Neurotensin modulates central muscarinic receptors, an effect which does not involve the high-affinity neurotensin receptor (NTS1)

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ABSTRACT

Neurotensin (NT) is a tridecapeptide distributed in central and peripheral nervous systems, which can behave as a neurotransmitter or neuromodulator at central and peripheral levels. Herein we tested the potential effect of this peptide on quinuclidinyl benzilate ($[^3H]$-QNB) binding to muscarinic receptor in rat CNS membranes. It was observed that NT decreased up to 50–70% ligand binding at 1×10^{-7} M–1×10^{-5} M concentration in cerebral cortex, cerebellum and striatum. In the hippocampus, NT exerted a biphasic effect, behaving as a stimulator in the presence of 1×10^{-12} M–1×10^{-10} M concentration but as an inhibitor at 1×10^{-8} M–1×10^{-5} M concentration. In order to test the involvement of high-affinity NT receptor (NTS1) in NT inhibitory effect, assays were carried out in the presence of 1×10^{-6} M NT and/or SR 48692 (Sanofi-Aventis, U.S., Inc.), a specific antagonist for this receptor, dissolved in dimethylsulfoxide (DMSO) 10% v/v. As controls, membranes incubated with DMSO and/or NT 1×10^{-6} M plus DMSO were processed. It was found that NT + DMSO decreased $[^3H]$-QNB binding to cerebral cortex, cerebellum and hippocampal membranes by 49%, 32% and 53%, respectively. This inhibition was not observed with the DMSO control group. Membrane preincubation with 1×10^{-6} M SR 48692 failed to alter NT effect on binding. SR 48692 at 1×10^{-6} M concentration decreased the binding by 50% only in cerebral cortex membranes, suggesting a possible direct effect of the antagonist on muscarinic receptors in this area. It was therefore concluded that the high-affinity NT receptor may not be involved in ligand binding inhibition to muscarinic receptor by NT.

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1. Introduction

Neurotensin (NT) is a tridecapeptide distributed in central and peripheral nervous systems, which can behave as neurotransmitter or neuromodulator, exerting diverse biological actions [1]. NT interacts with specific receptors, which can bind the peptide with high (NTS1) and low (NTS2) affinities. Structurally unrelated to these receptors, a third subtype, NTR-3/sortilin [2] and a fourth subtype NTR-4/SorLA [3] have been described.

The relationship between neurotensinergic and cholinergic systems has been documented in several ways [4,5]. The association of neurotensin binding sites with cholinesterase localization in neurons of the diagonal band, substantia innominata and nucleus basalis has been reported [6,7]. In basal magnocellularis nucleus, high-affinity neurotensin receptors (NTS1) were located in cell bodies and dendrites of cholinergic neurons [6], the most important input to neocortex and amygdala, two regions involved in alert, learning and memory [8]. NT differentially regulated evoked release of acetylcholine according to the brain area studied [9] and exerted excitatory effects on forebrain cholinergic neurons [10]. Binding and internalization of NT in hybrid cells derived from septal cholinergic neurons was demonstrated [11]. NT administered into the hippocampus produced a long lasting and concentration-dependent increase in the basal extracellular level of acetylcholine [12]. Microinjection of NT into the basal forebrain of freely-moving, naturally waking–sleeping rats induced neuron bursting. This effect is most likely a direct action on cholinergic neurons as evidenced by selective internalization of a NT-fluorescent ligand [13]. Most interestingly, the ability of cholinergic neurons to discharge in rhythmic bursts when activated by NT administered into the basal forebrain has been reported [14].

To deepen into the relationship between neurotensin actions and the cholinergic system, we studied NT effects on quinuclidinyl benzilate ($[^3H]$-QNB) binding to cholinergic muscarinic receptor in rat brain. The peptide invariably decreased the ligand binding to the cerebral cortex, cerebellar and striatal membranes whereas exerted a biphasic effect on hippocampal membranes. NT inhibitory effect on $[^3H]$-QNB binding seemed independent of NTS1 receptor since its specific antagonist SR 48692 [15] failed to block the effect. At present, potential involvement of NTS2 receptor cannot be disregarded.

