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Further characterization of neurotensin binding in the rat brain: levocabastine-displaceable neurotensin binding sites are not histamine-H_1 receptors

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Neurotensin was discovered in 1973 by Carraway and Leeman in extracts from bovine hypothalamus [1], and was found to be involved in numerous physiological processes both in the central nervous system and the periphery [2].

In radioligand binding studies, several lines of evidence suggested the existence of a high and a low affinity neurotensin binding site in rat brain membranes (NT_H- and NT_L-sites) [3, 4]. Levocabastine [5], a histamine-H_1 antagonist, structurally unrelated to neurotensin, was shown to inhibit selectively neurotensin binding to the NT_L-sites in rat, mouse and hamster brain, but not in other species including human. Levocabastine became a useful tool which enabled separate measurement of the labelling of high and low affinity neurotensin binding sites (K_D = 0.7 and 7.1 nM, respectively) [6].

NT_H-sites in rat brain displayed the characteristics of a neurotransmitter receptor, whereas the NT_L-sites have an as yet undefined role and are likely to be considered as chemical recognition sites for neurotensin or acceptor sites [6–8].

In the present report we will define additional biochemical properties of both neurotensin binding sites, in order to further scrutinize the distinction between NT_H and NT_L-sites in the rat brain and to analyse whether any relationship would exist between the low affinity neurotensin binding site and the histamine-H_1 receptor.

Material and methods

[^{3}H]*Neurotensin binding.* [^{3}H]Neurotensin binding (2 nM) was performed in 0.5 ml of Tris–HCl buffer (50 mM, pH 7.4) containing total particulate fraction corresponding to 5 mg original rat forebrain, 0.1% bovine serum albumin, 1 mM EDTA and 0.2 mM bacitracin. The incubation of 20 min at 25° was terminated by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. The filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the radioactivity retained on the filters was measured by liquid scintillation spectrometry.

Filters were soaked in 0.1% polyethylenimine for 2 hr prior to use, in order to reduce adsorption of the radioligand to the filters.

Selective binding to the NT_H-sites was measured in the presence of 1 μM levocabastine, which occludes the NT_L-sites. Nonspecific binding was determined by addition of 1 μM unlabelled neurotensin to the incubation medium.

Binding to NT_L-sites was calculated as the difference between total binding and binding in the presence of 1 μM levocabastine.

[^{3}H]*Levocabastine binding.* Binding experiments were performed in 1 ml of Tris–HCl buffer (50 mM, pH 7.4) containing total particulate fractions from rat cortex corresponding to 10 mg of original tissue per assay, 2 nM [^{3}H]levocabastine, 0.1% bovine serum albumin, 1 mM EDTA and 0.2 mM bacitracin. Incubation was run for 20 min at 25° and ended by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. Filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and retained radioactivity was counted by liquid scintillation spectrometry.

Filters were soaked in 0.1% polyethylenimine for 2 hr prior to use, in order to reduce adsorption of the radioligand to the filters.

Nonspecific binding was measured in the presence of 1 μM unlabelled levocabastine.

[^{3}H]*Pyrilamine binding.* [^{3}H]Pyrilamine binding (2 nM) was performed in total particulate fractions from guinea-pig cerebellum (10 mg original tissue/assay) diluted in 1.1 ml phosphate buffer (10 mN NaH_2PO_4, 40 mM K_2HPO_4, pH 7.5). Incubation was run for 30 min at 25° and terminated by rapid filtration under reduced pressure. Filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and radioactivity retained on the filters was counted by liquid scintillation spectrometry. Astemizole (1 μm) was used for the determination of the nonspecific binding.

Materials. [3,11-Tyrosyl-3,5-^{3}H(N)]neurotensin (40.9–74.5 Ci/mmol or 1.5–2.7 TBq/mmol) and [^{3}H]pyrilamine (27.9 Ci/mmol or 1.0 TBq/mmol) were purchased from New England Nuclear (Dreieich, F.R.G.). [^{3}H]Levocabastine (16.0 Ci/mmol or 0.6 TBq/mmol) was synthesized by C. Janssen (Janssen Research Foundation, Beerse, Belgium). Unlabelled neurotensin was obtained from U.C.B. (Braine-l'Alleud, Belgium) and unlabelled levocabastine was from Janssen Pharmaceutica (Beerse, Belgium). Instagel II scintillation fluid was purchased from Packard (Downers Grove, IL).

