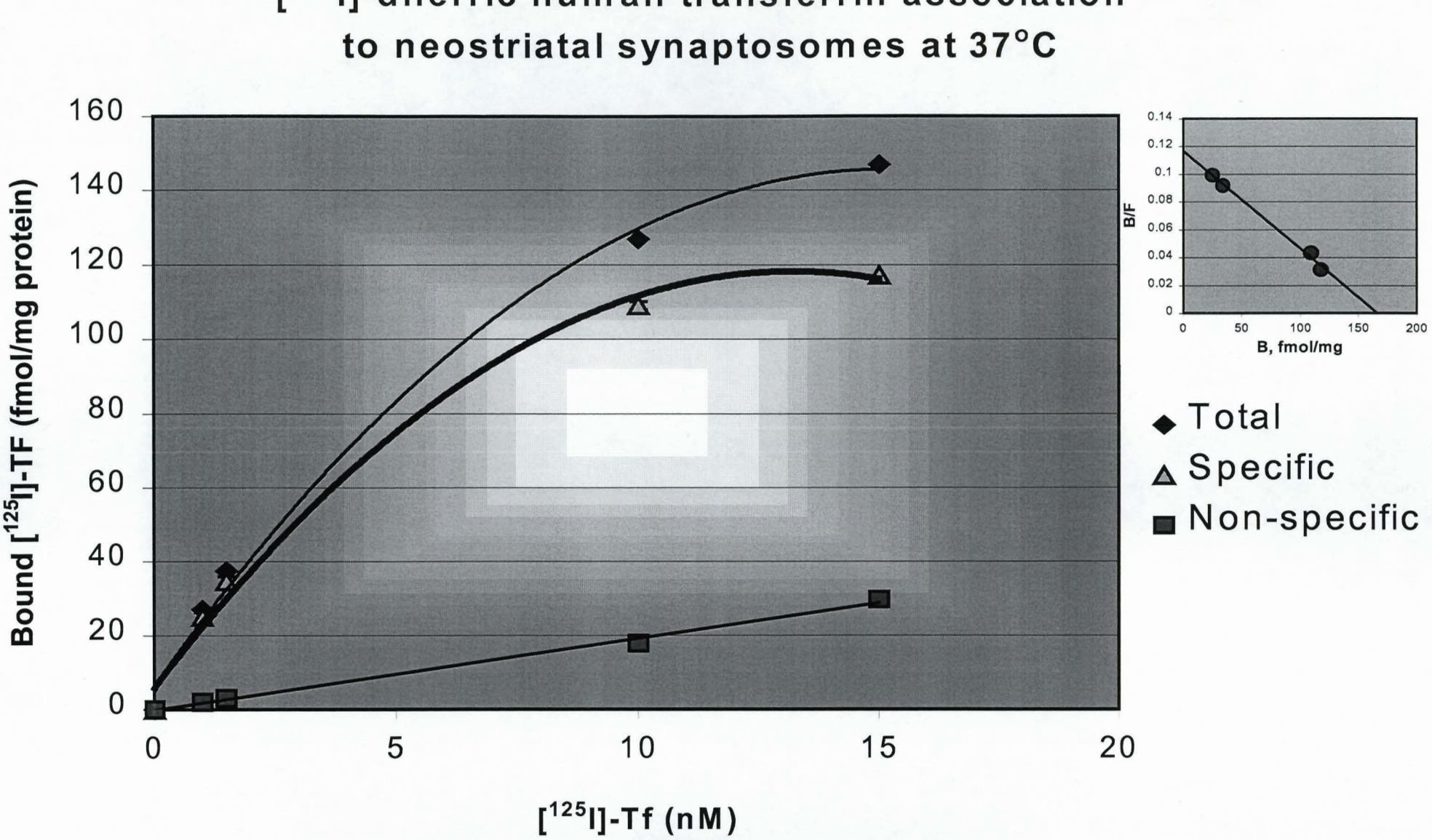


Figure 19: ***Saturation binding of [^{125}I]-Tf at 37°C.*** Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in increasing concentrations of [^{125}I]-Tf for 30 minutes at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled Tf (5μM). Specific binding was obtained by subtracting non-specific from total binding.

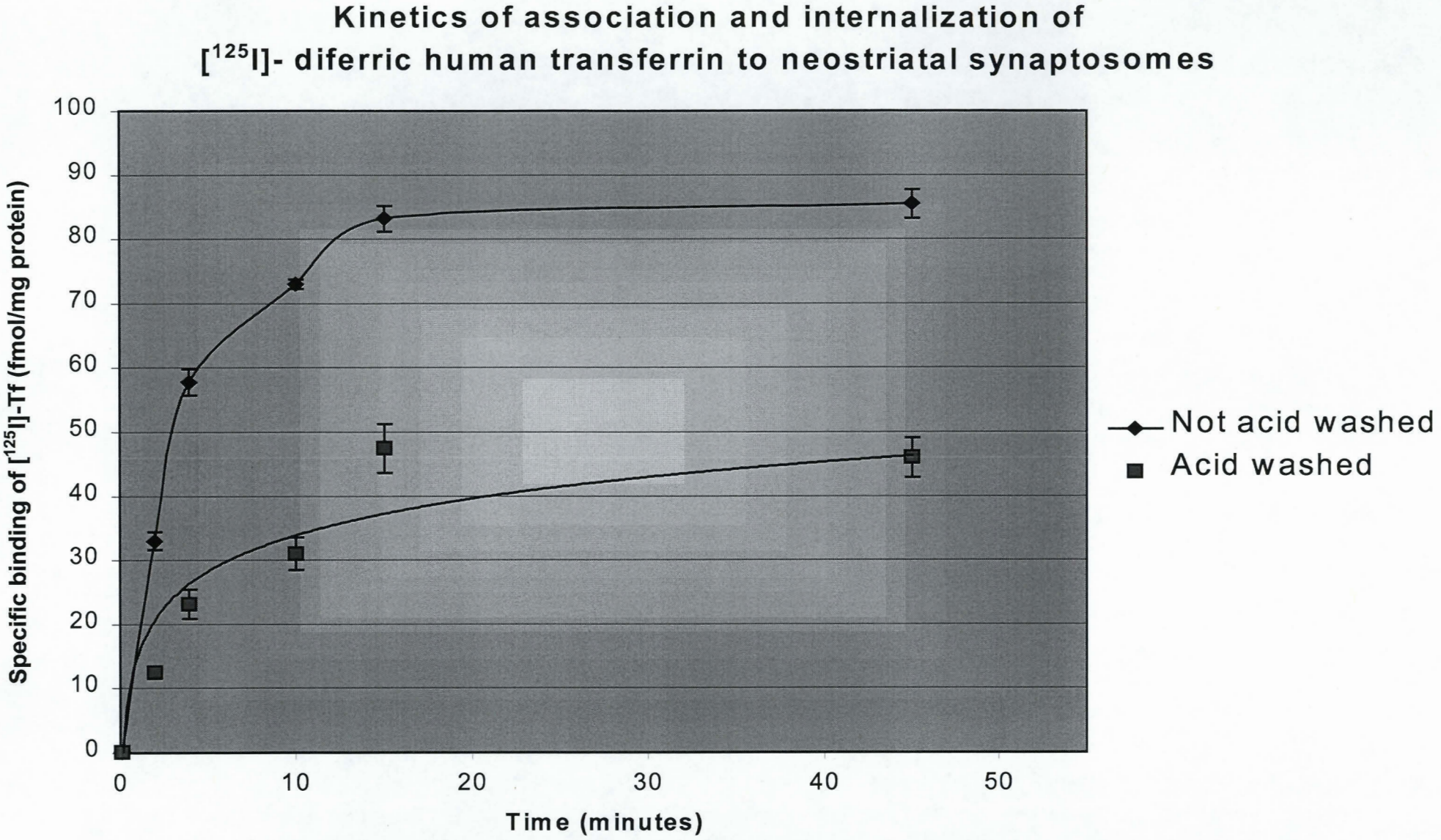


Figure 20: ***Kinetics of [^{125}I]-Tf association and internalization at 37°C.*** . Synaptosomal preparations were resuspended in Modified Earle's Buffer. Total binding was obtained by incubation in [^{125}I]-Tf (8nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled Tf (5μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 removes membrane-bound radioligands.

