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LOSS OF HIGH AFFINITY NEUROTENSIN RECEPTORS IN SUBSTANTIA NIGRA FROM PARKINSONIAN SUBJECTS

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Monoiodo(^{125}I-Tyr^{3})-Neurotensin binding was studied in post mortem substantia nigra from 17 control and 15 parkinsonian subjects. Binding to individual homogenates was decreased by 58 %, 49 % and 26 % at 0.36, 1.4, 5.5 M^{-9} concentration of ligand, respectively. Saturation analysis using pooled substantia nigra demonstrated an almost complete loss of the high affinity component of the neurotensin receptor complex, yielding a 24 % loss of the total binding capacity, with no alteration of the low affinity component. Similarly an important loss of binding was observed in monoiodo(^{125}I-Tyr^{3})-Neurotensin autoradiograms of two substantia nigra from parkinsonian subjects. These results support the hypothesis of neurotensin receptors occuring on dopamine cell bodies and/or dendrites in human substantia nigra. Role of neurotensin may be of importance in the regulation of dopamine pathway involved in parkinsonism.

Extensive evidence suggests that the tridecapeptide, neurotensin (NT), acts as a neurotransmitter or a neuromodulator in the central nervous system (1). In rat brain NT is unevenly distributed, and present in fibers, terminals and cell bodies (2). Specific NT binding sites have been characterized by radioreceptor assay (3, 4) and vizualized by autoradiographic techniques (5, 6). The high concentration of NT (2) and of NT receptors (5, 6) in the substantia nigra (SN) and in the ventral tegmental area (VTA) wherefrom

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ABBREVIATIONS : NT, neurotensin ; DA, dopamine ; SN, substantia nigra ; VTA, ventral tegmental area ; PD, Parkinson's disease ; monoiodo(^{125}I-Tyr^{3})-Neurotensin, ^{125}I-NT ; monoiodo(^{125}I-Trp^{11})-neurotensin, ^{125}I-Trp^{11}-NT.

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the nigrostriatal and mesolimbic dopaminergic pathways originate respectively, had raised the possibility that NT may act to modulate dopaminergic neurons. This hypothesis has been particularly investigated in rodents (see (7) for review). In human brain, the distribution of NT like immunoreactivity has been documented (8, 9) and recently (10), we characterized and visualized NT binding to receptor sites in human SN. These studies, using monoiodo (^{125}I-Tyr^{3})-NT (^{125}I-NT) showed that NT interacted, as in other species (11, 12), with two populations of binding sites.

Despite methodological pitfalls, Parkinson's disease (PD) offers a unique model of DA denervation for studying the relationship between dopaminergic and others neurons. Recently, Uhl and al. (13) reported decreased (^{3}H)-NT binding in SN from parkinsonian as compared to control subjects. However, they used a ligand with low specific radioactivity (60 Ci/mmol) and a rather semi-quantitative technique that did not allow them to definitely assess whether the 63 % loss of binding they observed resulted from affinity changes or from receptor loss.

The aim of our study was to compare the interaction of ^{125}I-NT, the only pure labelled ligand with high specific radioactivity (2000 Ci/mmol) and as active as the parent molecule (12), with NT receptor sites in SN homogenates from control and parkinsonian subjects. Moreover, results were confirmed by autoradioagraphic techniques using the same labelled ligand. Particular attention was paid to putative post mortem changes and overall, to determine whether decreased affinity or receptor loss could account for the changes observed.

MATERIAL AND METHODS

Monoiodo(^{125}I-Tyr^{3})-NT : greater than 95 % pure ^{125}I-NT, with specific radioactivity of 2000 and 100 Ci/mmol was used in these binding studies. This radioactive ligand, that has incorporated 1 atom of iodine per Tyr^{3} residue of the NT molecule, was prepared and purified as previously described (10).

Source of human brain tissues : human brain was obtained from 17 control subjects, with no known neurologic or psychiatric disorders and from 15 patients deceased with PD. The diagnostic of PD was based upon clinical and histopathological features along with a 80 % or more, loss of striatal DA content. SN were dissected from frozen brain, washed and stored at -70°C as previously reported (14) and either assayed individually or pooled before the

assay. For individual assays we used 10 control and parkinsonian SN (mean age 79.5 + 2.8 and 76.9 ± 2.1 years ; necropsy delay 10.1 ± 2.3 and 20.5± 5.7 hr, respectively). Pooled SN were prepared with SN from 7 control and 9 parkinsonian subjects (mean age 81.7 ± 1.3 and 74.1 ± 2.2 years ; necropsy delay 13.8 ± 2.8 and 18.5 ± 4.1 hr, respectively). Preterminal conditions were similar in these four groups of subjects. Autoradiographic studies were conducted with SN obtained from 2 control and 2 parkinsonian subjects (age 79 and 89 years ; necropsy delay 7 and 29 hr, and age 80 and 85 years, necropsy delay 24 and 19 hr, respectively).