Results and discussion

Association and dissociation of [^{3}H]*neurotensin binding.* Binding of [^{3}H]neurotensin to NT_H- and NT_L-sites was measured after different incubation times, between 1 and

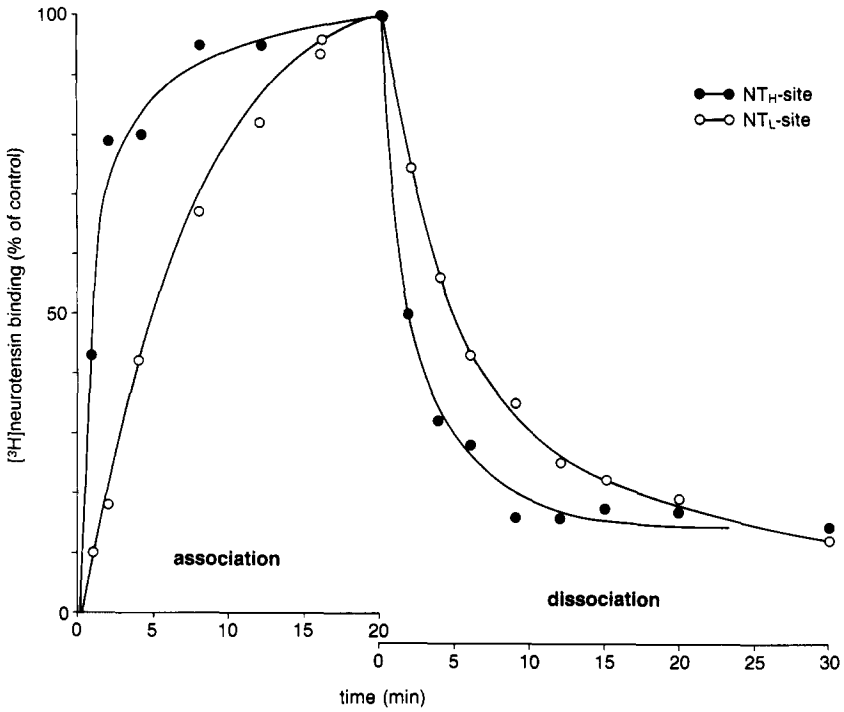

Fig. 1. Association and dissociation of [^{3}H]neurotensin binding to NT_H- and NT_L-sites in the rat forebrain, expressed in percentage of specific binding observed in the presence and in the absence of 1 μM levocabastine respectively. Binding of 2 nM [^{3}H]neurotensin to NT_H-sites was measured in the presence of 1 μM levocabastine. Dissociation of the ligand from the latter site was obtained by addition of 1 μm unlabelled neurotensin to the incubation medium, after having preincubated the ligand and the membranes for 20 min at 25°. For the dissociation of [^{3}H]neurotensin binding from NT_L-sites, 1 μM levocabastine was added after the preincubation period. Values are the mean of two independent determinations.

60 min. Figure 1 shows that [^{3}H]neurotensin reached 50% of its maximal binding to NT_H- and to NT_L-sites after 1 and 5 min, respectively, whereas maximal binding was obtained after 8 and 16 min, respectively. This maximal level of binding was maintained up to 60 min of incubation.

For the measurement of the dissociation rate of [^{3}H]neurotensin from the NT_H-sites, the radioligand was preincubated with the membrane preparation in the presence of levocabastine (1 μM) for 20 min at 25°. Then, unlabelled neurotensin (1 μM) was added to the incubation medium, and the membrane labelling was measured after different times.

Dissociation of [^{3}H]neurotensin binding from the NT_L-sites was measured by adding 1 μM levocabastine as a displacer after preincubation of the radioactive ligand and the membranes for 20 min at 25°.

[^{3}H]Neurotensin showed dissociation half-times from the NT_H- and NT_L-sites of 2 and 5 min, respectively (Fig. 1).

The rapid dissociation times for neurotensin for both the NT_H- and the NT_L-sites, will impose very short rinsing periods to wash off the non-bound radioligand in autoradiography.

Mutual affinity between neurotensin and levocabastine. Levocabastine was shown to inhibit about 60% of total [^{3}H]neurotensin binding in total particulate fractions from rat forebrain with an IC_{50}-value of 25 nM [6].

When a total particulate fraction preparation from rat cortex was incubated in the presence of 2 nM [^{3}H]levocabastine, unlabelled native neurotensin (1–13) and the carboxyterminal fragment neurotensin (8–13) were able to inhibit about 30% of total [^{3}H]levocabastine binding with an IC_{50}-value of 11 nM and 10 nM, respectively (Fig. 2).