Sucrose-pretreated synaptosomes yielded a similar [^{125}I]- Tf binding curve as untreated ones. [^{125}I]- Tf binding saturated after approximately 15 minutes to a value of 70,0 fmol/mg protein (Figure 21). This value corresponds to 83% of specific [^{125}I]- Tf binding capacity in the absence of sucrose. Sucrose pretreatment greatly reduced the amount of internalized [^{125}I]-Tf, as assessed after hypertonic acid wash (Figure 21). There was, however, a residual amount of internalized radioactivity, corresponding to 28,6% of specific [^{125}I]- Tf binding at saturation that was resistant to sucrose treatment (Figure 21).

By contrast, in PAO-treated synaptosomes, the proportion of Tf radiolabeling that was acid wash-resistant was as high as 65% (60 fmol/mg protein) at saturation (Figure 22). Saturation was reached within less than 5 minutes and the plateau was maintained for up to 45 minutes.

Figure 23 compares the internalization efficiency of [^{3}H]-NT and [^{125}I]-Tf as well as the proportion of internalized ligand that is sensitive to blockage with sucrose. In neostriatal synaptosomes, [^{125}I]-Tf and [^{3}H]-NT internalization differs in both their efficiency and their sensitivity to sucrose. [^{125}I]-Tf is internalized 2,5 times more efficiently than [^{3}H]-NT (52,6 as compared to 22,2 fmol/mg protein). Interestingly, only about half of [^{125}I]-Tf internalization is inhibited by sucrose whereas virtually all of [^{3}H]-NT internalization is inhibited by sucrose.

4.4 Association and internalization profile of [^{3}H]-NT in the absence of calcium

In COS-7 cells, NTS1 was found to internalize independently of Ca^{++} (Vandenbulcke et al, 2000). In nerve terminals (Henkel and Betz, 1995; Palfrey and Artalejo, 1998; Neves et al., 1999) as well a in synaptosomal preparations

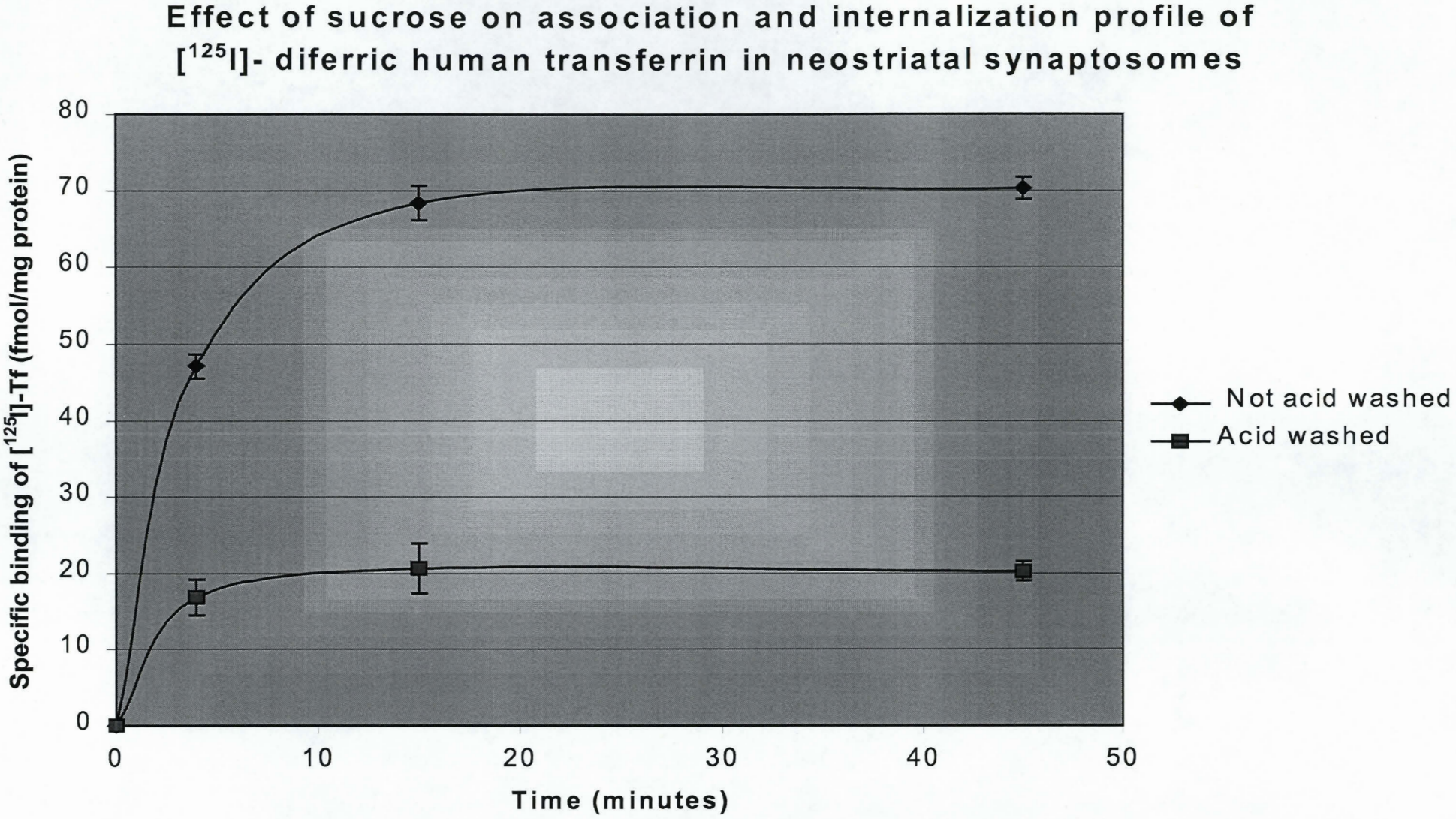


Figure 21: ***Kinetics of [^{125}I]-Tf binding and internalization in Sucrose buffer (0,32M).*** Synaptosomal preparations were resuspended in Modified Earle's Buffer containing 0,32M sucrose (Sucrose Buffer). Total binding was obtained by incubation in [^{125}I]-Tf (8nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled Tf (5μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH removes membrane-bound radioligands.