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2. Materials and methods

2.1. Animals and drugs

Male Wistar rats weighing 100–150 g were used. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory provided by the National Institutes of Health, USA. Reagents were analytical grade. Neurotensin acetate and atropine sulfate were purchased from Sigma Chemical Co., St. Louis, MO, USA. Peptide solutions in redistilled water were freshly prepared for each experiment. SR 48692 {2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy phenyl) pyrazol 3-yl) carbonylamino]tricyclo $(3.3.1.1^{3.7})$ decan-2-carboxylic acid} was kindly provided by Sanofi-Aventis, U.S., Inc. OptiPhase "Hisafe" 3 was purchased from Wallac Oy (Turku, Finland). L-[^{3}H]-QNB was from Du Pont Corp. New England Nuclear, Boston, MA, USA, specific activity 14,443 GBq/mmol. All other reagents were of analytical grade and purchased from local suppliers.

2.2. Membrane preparation

For each experiment, cerebellum, hippocampus, cerebral cortex and striatum from 3 to 4 rats were harvested and separately pooled. Tissues were rapidly homogenized at 10% (w/v), except for cerebral cortex at 4% (w/v), in 0.32 M sucrose neutralized with Tris base solution (0.4 mM Tris final concentration) in a Teflon glass Potter–Elvehjem homogenizer.

Homogenates were centrifuged at $900 \times g$ for 10 min and pellets discarded; resulting supernatants were diluted with 0.16 M sucrose to a final concentration of 0.25 M sucrose, centrifuged at $100,000 \times g$ for 30 min and membrane pellets stored at -70 °C until use.

2.3. [^{3}H]-QNB binding assay

[^{3}H]-QNB binding was determined according to the method described by Yamamura and Snyder [16] with slight modifications, in the presence of variable concentrations of NT. Membrane pellets were resuspended and later diluted in 50 mM sodium–potassium phosphate buffer (pH 7.4) to reach 0.1 mg protein per ml concentration.

Triplicate samples were incubated (2 ml final volume) at 30 °C for 60 min with 0.5 nM of [^{3}H]-QNB [16–18]. Non-specific binding was defined as tracer binding in the presence of 5 µM atropine sulfate. When indicated, 1×10^{-6} M SR 48692 disolved in dimethylsulfoxide (DMSO) 10% v/v [19] was included. After incubation, 3 ml of ice-cold sodium–potassium phosphate buffer was added and samples were vacuum-filtered through Whatman GF/B glass disks. Filters were washed twice with 3 ml of ice-cold buffer, placed in plastic vials and dried overnight at 70 °C. To each vial, 3 ml of OptiPhase "Hisafe" 3 was added and radioactivity quantified in a liquid scintillation counter.

Specific binding was calculated as the difference between the binding in the absence and presence of atropine sulfate, and represented 90–95% of total binding.

Binding assays were carried out by triplicate in membranes obtained in at least three different occasions.

2.4. Protein measurement

Protein was determined by the method of Lowry et al. [20] using bovine serum albumin as standard.

2.5. Data analysis

Data are presented as mean values $\pm$ SE of n experiments. To determine statistical significance of difference versus control, one-sample Student's t-test was employed. For comparison of different NT concentrations between groups, statistical significance of differences was assessed by one-way analysis of variance (ANOVA) followed by Conover test. The probability level indicative of statistical significance was set up $P < 0.05$. K_i values for competition of radioligand binding to a single binding site were obtained by non-linear regression, using GraphPad Prism Program version 4.0 (2003). Confidence intervals for K_i values were 95%.