Source of rat brain tissues : brains were obtained from male Sprague-Dawley rats (200-250 g)(Iffa-Credo, France).

Preparation of tissue homogenates and binding assays : thrice washed homogenates were prepared as previously reported (10), and were used on the day of the preparation for radioreceptor assay. Protein concentration was determined by the method of Hartree (15) with bovine serum albumin (BSA) as a standard. All binding studies were conducted for 20 min at 25°C in 50 mM Tris-HCl containing 0.2 % (w/v) BSA, 1 mM 1-10 phenanthroline, pH : 7.5. Brain homogenates (protein concentration 0.15-0.2 mg/ml) were incubated with ^{125}I-NT and subsequently bound ligand was separated from free using the filtration technique previously reported (11). All the data have been corrected for non specific binding, i.e. the amount of bound radioactivity measured in parallel experiments carried out in the presence of an excess (1 µ M) of unlabelled NT. Under these conditions the non specific binding was proportional to homogenates concentration, between 0.08 and 0.32 mg/ml of protein, and the degradation of the labelled ligand did not exceed 10 %.

Post mortem changes of the specific NT binding sites in rat striatum (St) : these studies were conducted with monoiodo (^{125}I-Trp^{11})-NT (^{125}I-Trp^{11}-NT) prepared as previously reported (11). The St was chosen as a reference area because of its size and of its high concentration of NT receptors in the rat (2). Influence of storage at deep cold on the stability of rat St specific NT binding sites was evaluated in the following way : 2 rats were killed, their brain removed and their St dissected, frozen in liquid nitrogen, and then stored at - 70°C for a week, until the day of homogeneization and radioreceptor assay. On this very day, 2 other rats were killed and their St immediately dissected, homogenized and assayed in a parallel experiment. The binding capacity of these two kinds of homogenates was determined at three different concentrations (0.16, 0.32 and 0.64 nM) of ^{125}I-Trp^{11}-NT (2000 Ci/mmol). Influence of necropsy delay on the stability of rat NT receptors was evaluated as follows : 18 rats were killed and their intact bodies were left at room temperature (20°C). At various time after death (0 to 48 hr) the brain was removed and st dissected and stored at - 70°C. Binding parameters were determined in saturation studies using increasing concentrations of ^{125}I-Trp^{11}-NT (100 Ci/mmol) in a unique set of experiments.

Autoradiographic studies : 32 µm sections of human brain were cut on a cryostat at - 15°C at the level of the SN according to the atlas of Olsewski and Baxter (20). These sections were processed and analyzed as previously described (10).

Analysis of data : curve fitting of curvilinear Scatchard plots was done according to a model depicting interaction of the ligand with two independant populations of binding sites (11). Results are expressed as mean ± standard error of the mean (SEM) ; statistical calculations (Student's t test) were obtained with a programable calculator (Hewlett-Packard, HP41C).

RESULTS

Post mortem changes of the specific NT binding sites in rat St.

(i) influence of storage at deep cold : the amount of ^{125}I-Trp^{11}-NT bound to the homogenates (fmol/mg of protein) at the three ligand concentrations

tested was : 6.41 ± 0.93, 10.28 ± 0.88, 21.07 ± 3.65 and, 7.75 ± 0.88, 12.8 ± 1.73, 19.9 ± 3.64 for the fresh and frozen-thawed St, respectively. These values were not significantly different and suggested that, at least in the rat, storage at deep cold did not alter significantly the binding capacity for NT of brain regions homogenates.

(ii) influence of post mortem delay : dissociation constant (Kd) and maximal binding capacity (Bm) were determined for homogenates prepared from St dissected after a post mortem delay of 0 hr (n = 8), 1 hr (n = 6), 6 hr (n = 6), 16 hr (n = 6), 24 hr (n = 6) and 48 hr (n = 4). Kd and Bm values were not significantly different from 0 to 16 hr. With longer post mortem delays they showed an upward tendency that did not reach the level of statistical significance, may be due to an important variability from one St to another (data not shown). These results, suggested that, at least in the rat, a post mortem delay of 16 hr or less had no detectable effects on binding parameters.

Human studies.

The lack of material (only a few mg of each SN was available) did not allow us to carry out a whole saturation analysis for each patient. Therefore, we determined, in triplicate, specific binding at three concentrations of ^{125}I-Tyr^{11}-NT (100 Ci/mmol) : 0.36, 1.4 and 5.5 nM. The amounts of specifically bound ligand determined at these three concentrations in 10 control and parkinsonian SN homogenates are listed in Table 1. Analysis of these values leads to two conclusions. First, there was, in both groups, a big variability from one SN to another from the same group and this variability could not be correlated to any clinical or post mortem features (age, sex pre-mortem conditions, necropsy delay). Second, there was a significant difference between control and parkinsonian at 0.36 nM ($p < 0.05$) and at 1.4 nM ($p < 0.05$), the difference was not significant at 5.5 nM, may be due to the increasing variability at this high concentration of ligand. Thus, it appeared that the parkinsonian SN had lossed 58 %, 49 % and 26 % of their ability to bind NT at the three concentrations tested, respectively.