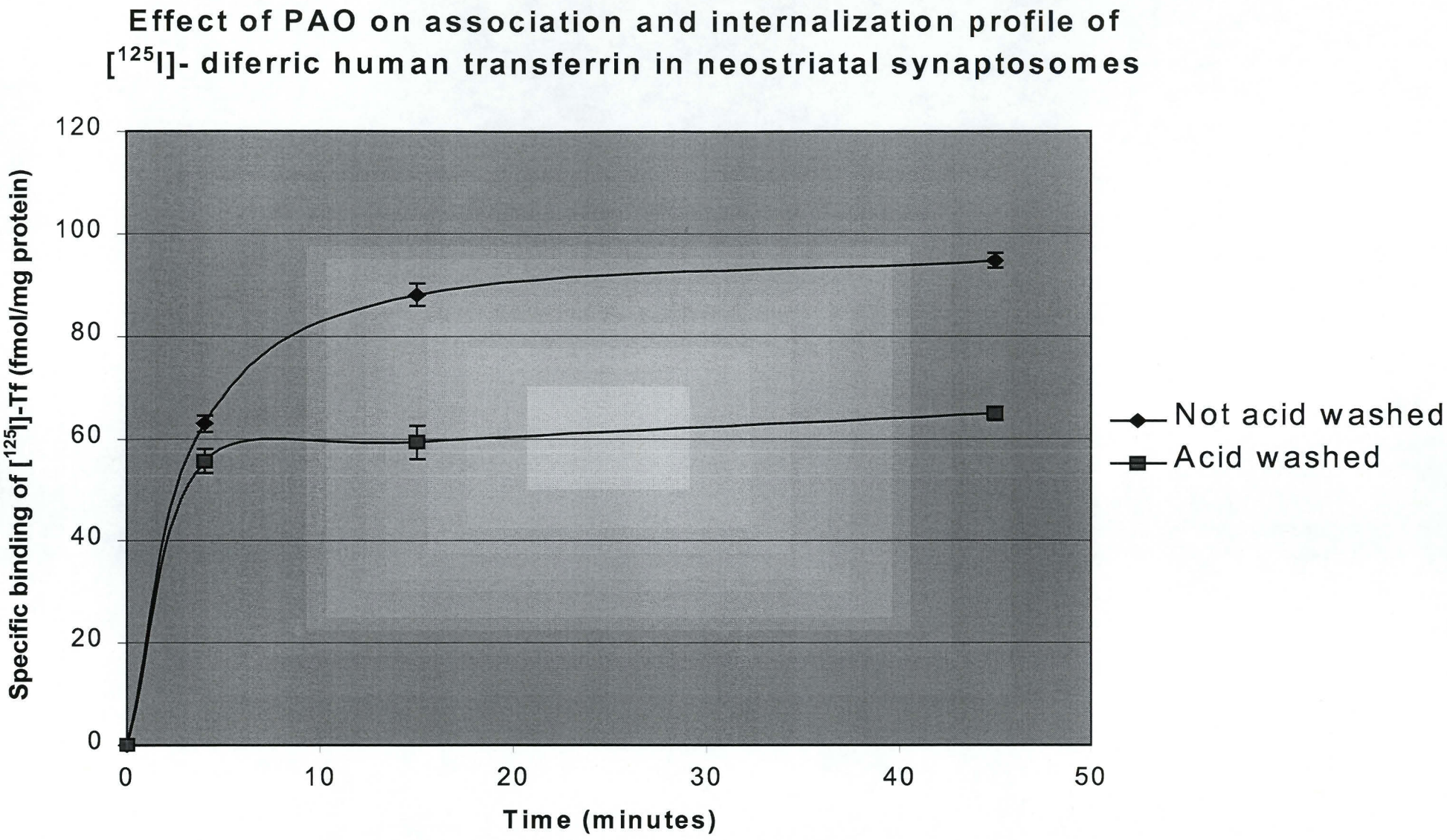


Figure 22: ***Kinetics of [^{125}I]-Tf binding and internalization in the presence of PAO (10μM).*** Synaptosomal preparations were resuspended in Modified Earle's Buffer containing 10μM PAO. Total binding was obtained by incubation in [^{125}I]-Tf (8nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled Tf (5μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 removes membrane-bound radioligands.

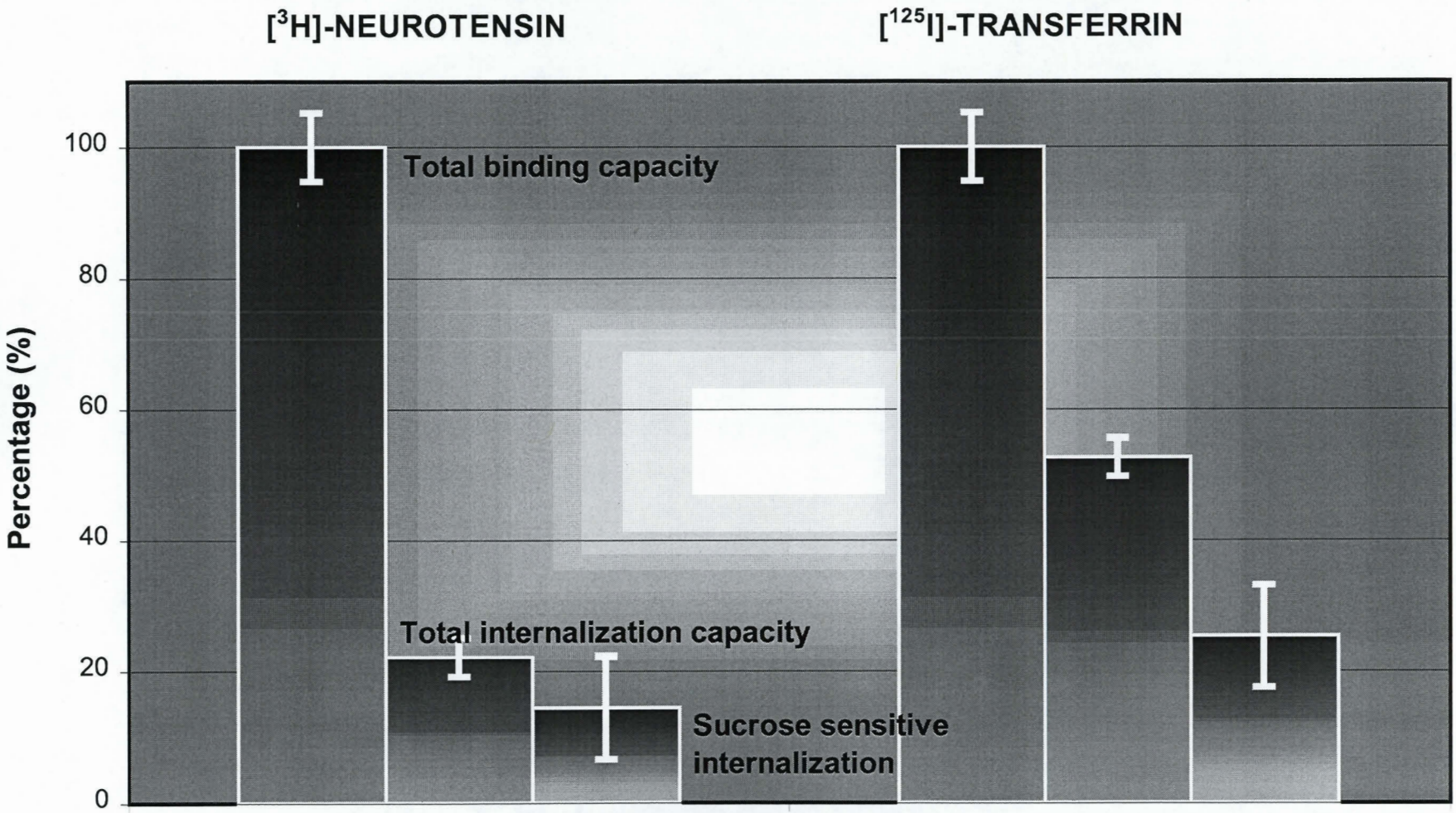


Figure 23: ***Comparison between the internalization properties of NT and Tf binding sites.*** Notice that about half of internalized [^{125}I]-Tf is not removed by acid wash following sucrose pretreatment whereas most if not all of internalized [^{3}H]-NT is sucrose-sensitive.

(Marks and McMahon, 1998), Ca^{++} ions are essential for endocytosis subserving plasma membrane retrieval in fast synaptic vesicle recycling.

To determine whether [^{3}H]-NT internalization in synaptosomes was likewise independent of the presence of Ca^{++}, time course experiments were performed in Ca^{++}-free medium containing EDTA, a Ca^{++} chelator. Acid wash was used to assess the amount of radioligand that had internalized (Figure 24).