3. Results

Muscarinic cholinergic receptor was studied by means of [^{3}H]-QNB binding to CNS membranes in the absence or presence of variable NT concentrations. In the absence of the peptide, ligand binding was 930 ± 5, 350 ± 6, 1140 ± 6 and 1070 ± 6 fmol per mg protein (mean values $\pm$ SE, $n = 3, 4$) for cerebral cortex, cerebellum, striatum and hippocampus membranes, respectively. [^{3}H]-QNB binding in the absence of additions (NT or SR 48692) was considered as 100%.

Ligand [^{3}H]-QNB binding to cerebral cortex, cerebellum and striatum membranes remained unaltered in the presence of 10^{-12}–10^{-8} M NT. Within the range of 10^{-7}–10^{-5} M concentration, the peptide invariably decreased [^{3}H]-QNB binding (Fig. 1A–C). [^{3}H]-QNB binding to cerebral cortex membranes decreased roughly 30% in the presence of 1×10^{-7} M NT; a similar drop was recorded with 1×10^{-6} M and 1×10^{-5} M NT (Fig. 1A).

In the case of cerebellar membranes, binding dropped 10–17% with 1×10^{-7} M and 1×10^{-6} M NT, without significant differences inter se; binding decrease reached 50% with 1×10^{-5} M NT (Fig. 1B). In the case of striatal membranes, binding dropped 45% with 1×10^{-7} M NT and 55% with 1×10^{-6} M and 1×10^{-5} M NT, without significant differences inter se (Fig. 1C).

In contrast, the peptide exerted a biphasic effect in membranes isolated from the hippocampus according to the concentration employed. NT enhanced 40%–50% of the ligand binding at 10^{-12}–10^{-10} M concentration, without significant differences between both conditions. The peptide diminished binding by 15%–25% at 10^{-8} M and 10^{-7} M concentration and roughly by 75% at 10^{-6}–10^{-5} M concentration (Fig. 1D).

In order to quantify the inhibitory ability of NT for every area, inhibition constants for NT versus [^{3}H]-QNB were determined. Results for K_i values and kinetic constants for [^{3}H]-QNB binding in control membranes were presented in Table 1.

To evaluate potential participation of NTS1 receptor in the inhibition of [^{3}H]-QNB binding by NT, new experiments were carried out in the presence of SR 48692, an antagonist of high-affinity NT receptors.

Rat hippocampal, cerebellar and cerebral cortex membranes were incubated with SR 48692 1×10^{-6} M disolved in DMSO 10% v/v, in the presence or absence of 1×10^{-6} M NT containing DMSO 10%. For each tissue, assays were carried out in the absence or presence of DMSO 10%, to demonstrate that this solvent failed to alter [^{3}H]-QNB binding (data not shown).

It was observed that NT plus DMSO decreased 53% [^{3}H]-QNB binding to hippocampal membranes, an effect which was not significantly modified by membrane preincubation with 1×10^{-6} M SR 48692. The single presence of 1×10^{-6} M SR 48692 had no effect on binding (Fig. 2).

NT plus DMSO decreased approximately 30% [^{3}H]-QNB binding to cerebellar membranes, an effect which was not significantly altered by membrane preincubation with 1×10^{-6} M SR 48692. Here again, the single presence of 1×10^{-6} M SR 48692 failed to modify ligand binding (Fig. 3).

