Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 206-213

CHARACTERIZATION AND VISUALIZATION OF NEUROTENSIN BINDING TO RECEPTOR SITES IN HUMAN BRAIN

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Received March 1, 1984

The binding of monoiodo [^{125}I-Tyr^3]-neurotensin to human brain was characterized and visualized using radioreceptorassay and autoradiographic techniques. Specific binding to homogenates of human substantia nigra at 25°C was maximal at 20 min, reversible and saturable. Scatchard analysis of equilibrium data indicated the existence of two populations of binding sites with Kd values of 0.26 nM and 4.3 nM. Corresponding binding capacities were 26 and 89 fmol/mg of protein. Neurotensin analogs inhibited the binding of iodinated neurotensin with relative potencies that demonstrated the crucial role of the C-terminal hexapeptide portion of neurotensin for binding to its receptors. Autoradiography of human substantia nigra sections incubated with iodinated neurotensin revealed high levels of specific binding in the nucleus paranigralis and substantia nigra, pars compacta, and low levels in the substantia nigra, pars reticulata.

Neurotensin (NT), a putative neurotransmitter peptide, has been shown using immunological techniques to be widely distributed in the brain of mammals (see (1) for review). Specific NT binding sites have been characterized and visualized in rat brain using radioreceptorassay and autoradiographic techniques. A large body of evidences indicates that NT modulates nigrostriatal and mesolimbic dopaminergic systems (see (2) for review).

The distribution of immunoreactive NT in human brain has been reported (3). However, apart from preliminary autoradiographic studies of [^{3}H]-NT binding in

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ABBREVIATIONS : NT, neurotensin ; SN, substantia nigra ; Ac, acetyl ; Cit, citrulline.

0006-291X/84 $1.50

human brain (2), there has been no detailed characterization of the properties and distribution of NT receptors in this tissue. In the present paper, the interaction of monoiodo [^{125}I-Tyr^3]-NT with NT receptors sites in human substantia nigra (SN) was characterized and visualized using radioreceptorassay and autoradiographic techniques. SN was chosen as a source of human brain tissue because this brain area wherefrom the nigrostriatal dopaminergic pathway originates is rich in NT receptors in the rat and other species (2).

MATERIALS AND METHODS

Peptides : NT(pGlu^1-Leu^2-Tyr^3-Glu^4-Asn^5-Lys^6-Pro^7-Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}-OH) and its analogs [Trp^{11}]-NT, NT(1-8), Ac NT(8-13), Ac[Cit^8]-NT(8-13), Ac[Cit^9]-NT(8-13), Ac[Phe^{11}]-NT(8-13) and Ac[Phe^{12}]-NT(8-13) were generous gifts from Drs. J. Van Rietschoten and C. Granier (Faculté de Médecine Nord, Marseille, France).

Monoiodo [^{125}I-Tyr^3]-NT :The preparation, purification, and chemical and biologic characterization of iodinated NT molecules that have incorporated 1 atom of iodine per Tyr 3 residue of the peptide will be reported in detail elsewhere. In the present study, greater than 95 % pure monoiodo [^{125}I-Tyr^3]-NT preparations (referred to as ^{125}I-NT) with specific radioactivity of 2000 and 100 Ci/mmol were used.

Source of brain tissues : Human brains were obtained from 7 patients (6 females, 1 male ; 73-98 years ; mean ± S.E.M. = 84 ± 3 years) with no known neurologic or psychiatric disorder. The mean interval between death and autopsy was 9 ± 3 h (range, 3-27 h).SN were dissected from frozen brains, crushed and stored at - 70°C as previously reported (4).

Preparation of tissue homogenates : Tissues were homogenized using a Polytron homogenizer (setting 6-7) for 20 sec in 20 vol of ice cold Tris buffer (50 mM Tris-HCl, pH 7.5). Homogenates were centrifuged (100,000 g, 15 min) and the resulting pellets were washed twice by resuspension in the initial volume of Tris buffer and centrifugation at 100,000 g for 15 min. The final pellets were resuspended in 1 ml of Tris buffer and were used on the day of the preparation for binding experiments after appropriate dilution in the binding assay buffer.

Binding assay : All experiments were carried out at 25°C in 50 mM Tris HCl buffer, pH 7.5, containing 0.2 % bovine serum albumin and 1 mM 1,10-phenanthroline . ^{125}I-NT at the concentrations and specific radioactivity indicated was incubated in 250 µl with SN homogenates (final protein concentration, 0.15 -0.2 mg/ml) and when necessary with various concentrations of unlabeled NT and analogs. Bound ligand was separated from free using the filtration technique previously described (5).