The profile of [^{3}H]-NT binding in the absence of Ca^{++} resembles that which was observed in medium containing Ca^{++} (Figure 13). Saturation was reached within a few minutes and was rapidly followed by a gradual decrease of binding over time. (Figure 24). However, the plateau value averaged about 7,8 fmol/mg protein which represents only 29% of [^{3}H]-NT binding detected in the presence of Ca^{++} (Figure 13).

The absence of Ca^{++} did not prevent [^{3}H]-NT internalization. Interestingly, internalization in this condition showed both the similar capacity as in the presence of calcium (3,7 fmol/mg protein) but saturation was more rapid (within 4 minutes or less) than in the presence of Ca^{++}.

5 FATE OF NTR1 RECEPTORS FOLLOWING [^{3}H]-NT INTERNALIZATION IN NEOSTRIATAL SYNAPTOSOMES

5.1 Effect of monensin on the kinetics of [^{3}H]-NT association and internalization

To determine whether [^{3}H]-NT dissociates from its receptor following internalization, neostriatal synaptosomes were pretreated with the ionophore monensin. Monensin prevents the acidification of endocytotic vesicles and ligand-receptor dissociation, an event essential for the recycling of many

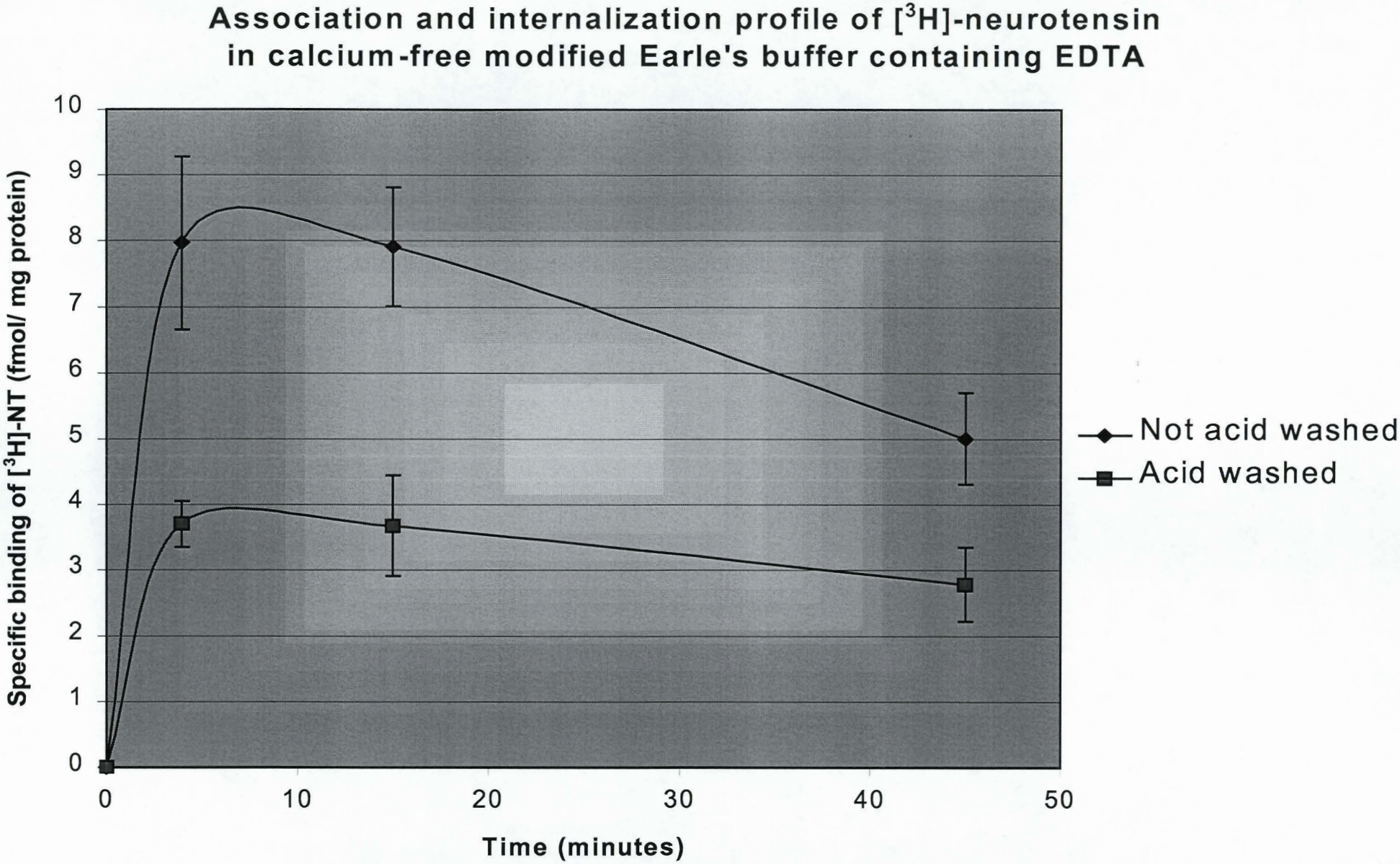


Figure 24: ***Kinetics of [^{3}H]-NT binding and internalization in the absence of calcium.*** Synaptosomal preparations were resuspended in calcium-free Modified Earle's Buffer containing 20mM EDTA. Total binding was obtained by incubation in [^{125}I]-Tf (8nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled Tf (5μM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 was used to removes membrane-bound radioligands.

receptors (Pressman et al., 1976; Tartakoff et al., 1983). Time course experiments followed by acid wash were performed on monensin-treated and untreated neostriatal synaptosomes (Figure 25).

Monensin-treated and untreated synaptosomes displayed similar [^{3}H]-NT binding curves but very distinct internalization profiles. Monensin treatment did not significantly alter [^{3}H]-NT binding properties. Like in untreated conditions, [^{3}H]-NT binding saturated within a few minutes to a value averaging 16 fmol/mg protein. This corresponds to 76% of [^{3}H]-NT binding in untreated conditions at saturation.

By contrast, monensin treatment greatly reduced the proportion of hypertonic acid-wash resistant [^{3}H]-NT. After 15 minutes, monensin treatment decreased the internalization capacity approximately 1/3 of the value obtained in monensin-free conditions, which corresponds to a value of 1,8 fmol/mg protein. Furthermore, in the presence of monensin, saturation of internalization was reached faster, taking only 5 to 10 minutes.