In the case of cerebral cortex membranes, NT plus DMSO decreased roughly 50% [^{3}H]-QNB binding either in the absence or presence of 1×10^{-6} M SR 48692. The single presence of 1×10^{-6} M SR 48692 diminished 40% [^{3}H]-QNB binding to cerebral cortex membranes (Fig. 4).
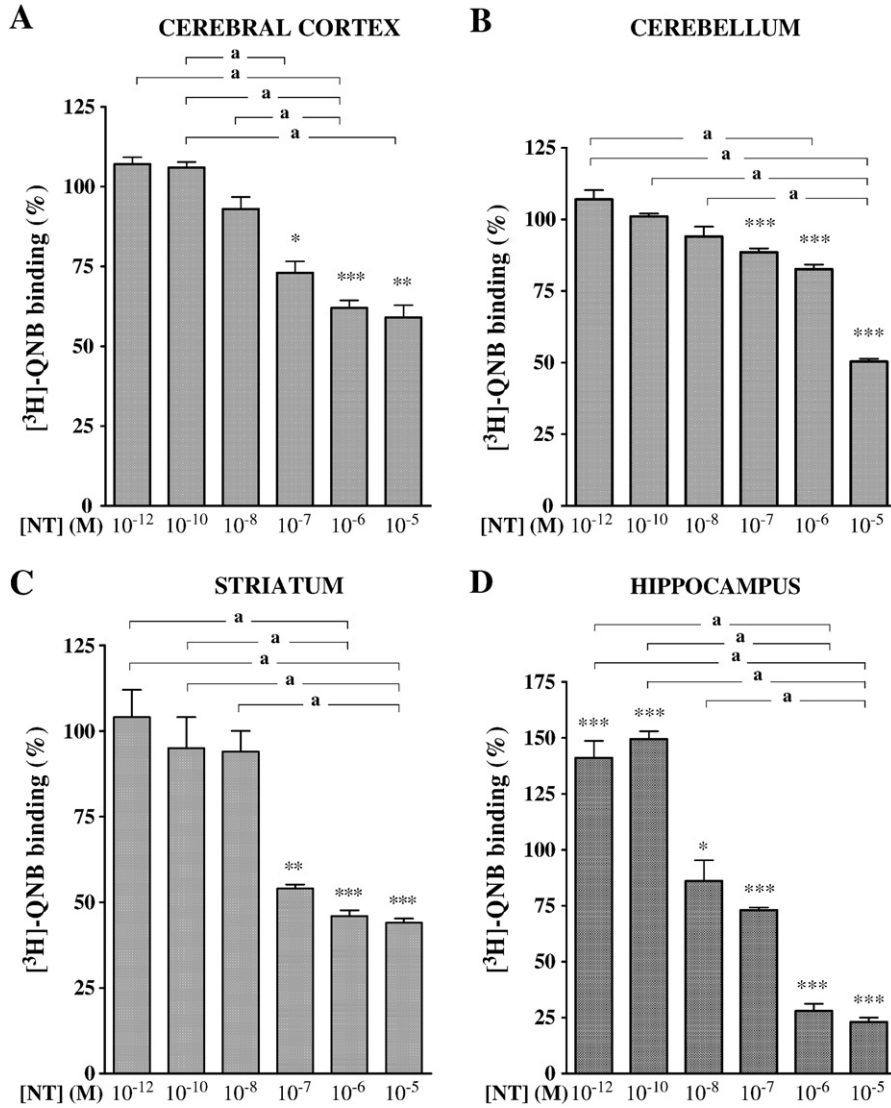
Fig. 1. Neurotensin effect on specific [³H]-QNB binding to rat CNS membranes. [³H]-QNB binding to membranes was assayed in the absence (control) or presence of NT at the concentrations indicated. Results are expressed as percent binding versus control without additions and are mean values ± SE from 3 to 4 assays performed by triplicate. A) Cerebral cortex; B) cerebellum; C) striatum; D) hippocampus. *$P<0.05$; **$P<0.005$; ***$P<0.001$ versus control without additions by one-sample Student's t-test. [a]$P<0.05$ by one-way analysis of variance (ANOVA) followed by Conover test.

4. Discussion

The relationship between neurotensinergic and cholinergic systems has been documented in several ways (see Introduction). As an approach to deepen into such interaction we studied the effect of NT on [³H]-QNB binding to muscarinic receptor in rat CNS membranes. It was observed that the peptide invariably decreased binding to

Table 1
K_i values for [³H]-QNB binding inhibition by NT and [³H]-QNB binding characteristics in control membranes.