For dissociation kinetics, ^{125}I-NT and SN homogenates were incubated for 20 min, the incubation mixture was then diluted 40-fold with incubation buffer containing 0.1 µM unlabeled NT. At varying times following dilution, 10 ml portions of the incubation medium were filtered (5). All the data have been corrected for nonspecific binding, i.e. the amount of radioactivity bound in the presence of an excess (1 µM) unlabeled NT. The specific binding was proportional to homogenate concentrations between 0.08 and 0.32 mg/ml of protein. In the experiments reported here, degradation of labeled ligand did not exceed 10 %.

Autoradiographic studies : 32 µm sections of frozen human brain were cut on a cryostat at -15°C at the level of the SN according to the atlas of Olsewski and Baxter (6). They were mounted onto gelatin-coated slides, stored overnight at -25°C and then kept at -80°C until assay. The slide-mounted sections were incubated for 60 min at 4°C with 0.1 nM ^{125}I-NT (2000 Ci/mmol) in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 0.2 % bovine serum albumin and 0.02 mM

bacitracin. Nonspecific binding was obtained in parallel incubations with 500 nM unlabeled NT and corresponded to 20 % of total binding. After incubation, the sections were washed 4 times for 2 min in ice cold buffer, dipped in distilled water and dried. Autoradiograms were obtained by apposition of the sections to ^{3}H-Ultrofilm (LKB, France) for 2 weeks at room temperature in the dark.

RESULTS

Time dependence of ^{125}I-NT binding : Figure 1 shows that the specific binding of ^{125}I-NT to SN homogenates increased with time, reached a plateau by 20 min and remained stable for another 50 min period. At this time, specifically bound ligand represented less than 2 % of total added ligand. For this reason, the association kinetic can be analyzed as a pseudo first order reaction according to the equation $LnX = (k_1[L] + k_{-1})t$ where $X = [Beq]/([Beq]-[B])$, $[Beq]$ is the concentration of bound ligand at equilibrium, $[B]$ the concentration of bound ligand at a given time t, $[L]$ the concentration of total ligand, k_1 and k_{-1} the rate constants of association and dissociation, respectively. The left inset in Fig. 1 shows that the plot of LnX versus time was linear with a slope $k = k_1[L] + k_{-1} = 3.2\ 10^{-3}\ sec^{-1}$ where $[L] = 73$ pM.

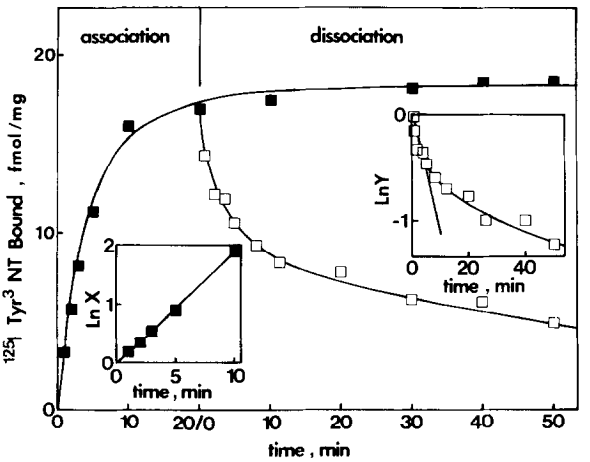

Figure 1 . Association (■) and dissociation (□) kinetics of ^{125}I-NT binding to SN homogenates. The binding of ^{125}I-NT (73 pM, 2000 Ci/mmol) was measured as explained in "Materials and Methods". Only the specific binding has been plotted. Left inset : pseudo first order representation of the association data (see "Results" for details). Right inset : first order representation of the dissociation data in which $Y = [B]/[B_0]$ where $[B]$ and $[B_0]$ represent the concentration of bound ligand at a given time t and at time 0 of dissociation, respectively. The straight line corresponds to the initial rate of dissociation and, from its slope value, the rate constant of dissociation k_{-1} was derived ($LnY = - k_{-1}\ t$).

After 20 min of association, dissociation of bound ligand was induced as explained in "Materials and Methods". As shown in Fig. 1, dissociation proceeded quickly during the first ten minutes and slowly thereafter. A logarithmic representation of the data is shown in the right inset of Fig. 1. The plot was biphasic. Only the initial rate of dissociation was taken into account for the calculation of k_{-1} for which a value of $1.8\ 10^{-3}\ sec^{-1}$ was obtained. Introduction of k_{-1} in the expression of k(see above) yielded a value of $1.92\ 10^{7}\ M^{-1}\ sec^{-1}$ for k_1. From these kinetic parameters, an estimate of 0.1 nM was calculated for the dissociation constant $K_D = k_{-1}/k_1$.