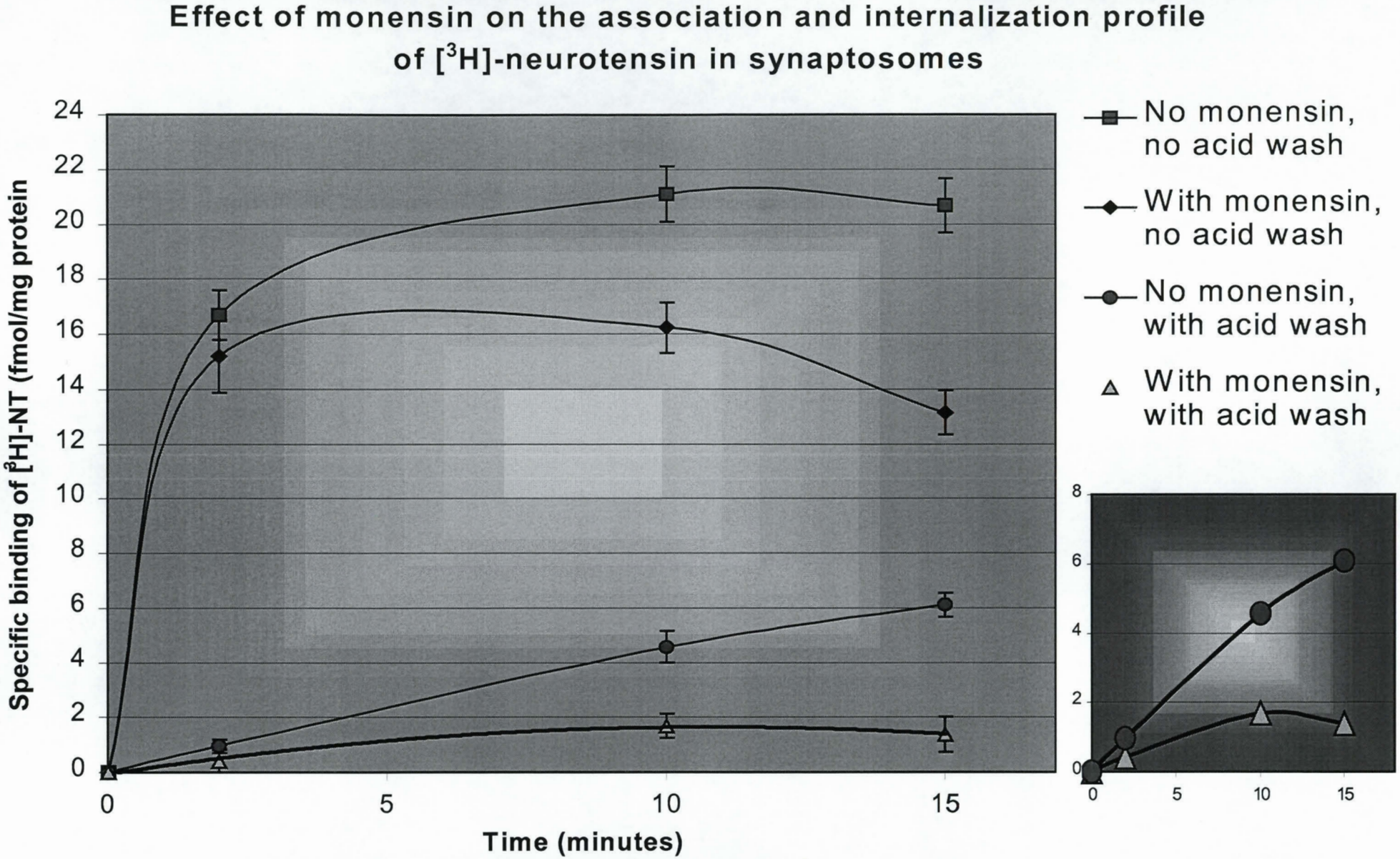


Figure 25: ***Kinetics of [^{3}H]-NT binding and internalization in the presence of monensin (25 µM).*** Synaptosomal preparations were resuspended in Modified Earle's Buffer containing 25 µM monensin. Total binding was obtained by incubation in [^{3}H]-NT (1nM) for increasing time intervals at 37°C. Non-specific binding was determined in the presence of an excess of unlabeled NT (1µM). Specific binding was obtained by subtracting non-specific from total binding. Hypertonic acid wash pH 4 effectively removes membrane-bound radioligands without extracting internalized ones.

DISCUSSION

1 OPTIMIZING SYNAPTOSOME PREPARATION FROM THE RAT NEOSTRIATUM

Three methods to isolate synaptosomes from the neostriatum have been tested: Method I, II and III (see Material and Methods). Method I gave a crude synaptosomal preparation and involved three centrifugation steps. Both Methods II and III gave a purified preparation of synaptosomes using an additional step that involves discontinuous gradients of sucrose and Percoll/sucrose, respectively.

Synaptosomes prepared from Method I were shown to contain clathrin-coated pits and vesicles whereas only the latter was observed in synaptosomes prepared by Method II. The presence of these structures in the preparation of nerve terminals strongly suggests a bi-directional intracellular trafficking of molecules between the membrane and the cytoplasm. This indicates that much like cell bodies, nerve terminals are endowed with clathrin-dependent internalization machinery.

Furthermore, synaptosomes from Method I and II contain three elements essential for receptor endocytosis and its biochemical detection: mitochondria that provide energy (ATP), a large intracellular space to accommodate endocytotic vesicles and an intact plasma membrane to prevent leakage. These elements are rarely observed (if at all) in synaptosomes isolated from Method III.

Method I was selected over Method II on the basis of its high protein concentration yield which was sufficient for biochemical studies as well as the fact that it was more efficient as it took much less time to prepare. Moreover, this

method perhaps assured a better representation of endogenous synaptic terminals.

2 PROPERTIES OF [^{3}H]-NT BINDING SITES IN NEOSTRIATAL SYNAPTOSOMES

It is well established that dopaminergic neurons in the substantia nigra send projections to the neostriatum and that these nerve terminals display high affinity NT receptors (NTS1) at the level of their plasma membrane. Using synaptosomal preparations from the neostriatum, this study constitutes the first attempt ever made to pharmacologically detect and characterize the internalization mechanism of pre-synaptic G-coupled receptors, specifically high affinity NT receptors (NTS1)-like. This study also looked at the pathways taken by both the receptor and its ligand at the level of nerve terminals after internalization.

This study confirms the presence of membrane-bound high affinity NT receptors (NTS1)-like in nerve terminals isolated from the rat neostriatum based on both its distinct pharmacological properties and its regional localization. It should be mentioned that synaptosomes prepared from the neostriatum contain not only terminals from dopaminergic neurons, but also most problaby a large number of GABAergic and glutamatergic terminals arising from other pathways that also innervate this region. In this study, NTS1-like binding sites are though to be associated with the dopaminergic elements on the light of evidences previously presented, demonstrating the exclusive association of high affinity binding sites with dopaminergic terminals in the neostriatum.

Unless mentioned, all binding experiments were performed in the presence of levocabastine, an inhibitor of low affinity NT binding sites (NTS2)-like. Saturation experiments showed that synaptosomal preparations whether resuspended in sucrose medium or modified Earle's buffer are endowed with NT binding sites that display binding properties corresponding to that previously reported for NTS1 in synaptic membrane preparations from whole rat brain (Mazella et al, 1983, Sadoul et al., 1984), cultured neurons (Dana et al., 1991; Brouard et al., 1992; Vanisberg et al., 1997), brain sections (Kitabgi et al., 1987) and purified synaptosomes (Awad et al., 1989).

Both the affinity (Kd) and capacity (B_{max}) of NT binding sites found in neostriatal synaptosomal preparations closely approximate the ones reported in membrane preparations (0,20-0,42 nM and 25,3-22,8 fmol/mg protein in this study as compared to 0,1-0,3 nM and 12-26 fmol/mg protein in membrane preparations) (Mazella et al., 1983). In cultured neurons, brain sections and synaptosomes, NT binding affinity was similar (0,29, 0,19 and 0,084 nM, respectively) although the capacities varied greatly.

In rat embryonic neurons, the NT binding capacity was about 5 and 20 times higher, reaching up to 123 and 474 fmol/mg protein for 4 and 10 day-old cultures, respectively (Vanisberg et al., 1997). This was expected as culture conditions yield much more NTS1 protein than whole brain homogenates, which contain high level of glial contaminants. Furthermore, it is well documented that NTS1 expression increases with the age of neuronal cultures (Dana et al., 1991; Vanisberg et al., 1997). By contrast, NT binding capacity is reduced in rat brain sections (Kitabgi et al., 1987) and in preparations of neostriatal synaptosomes

purified by Percoll/sucrose gradients (Awad et al., 1989). Values were reported to be as low as 7,7 and 1,2 units, respectively, and can easily be explained. In brain sections, membranes bearing NT receptors were embedded in their natural cellular environment, which precludes extensive interaction with the ligand. In the present study, the low capacity observed in purified synaptosomal preparations may be explained by the very delicate technique used to purify them. Percoll/sucrose gradient centrifugation is a method that has previously been demonstrated to be very destructive (see results, Method III). However, it is the only method to date that has characterized the properties of NT binding in synaptosomal preparations from the neostriatum and cerebral cortex (Awad et al., 1989).