Area	K_i	K_d (pM)	B_{max} (pmol mg prot^{-1})	Hill number	Reference
Cerebral cortex	6.46×10^{-10} M	113.7	1.78	0.97	[25]
Hippocampus	5.47×10^{-9} M	115.4	2.31	0.97	[18]
Striatum	2.29×10^{-8} M	681.6	8.78	0.93	[17]
Cerebellum	8.17×10^{-8} M	197.5	0.36	1.01	[18]

To calculate K_i values all data shown in Figs. 1–3 were processed. In the case of the hippocampus (Fig. 4), only data recorded within the 1×10^{-8} M to 1×10^{-5} M NT concentration range were processed. [³H]-QNB binding constants were from references indicated.
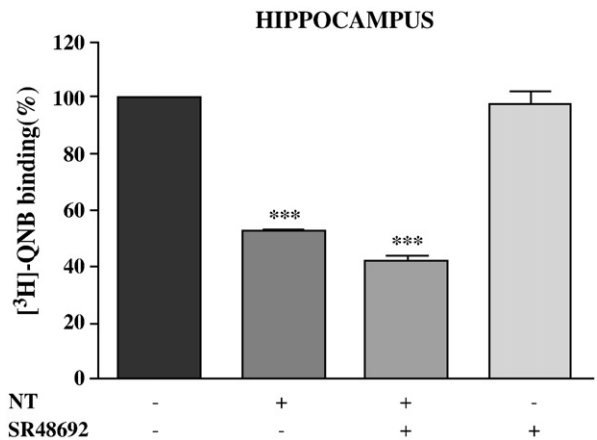
Fig. 2. [³H]-QNB binding to rat hippocampus membranes in the presence of 1×10^{-6} M NT and 1×10^{-6} M SR 48692, antagonist for NTS1 receptor. In all cases, DMSO 10% was present. Results are expressed as percent binding versus control without additions. ***$P<0.001$, by one-sample Student's t-test.
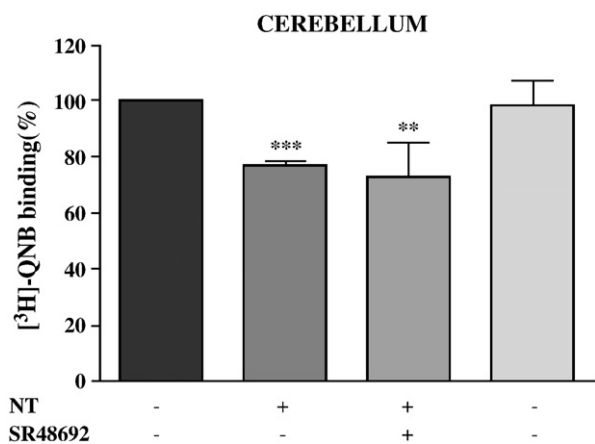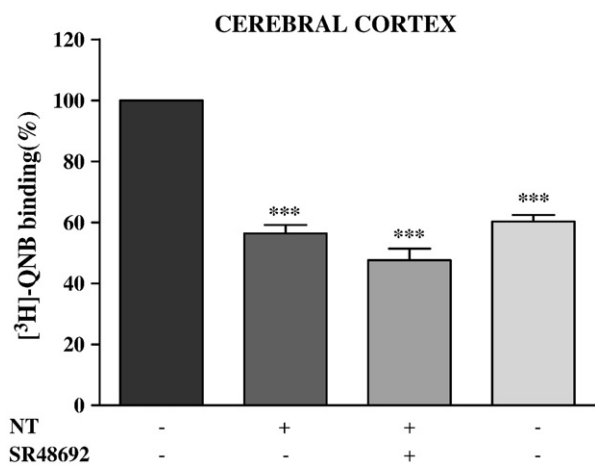
CEREBELLUM

Fig. 3. [^{3}H]-QNB binding to rat cerebellum membranes in the presence of 1×10^{-6} M NT and 1×10^{-6} M SR 48692, antagonist for NTS1 receptor. In all cases, DMSO 10% was present. Results are expressed as percent binding versus control without additions. *$P<0.05$; ***$P<0.001$, by one-sample Student's t-test.

muscarinic receptor in cerebellum, cerebral cortex and striatum membranes, whereas a biphasic effect was recorded in hippocampal membranes. Inhibitory effect of NT on binding was not impaired by membrane preincubation with SR 48692, a specific antagonist for high-affinity NT receptor (NTS1).