Concentration dependence of ^{125}I-NT binding : Figure 2,A shows the binding of increasing concentrations of ^{125}I-NT to SN homogenates. The nonspecific binding increased linearly as a function of ligand concentration. The specific binding reached a plateau at ligand concentrations greater than 2 nM, indicating that this binding component was saturable. Figure 2,B illustrates a Scatchard analysis obtained from three separate experiments such as that shown in Fig.2,A. The Scatchard plot was curvilinear with an upward concavity. This was interpreted as resulting from an interaction of the ligand with 2 populations of NT binding

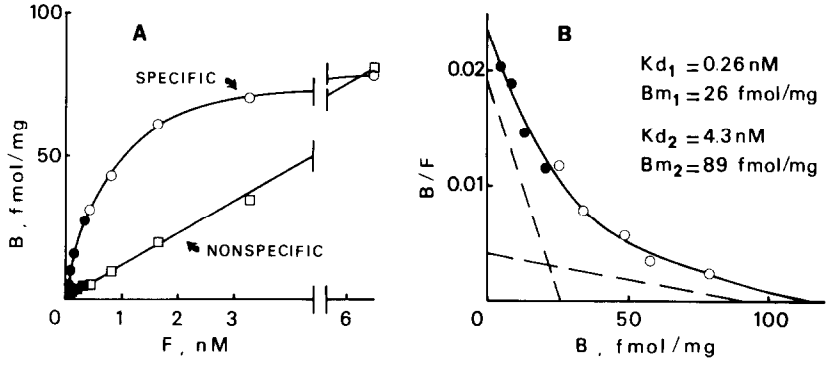

Figure 2 . Binding of ^{125}I-NT to SN homogenates as a function of ligand concentration. Varying concentration of ^{125}I-NT at 2000 Ci/mmol (● , ■) or 100 Ci/mmol (○ , □) were incubated for 20 min with SN homogenates and the binding was measured as described in "Materials and Methods".A, typical experiment showing the specific and the nonspecific binding of ^{125} I-NT as a function of ligand concentration. B, Scatchard analysis of the data obtained from three independent experiments like that shown in A. The data were fitted by computer according to a model describing the interaction of one ligand with two independent binding sites, as previously described (5). This yielded best estimates of the dissociation constants Kd and maximal binding capacities B_M for each class of sites. These values are shown in the Figure. The dashed lines represent the contribution to binding of each class of sites.

sites. Best fit of the experimental data according to this model yielded for each class of sites estimates of the dissociation constant (Kd) and maximal binding capacity (B_M). From the values shown in Fig. 2,B it was calculated that at 0.1 nM ^{125}I-NT, about 80 % of specifically bound ligand was bound to the population of high affinity sites. Note that the Kd value for these sites (0.26 nM) was close to the Kd value (0.1 nM) derived from kinetic experiments that were carried out at ligand concentrations close to 0.1 nM.

Competition experiments with NT related and unrelated peptides : NT partial sequences and analogs competed in a concentration-dependent manner for the binding of ^{125}I-NT to SN homogenates with IC_{50} and potency values shown in Table 1. The data indicate that the C-terminal hexapeptide sequence of NT contained almost all the structural requirements necessary for interacting with NT binding sites. In contrast the N-terminal octapeptide sequence had no binding activity. Substitution of Tyr 11 in NT by Trp resulted in a 10-fold decrease in binding potency. In general, substitutions at positions 9, 11 and 12 of the NT amino acid sequence led to important losses of binding activity (Table 1). Substitution of Arg 8 by Cit produced only a slight decrease in activity. For comparison, previously reported potencies (5) of NT analogs in the rat brain membrane radioreceptorassay have been included in Table 1.

Table 1 : Inhibition of ^{125}I-NT binding by NT analogs

PEPTIDES	HUMAN BRAIN[1]		RAT BRAIN[4]
	IC_{50} (nM)[2]	POTENCY[3]	POTENCY[3]
NT	0.33	100	100
[Trp^{11}]-NT	3.4	9.7	100
Ac NT(8-13)	0.62	53.2	133
Ac[Cit^{8}]-NT(8-13)	0.96	34.4	1.9
Ac[Cit^{9}]-NT(8-13)	5.5	6.0	2.0
Ac[Phe^{11}]-NT(8-13)	51	0.65	8
Ac[Phe^{12}]-NT(8-13)	200	0.16	0.54
NT(1-8)	> 20000	< 0.001	

1. SN homogenates were incubated for 20 min with 0.1 nM ^{125}I-NT (2000 Ci/mmol) and varying concentrations of unlabeled NT and analogs.
2. IC_{50} : concentration of unlabeled peptide that inhibits 50 % of ^{125}I-NT binding.
3. Potencies were calculated as 100 x IC_{50}(NT)/IC_{50} analog.
4. Potencies in the rat brain radioreceptorassay were taken from ref.(5).