[^{3}H]-NT binding in neostriatal synaptosomes was efficiently displaced by low concentrations of SR48692 corresponding to an IC_{50} of 3,76 nM. Similarly, in rat brain homogenates, SR48692 specifically recognized NTS1 with an IC_{50} of 5 nM, which corresponds to an affinity of about 15 to 600 times higher than NTS2 and NTS3, respectively (Gully et al., 1993). Interestingly, the fact that SR48692 was unable to displace a remaining 15% of [^{3}H]-NT labeling suggests that this proportion corresponds to internalized radioligands.

Further biochemical evidence that suggests NTS1 is the major NT binding site in neostriatal synaptosomes comes from a set of experiments in which sodium (regular Earle's buffer) and low temperature (4°C) were found to inhibit NT binding sites. In this study, introducing 140 nM of sodium to the incubation medium reduces [^{3}H]-NT binding to about 40% of its value obtained in absence of the ion. NT binding to NTS1 is sensitive to sodium whereas binding to NTS2 is

not affected by sodium ions (Martin et al., 1999). Moreover, previous studies on membrane preparations from whole rat brain reported a similar reduction upon exposure to sodium (Uhl et al., 1976; Goedert et al., 1984d). In the present study, both the capacity and affinity of NT binding sites were found to decrease in low temperature conditions (4°C), something that is well documented for NT receptors in rat brain membrane preparations (Kitabgi et al., 1987) and cultured neurons (Vanisberg et al., 1991, Chabry et al., 1993). Much like synaptosomes, these two models display a near linear NT binding profile at 4°C.

Saturation experiments were performed on neostriatal and cerebellar synaptosomal preparations in the absence or presence of 1 mM levocabastine, a specific blocker of low affinity NT binding sites (NTS2). In the presence of levocabastine, specific [^{3}H]-NT binding is attributed to NTS1 whereas in its absence, both NTS1 and NTS2 were detected. In the absence of levocabastine, a decrease in NT binding affinity and an increase in capacity was observed in synaptosomal preparations from both brain regions. For the neostriatum, these values were 1,3 nM and 106 fmol/mg protein, respectively. This is in keeping with previous studies performed on synaptic membrane preparations from whole brain reporting values of 2 - 4,7 nM and 126-135 fmol/mg protein, respectively (Kitabgi et al., 1977, Sadoul et al., 1984, Mazella et al., 1984) and with the concept that NTS2 is a low affinity and high capacity NT binding site. For reasons already discussed, purified synaptosomal preparations and membrane preparations from cultured neurons gave similar affinity (1,5 and 1,05 nM respectively) but different capacity (7,7 and 474 fmol/mg protein, respectively) (Awad et al., 1989; Hermans et al., 1997).

Although the total number of NT receptors was similar in the neostriatal and cerebellar synaptosomal preparations, NTS1 was about 3 times more concentrated in the neostriatum than in the cerebellum, while NTS2 is the predominant NT receptor subtype and accounts for 6/7 of [^{3}H]-NT binding in the cerebellum. These proportions were reported in experiments using purified neostriatal and cortical synaptosomal preparations (Awad et al., 1989) and are in keeping with other radioligand binding, autoradiographic, immunohistochemical and in situ hybridization studies. All of these studies detected an enrichment of NTS1 in the neostriatum and of NTS2 in both cerebral and cerebellar cortices, two regions reported to express low level of NTS1 (Schotte et al., 1986; Kitabgi et al., 1987; Boudin et al., 1996, Mazella et al.,1996).

The present results therefore clearly demonstrated the presence of NTS2 in synaptosomal preparation. It is unclear, however,if these sites are associated with axon terminals and/or with glial sacs known to be present in synaptosomal preparations. Previous in situ hybridization studies have demonstrated high concentrations of NTS2 mRNA within granular cells of the cerebellar cortex. As these neurons project densely to the molecular layer and as virtually no NTS2 mRNA was found in association with glial cells in normal adult brain, it is likely that the NTS2 receptors detected in cerebellar preparations were mainly presynaptic. Because glial expression of NTS2 was also found to be only exceedingly sparse in unlesioned rat (Nouel et al., 1999) and mouse (Sarret et al., 1998) neostriatum, it is also likely that most of NTS2 binding detected in the neostriatum can be accounted for by terminal labeling. It is unlikely, however, that the terminals bearing NTS2 binding sites are the same than the ones that

express NTS1. Indeed, in preparations from this brain region, there was no loss of NTS2 binding activity following neurotoxic destruction of nigrostriatal projections by 6-hydroxydopamine (Schotte et al.1988).

3 KINETICS OF [^{3}H]-NT ASSOCIATION AND INTERNALIZATION AT PERMISSIVE TEMPERATURE (37^{o}C) IN NEOSTRIATAL SYNAPTOSOMES

The kinetics of [^{3}H]-NT association in neostriatal synaptosomal preparations resembles to those previously documented from brain membrane preparations, but its kinetics of internalization differs markedly from those seen in neurons in culture.

In neostriatal synaptosomal preparations at 37°C, [^{3}H]-NT association with NTS1 was very rapid, reaching saturation within 2 minutes. This is consistent with previous binding studies performed on purified synaptosomal preparations from the neostriatum which reported a saturation at 4 minutes following incubation with [^{3}H]-NT at 25°C (Awad et al., 1989). This result is also in keeping with those of studies on rat brain synaptic membranes and rat brain homogenates (Uhl et al., 1976; Mazella et al., 1983; Goedert et al., 1983; Schotte et al., 1989). However, in intact cultured neurons (SN17 cells, cultured embryonic mouse brain and rat mesencephalic cells), the kinetics of NT binding was consistently slower than observed here is synaptosomes and saturated at only 45 to 60 minutes (Dana et al., 1991; Mazella et al., 1991; Vanisberg et al., 1991; Faure et al., 1995).

In neostriatal synaptosomal preparations, saturation was followed by a gradual reduction of specific binding over time down to about 76% of the initial value. The decrease was faster during the first 15 minutes and remained approximately constant during the rest of the incubation. Vanisberg and colleagues (1991) similarly reported a gradual reduction in [^{3}H]-NT over time in rat primary culture, the profile of which closely resembling what is reported in the current study. This loss of membrane-bound radioligand over time was interpreted as being due to its intracellular sequestration. However, this interpretation is unlikely to account for our observations in synaptosomal preparations, since neither the amount of internalized radioligand nor the rate of ligand internalization (see below) fitted the loss of specific binding observed. The observed decreased, therefore, more likely reflects a degradation of surface-bound ligand, congruent with the presence of NT-degrading activity in synaptosomal preparations (McDermott et al., 1983). HPLC analysis of enzymatic products over time revealed that within synaptosomes, degradative activities were both intracytoplasmic and membrane-bound, and was maximal following 10-15 minutes of exposure of the synaptosomes to NT (McDermott et al., 1983). The major cleavage was detected to occur in the C-terminal region which is critical for the biological activity of this peptide (Kitabgi et al., 1980; St-Pierre et al., 1981; Kitabgi et al., 1984). The major inactivating sites of NT were shown to be cleaved by endopeptidase 24 - 11 and 24 – 15 (Checler et al., 1983, 1985), two enzymes abundantly concentrated in the neostriatum (Checler et al., 1983, 1985). It was also found that only 81% of NT's degradative activity was inhibited by 1,10-phenanthroline at a concentration of 1 mM, a concentration

used to block [^{3}H]-NT degradation in the present study. When this concentration was reduced by 10 times (0,1 mM), a dramatic loss of [^{3}H]-NT binding over a short time was observed in synaptosomal preparations from the neostriatum (results not shown), confirming the importance of including peptidase inhibitor to study [^{3}H]-NT binding and internalization in synaptosomal preparations.