Neurotensin at micromolar range (3×10^{-8} M to 3×10^{-6} M concentrations) inhibited synaptosomal membrane Na$^+$, K$^+$-ATPase activity [19] and at nanomolar range concentrations was effective to enhance phosphoinositide metabolism in brain stimulated by muscarinic agonist carbachol [4] and to reduce the affinity of dopamine agonist binding to subcortical limbic sites [21]. Taken jointly, these findings led us to employ NT at a concentration range of 10^{-12} to 10^{-5} M to test its potential effect on CNS cholinergic muscarinic receptor.

Several differences in [^{3}H]-QNB binding among the four CNS areas studied were observed, including the data for K_d and B_{max} values [17,18,22]. Present results showed significant decreases in [^{3}H]-QNB binding to muscarinic receptor in cerebral cortex and striatum membranes, though the responses were not strictly concentration-dependent. In cerebellum, binding inhibition was concentration-dependent whereas in hippocampus a biphasic effect was recorded. K_i

CEREBRAL CORTEX

Fig. 4. [^{3}H]-QNB binding to rat cerebral cortex membranes in the presence of 1×10^{-6} M NT and 1×10^{-6} M SR 48692, antagonist for NTS1 receptor. In all cases, DMSO 10% was present. Results are expressed as percent binding versus control without additions. ***$P<0.001$, by one-sample Student's t-test.

values recorded for the CNS areas studied indicated that receptor sensitivity to NT is not related to either receptor affinity or the binding site density.

Receptor phosphorylation is one of the cellular events probably mediating muscarinic receptor modulation [23,24]. Moreover, intracellular factors may be involved in agonist-induced receptor changes. Ligand binding to cholinergic muscarinic receptor is modified by diverse endogenous agents, including guanine nucleotides and choline (see [25]), as well as by peptides like calcitonin [26] and NT ([4,5], present results). Modulation of radioligand binding to muscarinic receptors by several drugs has suggested the existence of an allosteric regulatory site [27]. The acetylcholine-binding site (orthosteric site) presents a high sequence homology across all muscarinic receptor subtypes (M$_1$–M$_5$). Most interestingly, the extracellular allosteric binding sites (one or two) can recognize small molecule allosteric modulators to regulate the binding [28]. With respect to receptor subtypes, the majority of muscarinic receptors in the brain belong to the M$_1$ subtype and the subtype receptor proteins are detected in the four areas here analyzed, though their relative contribution differ considerably in the individual regions [29].

Neurotensin action on ligand [^{3}H]-QNB binding to muscarinic receptor here described may well offer a tool to detect and characterize the possible allosteric effects that can occur when two ligands simultaneously bind to this receptor. By means of ligand [^{3}H]-QNB it is not possible to disclose if NT effect differs for different muscarinic cholinergic subtypes. It would be of interest to extend this study to the employment of other ligands, specific for each of the various subtypes, which may help to clarify this subject.

Herein we observed that NT exerted a biphasic effect on [^{3}H]-QNB binding to hippocampal membranes. In agreement, in several in vitro [30–32] and in vivo [33,34] experimental models, NT behaved as a stimulator or as an inhibitor according to the dose employed. On the other hand, cholinergic modulation of hippocampal cells and circuits has been demonstrated. A great number of actions contributed to the oscillogenic properties of acetylcholine in this area, mainly induced by activation of muscarinic receptors [35]. The biphasic effect of neurotensin on ligand binding to muscarinic receptor described herein may well contribute to this modulation.