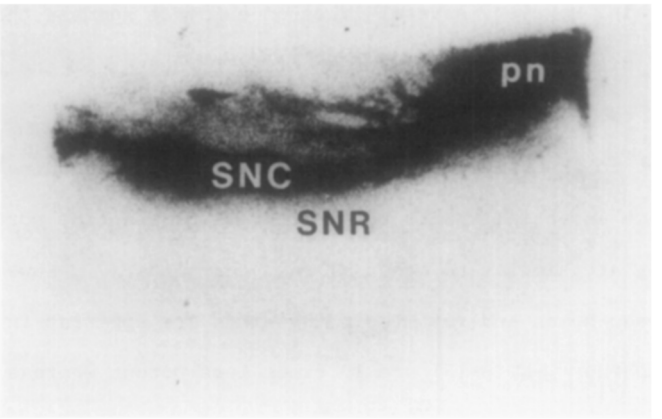

Figure 3 . Autoradiogram of ^{125}I-NT binding to a human brain SN section. The section was incubated with ^{125}I-NT and processed as described in "Materials and Methods". SNC, substantia nigra, pars compacta ; SNR, substantia nigra, pars reticulata; pn, nucleus paranigralis.

Peptides unrelated to NT like bradykinin, somatostatin, thyroliberin, insulin and apamin at µmolar concentrations had no effect on the binding of ^{125}I-NT to SN homogenates (not shown).

Autoradiography of ^{125}I-NT binding : Autoradiograms of human SN sections that were incubated in the presence of 0.1 nM ^{125}I-NT showed high levels of NT binding sites in the nucleus paranigralis and in the SN, pars compacta (Fig. 3). In contrast, low levels of binding sites were found in the SN, pars reticulata (Fig. 3). Nonspecific binding in adjacent sections that had been incubated with ^{125}I-NT in the presence of 500 nM unlabeled NT was homogenous and indistinguishable from the film background (not shown).

DISCUSSION

This study represents the first characterization of monoiodo[^{125}I-Tyr^{3}]-NT binding sites in human brain tissues using radioreceptorassay and autoradiographic techniques. The specific component of ^{125}I-NT binding was time dependent, reversible and saturable. Scatchard analysis of equilibrium data revealed the existence of 2 populations of NT binding sites with Kd values of 0.26 nM and 4.3 nM. Two populations of NT binding sites with similar Kd values (0.1 nM and 4.7 nM) were also recently characterized in rat brain (5). Further experiments are needed to understand the significance of two types of NT binding sites in

rat and human brain. However, several lines of evidence suggest that in the rat, the two types of sites represent different affinity states of the same NT receptors rather than two distinct NT receptors (5).

With respect to their structural requirements, the NT binding sites in human brain interact essentially with the C-terminal hexapeptide portion of NT. In that regard, they are similar to other NT receptor sites (7). However, some differences between human and rat NT binding sites are apparent in Table 1. Thus, [Trp ^{11}]-NT and Ac[Phe ^{11}]-NT(8-13) are 10 times less potent whereas Ac[Cit ^{8}]-NT (8-13) is 20 times more potent in the human than in the rat brain radioreceptor-assay. These differences may reflect species-related variations in NT receptor sites. It is interesting that [Trp^{11}]-NT which is as active as NT in the rat was found to be 10 times less potent than NT in other species like guinea-pig (8) and man (present study). Consequently, monoiodo[^{125}I-Tyr^{3}, Trp^{11}]-NT which has been successfully used as a labeled ligand for characterizing NT receptor binding sites in murine species (5, 9) would represent a poor ligand of human NT receptors.

The distribution of ^{125}I-NT binding sites in human SN, as visualized by autoradiography, is similar to that previously observed with [^{3}H]-NT in the rat and other species (2, 10, 11). i.e., high density of binding sites in the SN, pars compacta and low density in the pars reticulata.

The present work demonstrates that monoiodo[^{125}I-Tyr^{3}]-NT represents a useful ligand for studying human brain NT receptor sites using radioreceptor and autoradiographic techniques. These techniques will make it possible to map the distribution of NT receptors in human brain and to investigate possible alterations of NT binding in neurologic diseases like Parkinson's disease which are characterized by a degeneration of nigrostriatal dopaminergic neurones.

ACKNOWLEDGMENTS

We wish to thank Miss G. Clénet for secretarial assistance. This work was supported in part by Grant 81E0542 from the Délégation Générale à la Recherche Scientifique et Technique and by the Fondation pour la Recherche Médicale.

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