Previous studies have demonstrated that internalized radiolabeled-NT can be detected by the use of hypertonic acid wash, pH 4, to remove membrane-bound ligand (Botto et al., 1998; Nouel et al., 1997). Hypertonic acid wash, pH 4, has been used in the present study and internalization of [^{3}H]-NT was detected in neostriatal synaptosomal preparations. However, the kinetics of this internalization differed in many points from those reported in previous studies in SN17 cells (Faure et al., 1995), cultured mouse neurons and rat brain (Mazella et al., 1991; Chabry et al, 1993). In these earlier studies, NT internalization was found to be highly efficient (60-70%) and to closely parallel its association, saturating at about the same time (45-60 minutes). In synaptosomal preparations from the neostriatum, [^{3}H]-NT internalized with an apparent efficiency of only 20% and only saturated within 15 minutes. This proportion might be higher as synaptosomal preparations also contain disrupted synaptosomes that offer [^{3}H]-NT binding sites but does not internalize the ligand. After saturation, there was no decrease in the amount of radioactivity detected after hypertonic acid wash, indicating that it was not destructive to synaptosomes. Furthermore, the profile of [^{3}H]-NT internalization was divided in two consecutive phases, which form an almost linear slope up to saturation. This suggests that the internalization rate was relatively constant. The first phase of [^{3}H]-NT internalization was

characterized by a high internalization rate and was short-lasting whereas the second is longer and proceeds at a slower rate. This finding is consistent with the concept of receptor-mediated internalization of [^{3}H]-NT. Briefly, as bound NTS1 internalizes from the plasma membrane the concentration of NT receptors at the membrane drops, thus reducing its internalization rate.

In brief, the kinetics of NT internalization in synaptosomal preparations differs greatly from that reported of cultured neurons. This observation indicates that NT internalization in nerve terminals may proceed through a different mechanism than in the perikaryal domain. In fact, in contrast to NT internalization in cultured neurons, which closely parallel its association, NT internalization in nerve terminals seems to proceed independently of its binding kinetics or of the number of occupied receptors.

4 ENDOCYTOTIC MECHANISMS OF [^{3}H]-NT INTERNALIZATION IN NEOSTRIATAL SYNAPTOSOMES

Many lines of evidence suggest that in non-neuronal cells, as well as in the somatodendritic domain of neuronal cells, NTS1 internalizes through a clathrin-dependent pathway. To determine whether the same mechanism underlies internalization of NTS1 in nerve terminals, synaptosomal preparations were subjected to different treatments known to interfere with clathrin-mediated internalization. These included incubation at low temperature (4°C) or in the presence of sucrose or phenylarsine oxide (PAO). Surprisingly, only low temperature and sucrose were found to completely block [^{3}H]-NT internalization, whereas PAO had no effect at all.

Consistent with the present findings, low temperature was reported to prevent NTS1 internalization in neuronal cells in culture (SN17 cells, cultured embryonic mouse brain and rat mesencephalon) (Mazella et al., 1991; Vanisberg et al., 1991; Faure et al., 1995). As seen here, and as reported before (Goedert et al., 1983), this loss of NT internalization was accompanied by a reduction in NT binding capacity in rat brain membranes. As observed here in synaptosomes, other studies had found a similar inhibitory effect of sucrose on NTS1 internalization in neuronal (Botto et al., 1998) and non-neuronal cells (Vandenbulcke et al., 2000). In synaptosomal preparations subjected to low temperature or incubated in the presence of sucrose, total specific [^{3}H]-NT binding was not found to decrease following saturation, indicating that these treatments somehow prevented [^{3}H]-NT degradation.

In contrast, PAO pretreatment did not prevent [^{3}H]-NT internalization nor did it protect [^{3}H]-NT from degradation in synaptosomal preparations. In fact, whether treated with PAO or not, synaptosomal preparations displayed almost indistinguishable [^{3}H]-NT binding and internalization profile. The lack of internalization blocking effects of PAO on NT internalization could not be attributed to artifactual disruption of synaptosomal integrity as EM microscopy showed good morphological preservation of synaptosomes after PAO treatment, both before and after hypertonic acid wash. In fact, even prolonged acid washing (up to 6 minutes) was unable to remove a small proportion of internalized radioligand.

The insensitivity of [^{3}H]-NT internalization to PAO treatment in isolated nerve terminals contrasts sharply with what has been reported in transfected

non-neuronal cells (Chabry et al., 1995; Hermans et al., 1994) as well as in neuronal perikarya (Mazella et al., 1991, Chabry et al., 1993) where PAO treatment was found to effectively inhibit NT internalization.

To determine whether the lack of effect of PAO was specific to [^{3}H]-NT or reflected the existence of a PAO-independent pathway for receptor internalization in nerve terminals, the effect of PAO was tested on the internalization of transferrin receptors (TfR), which has been extensively documented to be clathrin-dependent in both non-neuronal (CHO and HeLa) (Sturrock et al., 1990; Martys et al., 1995; Warren et al., 1997) and neuronal cells (Prekeris et al., 1999). Sucrose treatment was included as a control. Whereas TfR is predominantly localized in the somatodendritic domain in neuronal cultures (Cameron et al., 1992; Parton et al 1992), evidence also points to its localization in nerve terminals, in vivo. In the neostriatum, dopaminergic terminals were shown to be endowed with TfR as a decrease in TfR binding density was observed in neostriatum from Parkinsonian patients and from MPTP-treated mice (Mash et al. 1991). MPTP produces active metabolites that kill nigral neurons by radical-induced lipid peroxidation (Dexter et al., 1989).

In synaptosomal preparations, [^{125}I]-Tf binding was specific, with an affinity of 5,1 nM and a capacity of 166,7 fmol/mg protein. These values correspond to values reported for Tf binding to that receptor in rat and human brain (Mash et al., 1991; Roskams et al., 1992). Fifty-five percent of total associated membrane-bound TfR internalized at saturation and sucrose treatment blocked over 50% of that internalization. It should be noted that the efficiency of TfR internalization may not be uniquely attributed to nerve terminals as glial cells (oligodendrocytes)

were also found to express TfR (Espinosa and de Vellis, 1988a,b; Fishman et al.,1990). However, it is probable that the major proportion of [^{125}I]-Tf detected in synaptosomal preparations is associated with nerve terminals rather than glial processes given that MPTP destruction of DA nigrostriatal neurons in mice, results in a 70% decrease in [^{125}I]-Tf binding in the neostriatum. Our results therefore suggest that like NTS1, TfR internalize in synaptosomal preparations and that this internalization is sensitive to sucrose. The fact that sucrose only partly blocked TfR internalization suggests that either there is incomplete loss of cell surface binding following acid wash (Sturrock et al., 1990) or that there exists a sucrose-independent pathway in synaptosomal preparations.

However, like for NTS1 internalization, treatment with PAO had no effect on TfR internalization. Here again, PAO had nonetheless been reported to be efficient in inhibiting TfR internalization in non-neuronal cells (Sturrock et al., 1990) and in neuronal cells at the level of their cell bodies (Takeuchi et al., 1992). Taken together, the present findings suggest that the endocytotic machinery present in cell bodies and in nerve terminals is not exactly the same. Nerve terminals possess a mechanism for receptor internalization that appears to be clathrin-dependent, on the basis of its sensitivity to sucrose, but is distinct from classical clathrin-dependent pathways in its insensitivity to PAO. The pharmacological properties of this pathway may be better understood by comparing the mechanism by which sucrose and PAO interfere with receptor endocytosis.