NT actions occurring through the NTS1 receptor were blocked by non-peptidic antagonist SR 48692 [15]. In vitro, this compound competitively inhibited ^{125}I-labeled neurotensin binding to the high-affinity binding site (NTS1) present in brain tissue from various species, and antagonized NT effects in a variety of experimental models [15,36]. Previous work from this laboratory has shown that phosphoinositide turnover stimulation by muscarinic agonist carbachol, either alone or plus NT, was partially or totally blocked by SR 48692 [37]. These findings, which indicated potential involvement of NTS1 receptor in NT effect, supported the relationship between neurotensinergic and cholinergic systems. On the other hand, SR 48692 failed to antagonize hypothermia and analgesia induced by NT administration into the CNS, suggesting that the effect is mediated through a NT receptor subtype which is insensitive to SR 48692 [38].

In order to study direct actions of NT in diverse experimental models, several NT receptor antagonists have been introduced (see [36]). Among them, both SR 48692 and SR 142948A are suitable antagonists for the neurotensin receptor. For the present study, which was carried out in the rat brain, we have chosen the former because available information regarding its effect on neurotransmitter systems at CNS was more abundant than that recorded with SR 142948A. On the other hand, whereas SR 142948A proved effective to block both NTS1 and NTS2 receptors, the selectivity of SR 48692 to block NTS1 receptor in rodent brain was demonstrated [39].

To test whether NTS1 receptor was involved in ligand binding inhibition by NT, experiments were carried out in the presence of antagonist SR 48692 and NT, both at 1×10^{-6} M concentration. This condition prevented the inhibition of synaptosomal membrane Na$^+$,

K^+-ATPase activity by NT [19]. At variance, present results showed that the decrease of ligand binding to muscarinic receptor by NT was not impaired by SR 48692, most likely indicating that this effect was not associated with the NTS1 receptor. NT affinity is 0.1–0.3 nM for NTS1 receptor and 3–5 nM for NTS2 receptor [2]. According to the NT concentration range employed herein (10^{-12} to 10^{-5} M concentration) NT may well occupy not only NTS1 but also NTS2 receptors. It should be recalled that NTS2 receptor has been localized in neurons both at presynaptic and postsynaptic sites [40,41], and that it is involved in analgesia at CNS level [42].

Therefore, at present potential role of NTS2 receptor cannot be ruled out. The employment of another antagonist, specific for NTS2 receptor, might clarify this subject. Another plausible interpretation for the present findings is the possibility that a direct effect – not mediated by receptors – on binding site for ligand QNB in muscarinic receptor may take place.

The single presence of SR 48692 failed to alter [^{3}H]-QNB binding to hippocampus, cerebellum and striatum membranes whereas it exerted an inhibitory effect on ligand binding to cerebral cortex membranes. This result may indicate a novel, area specific effect for the antagonist, which may be due to a different area distribution of muscarinic receptor subtypes. Another possible hypothesis could involve the participation of a natural ligand for NTS1 receptor which is different to NT [43]. Therefore, our finding of the SR 48692 inhibitory effect, which was only observed in cerebral cortex membranes, may be attributed to a differential regional distribution of such endogenous ligand. However, the possibility that the receptor environment in isolated cerebral cortex membranes differed from other CNS membranes cannot be disregarded.

Although research about NT and their receptors received a great deal of attention during the last decades, many questions regarding neurotensinergic system role at CNS remain to be elucidated.

To summarize, these results point to a modulation of cholinergic muscarinic receptor functionality by NT. It can be concluded that NT mainly decreased ligand [^{3}H]-QNB binding to CNS membranes whereas an exception was recorded in hippocampal membranes, where at low concentration, NT enhanced the binding. Whereas NT inhibitory effect on binding seemed independent of NTS1 receptor, at present, potential involvement of NTS2 receptor cannot be disregarded.

Acknowledgements

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