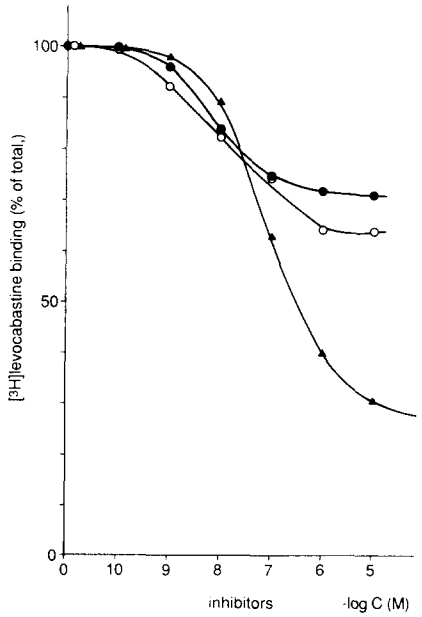

Fig. 2. Inhibition of 2 nM [^{3}H]levocabastine binding by neurotensin (1–13) (closed circles), neurotensin (8–13) (open circles) and by unlabelled levocabastine (closed triangles) in the rat cortex, expressed as percentage of total binding. Values are the means of four independent measurements performed in triplicate. SE values were less than 10%.

Table 1. Comparison of the affinities of levocabastine-isomers [5, 6] for histamine-H_1 receptors and for the NT_L-sites

Isomers	pIC_{50} [^{3}H]Pyrilamine binding to H_1 receptors in guinea-pig cerebellum	pIC_{50} [^{3}H]Neurotensin binding to NT_L-sites in rat forebrain
Levocabastine (−){3S[1(*cis*),3α,4β]}	7.0	7.6
Dextrocabastine (+){3R[1(*cis*),3α,4β]}	7.0	7.3
R-61816 (−){3S[1(*trans*),3α,4β]}	6.1	6.0
R-64034 (−){3S[1(*cis*),3α,4α]}	7.0	5.4
R-64035 (+){3R[1(*cis*),3α,4α]}	7.2	<5.0

Unlabelled levocabastine (10 μM) displaced 70% of total [^{3}H]levocabastine binding with an IC_{50}-value of 80 nM (Fig. 2). In ligand binding studies it is common to observe a larger inhibition when a radioligand is displaced by the same drug in its unlabelled form.

The reciprocity in the binding affinities between levocabastine and neurotensin in rat brain allows to assume that conformational similarities may exist between neurotensin and levocabastine and suggest that neurotensin and levocabastine should therefore be able to bind to a common site, in spite of their important chemical dissimilarity. This hypothesis is reinforced by the fact that neurotensin could no more displace [^{3}H]levocabastine binding after addition of 1 μM levocabastine to the incubation medium, indicating clearly that levocabastine and neurotensin bind indeed to one common binding site.

In addition, it appeared that the carboxyterminal portion of neurotensin was concerned with the inhibition of [^{3}H]levocabastine binding by the peptide.

Kitabgi *et al.* [9] assumed that levocabastine bound to a separate site which could modulate neurotensin binding to the low affinity sites. The mutual inhibition between neurotensin and levocabastine rather indicates that both might bind to the same site, which can be a chemical recognition site common to both neurotensin and levocabastine.

The [^{3}H]levocabastine binding in the rat brain was not affected by 1 μM concentrations of the chemically different H_1-antihistamines pyrilamine, astemizole, terfenadine, ketotifen and noberastine suggesting that the [^{3}H]levocabastine binding in the rat cortex does not occur to histamine-H_1 receptors. This is in accordance with the fact that histamine-H_1 receptor density in the rat brain is very low, and barely detectable using various labelled histamine-H_1 receptor ligands.

Neurotensin showed no affinity at all for the histamine-H_1 receptors, since the peptide did not inhibit [^{3}H]pyrilamine binding in the guinea-pig cerebellum (not shown).

Table 1 shows that no correlation exists between the inhibition of [^{3}H]neurotensin binding to the NT_L-sites by various levocabastine isomers and the potencies of these compounds to inhibit [^{3}H]pyrilamine binding to histamine-H_1 receptors. Some isomers showed the same antihistaminic potency as levocabastine, but had lost totally the affinity for the NT_L-sites.

From the above data it can be concluded that no interference exists between the levocabastine-displaceable neurotensin binding sites and the histamine-H_1 receptors.

In summary, [^{3}H]neurotensin binding to high and low affinity neurotensin binding sites (NT_H- and NT_L-sites) is essentially reversible. [^{3}H]Neurotensin binding to both the NT_H- and NT_L-sites rapidly associates and dissociates.

Our results support the hypothesis that neurotensin and levocabastine bind to one and the same binding site. The NT_L-sites are distinct from the histamine-H_1 receptors since the antihistamine drugs pyrilamine, astemizole, terfenadine, ketotifen and noberastine do not interfere with the NT_L-sites and since no correlation existed between the antihistamine properties of levocabastine isomers and their potencies for the inhibition of neurotensin binding to the NT_L-sites. [^{3}H]Levocabastine did not bind to histamine-H_1 receptors in the rat brain. Moreover, neurotensin showed no affinity for the histamine-H_1 receptors in the guinea-pig cerebellum.

Janssen Research Foundation
Department of Biochemical Pharmacology
B-2340 Beene
Belgium

ALAIN SCHOTTE
JOSÉE E. LEYSEN

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