Even though it is not fully understood, evidence indicates that sucrose has a broader spectrum of action than PAO. Sucrose treatment has been reported by

an EM study to result in the disappearance of clathrin coated-pits and the appearance of atypically small coated-pits ("microcages") which fail to internalize (Heuser and Anderson, 1989). For PAO, the block of receptor internalization is achieved more specifically through interaction of the drug with vicinal sulfhydryl groups, resulting in the formation of stable ring structures which inhibit receptor clustering and internalization (Hertel et al., 1985). Considering these facts, in nerve terminals, the sensitivity of receptor internalization to sucrose suggests that it proceeds through the formation of vesicular compartments but its insensitivity to PAO strongly suggests that this process does not require the participation of vicinal sulfhydryl groups.

Further support for the existence of a PAO-insensitive, clathrin-dependent internalization pathway comes from studies on epidermal growth factor receptor (EGFR) in rat hepatocytes (Kato et al., 1992). In this cell type, a component of EGFR internalization, which is well documented to be clathrin-mediated (Hanover et al., 1985), was found to be PAO-independent and to strikingly resemble that demonstrated here for NTS1 in nerve terminals, in both its binding and internalization profile. Indeed, like NTS1, EGFR internalizes with an efficiency of 20%, in a linear fashion and saturates within 15 minutes whereas a slow decrease over time was observed in its binding profile (Sato et al., 1990).

To determine whether [^{3}H]-NT internalized in nerve terminals through the same clathrin-dependent mechanism as involved in the retrieval of plasma membrane during fast synaptic vesicles recycling, we repeated the experiments after removing Ca^{++} from the incubation medium and adding EDTA to chelate endogenous ions. Indeed, synaptic vesicle recycling has been abundantly

documented to be dependent on Ca^{++} (Palfrey and Artalejo, 1998; Neves et al., 1999). As previously reported (Lazarus et al., 1977), Ca^{++} removal was found to greatly reduce [^{3}H]-NT binding capacity (by more than half). However, it was without effect on [^{3}H]-NT internalization. In fact, the latter proceeded with the same capacity as when Ca^{++} was present, but with a higher internalization rate saturating within 5 instead of 15 minutes.

The insensitivity of [^{3}H]-NT internalization to Ca^{++} removal indicates that this internalization process operates through mechanisms distinct from synaptic vesicle recycling. The present set of experiments also suggests that NTS1 internalization capacity is dissociated from total specific [^{3}H]-NT binding capacity. Indeed, two very different concentrations of membrane-bound NTS1 (in the absence vs. presence of Ca^{++} in the incubation medium) were found to result in the same maximal internalization capacity.

[^{3}H]-NT internalization rate is faster in the absence of Ca^{++} than in its presence and resembles that of TfR in the presence of this ion. This observation suggests that the rate of NTS1 internalization is negatively regulated by Ca^{++}. This negative regulation could be the consequence of Ca^{++}-dependent phosphorylation of a number of proteins, shown to be induced by NT stimulation in synaptosomal preparations and brain slices from the neostriatum (Cain et al., 1988, 1991, 1992; Kaschow et al., 1991). One could speculate that Ca^{++} /calmodulin, a well known Ca^{++}-activated regulatory protein which has been suggested to mediate NT phosphorylative action in the neostriatum (Kaschow et al., 1991), might play a role in this process.

5 FATE OF NTS1 FOLLOWING [^{3}H]-NT INTERNALIZATION IN NEOSTRIATAL SYNAPTOSOMAL PREPARATIONS

To determine the fate of NTS1 following ligand-induced internalization in axon terminals, we pretreated synaptosomal preparations with monensin, a carboxylic ionophore that prevents the [^{3}H]-NT-NTS1 complex from dissociating, without interfering with receptor internalization nor recruitment of newly synthesized receptors to the plasma membrane (Chabry et al., 1993, Botto et al., 1998). Monensin is well known to block recycling of many receptors such as the LDL and atrial-natriuretic-factor receptor (Basu et al., 1981; Rathinavelu and Isom, 1991). NTS1 recycling to the plasma membrane may explain many particularities in this study.

Monensin treatment was found to reduce both the capacity and saturation time of [^{3}H]-NT internalization in neostriatal synaptosomes, as compared to controls. This finding indicates that following internalization, [^{3}H]-NT normally dissociates from its receptor. It also suggests that under normal conditions, NTS1 are efficiently recycled i.e. are targeted back to the plasma membrane, making them available for further binding and internalization. Again, this situation is very different from that reported to occur in epithelial cells or at the somatodendritic level in neurons, where dissociated NTS1 was reported to be targeted to lysosomes for degradation (Botto et al., 1998, Vandenbulcke et al., 2000).

Dissociation of NT from its high affinity receptor (NTS1) following its internalization was previously documented in mesencephalic neurons in culture, by demonstrating that internalized ligand-receptor complexes were abnormally trafficked if covalently cross-linked prior to internalization (Nouel et al., 1997).

Hypothetically, if internalized NTS1 goes through multiple rounds of recycling to the plasma membrane, the following features must be observed in the internalization kinetics of [^{3}H]-NT:

1) In the absence of monensin, [^{3}H]-NT internalization should take longer and should saturate at a greater capacity than if monensin was present.

2) Due to the cyclic (repetitive) nature of receptor recycling, when receptor recycling is not blocked, the rate of [^{3}H]-NT internalization should be constant; [^{3}H]-NT internalization curve must be linear.

Indeed, synaptosomal preparations that were treated with monensin displayed a lower [^{3}H]-NT internalization capacity and took less time to saturate than those that were not treated (Figure 25). Also, in untreated synaptosomes, [^{3}H]-NT internalization was previously found to be relatively constant (Figure 13). This internalization was previously described as being divided into two phases: high rate, short-lasting and a slow rate, long-lasting. This particularity may be explained by the fact that receptors are initially located on the plasma membrane and following interaction with their radioligand, internalize at a rate that accounts for the first phase. However, within 5 minutes or so, internalized receptors are recycled back to the plasma membrane and the full recycling pathway has kicked in. Now, the concentration of membrane-bound receptors is reduced as a constant pool of receptors flows inside the synaptosomes that await dissociation from their ligand and subsequent recruitment to the plasma membrane. This results in a decrease in the rate of receptor internalization and accounts for the second phase. Within 15 minutes or so, saturation is reached, probably as a consequence of energy depletion. Receptor endocytosis is an energy-dependent

process and synaptosomal preparations are only given limited concentrations of substrate such as glucose (5 mM) from which they produce ATP (Bradford et al., 1969).

In summary, this study has shown that:

1) In nigrostriatal synaptosomes, NT binding sites are characterized by high affinity (0,20-0,42 nM) and low capacity (22,76-25,3 fmol/mg protein), and is efficiently displaced by SR48692 (IC_{50}= 3,2 nM) with values that correspond to the binding properties of the high affinity NT receptor, NTS1.
2) Following binding of [^{3}H]-NT to NTS1, the complex [^{3}H]-NT-NTS1 is internalized. The rate of this internalization is relatively constant as indicated by the linear slope of [^{3}H]-NT internalization. [^{3}H]-NT internalization saturates within 15 minutes.
3) In isolated nerve terminals, [^{3}H]-NT-NTS1 internalizes through a mechanism that differs from the classical clathrin-dependent pathway occurring in non-neuronal and in somatodendritic regions of neuronal cell by its insensitivity to PAO treatment. Such a PAO-independent internalization pathway has been previously reported to exist for the EGF receptor in rat hepatocytes.
4) This pathway of [^{3}H]-NT-NTS1 internalization operates through a mechanism distinct from synaptic vesicles recycling, as suggested by the insensitivity of [^{3}H]-NT internalization to Ca^{++} removal, Ca^{++} being an ion critical in synaptic vesicles recycling.

5) The fact that [^{3}H]-NT internalization was partially prevented by monensin treatment suggests that following internalization of the [^{3}H]-NT-NTS1 complex, NTS1 dissociates from its radioligand and is targeted back to the plasma membrane, where it is available to bind and internalize more ligand molecules.

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