Table 1 : ^{125}I-NT binding in individual SN homogenates from control and parkinsonian subjects

CONTROL				PARKINSONIAN			
Subject Number	Ligand 0.36	Concentration 1.4	(nM) 5.5	Subject Number	Ligand 0.36	Concentration 1.4	(nM) 5.5
1	7.6[a]	12.78	34.99	18	8.05	19.55	48.79
2	55.27	101.31	126.02	19	7.02	16.57	47.97
3	10.64	35.27	43.87	20	28.11	54.78	62.17
4	30.85	61.88	90.44	21	1.38	6.72	b
5	21.52	42.29	88.57	22	b	13.72	b
6	11.05	15.23	34.99	23	b	38.99	61.67
7	25.86	39.53	70.44	24	13.49	25.85	38.73
8	25.6	44.97	43.51	25	6.35	10.97	35.16
9	11.19	47.46	68.65	26	9.11	21.18	58.99
10	17.58	23.61	52.4	27	3.57	8.69	32.94
Mean	22.72	42.43	65.39	Mean	9.64*	21.79*	48.30[0]
S.E.M.	4.10	7.68	8.85	S.E.M.	2.74	4.52	4.20

a. Concentration of specifically bound ^{125}I-NT in fmol/mg of protein in the homogenates
b. Unreliable data (anarchic triplicates)
*. $p < 0.05$ as compared with control
0. Non significantly different from control.

Despite these significant results we were unable, at that point, to determine whether this "loss of binding" was either due to an impaired affinity, or to a decreased number of receptor, or both.To further examine this question it was necessary to carry out saturation studies with high and low specific radioactivity labelled ligand as was previously done with control SN (10). Because of the small amount of tissue available for each subject these studies were conducted with homogenates prepared from pools of control and parkinsonian SN. Binding isotherms were determined in three separate experiments and within each experiment, control and parkinsonian SN were analyzed in parallel. Fig. 1 illustrates a Scatchard plot (17) obtained from one representative experiment. In each case Scatchard plots were curvilinear with an upward concavity and were interpreted as resulting from an interaction of the ligand with two populations of binding sites (11, 12). Best fit of the experimental data according to this model yieldied, for each class of sites, estimates of the dissociation constant (Kd), and maximal binding capacity (Bm). Total binding capacity (B_T), the sum of Bm for each class of sites, was derived

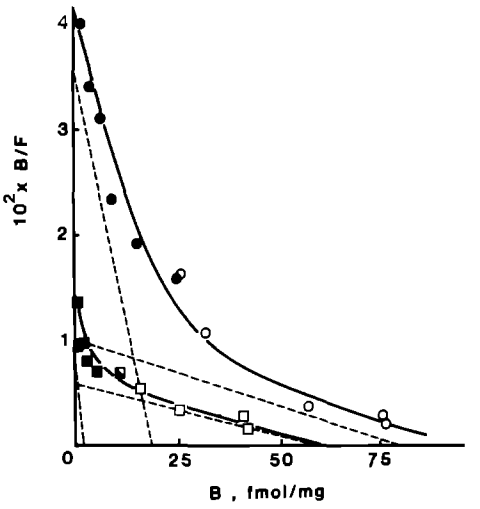

Figure 1. Scatchard plots for the binding of ^{125}I-NT to an aliquot of pooled S.N. homogenates from control (●,○) and parkinsonian subjects (■,□). Homogenates (0.32 mg of protein/ml) were incubated at 20°C for 20 min, with increasing concentrations of ^{125}I-NT at 2000 Ci/mmol (●,■) or 100 Ci/mmol (○,□) and the binding was measured as described in "Material and Methods". Parallel experiments were run in triplicate for each group of patient. The data were fitted by computer according to a model describing the interaction of one ligand with two independant populations of binding sites, as previously described (11). This yielded best estimates of the dissociation constants (Kd) and maximal binding capacities (Bm) for each class of binding sites. B and F are the bound and free concentrations of labelled ligand, respectively. The dashed line represents the contribution to binding of each class of binding sites.

from these values. As can be seen in Table 2, difference between the two groups were significant for K_1 ($p < 0.01$), Bm_1 ($p < 0.01$) and B_T ($p < 0.05$). These results suggested that, in parkinsonian SN, the alteration of NT binding to specific sites could be accounted for by an almost complete loss of high affinity state receptors and consequently at 24 % decrease in total binding capacity with no detectable change of the low affinity state receptors.

Further comparison of NT binding in SN from control and parkinsonian subjects was performed by autoradiography of SN sections that were incubated in the presence of 0.1 nM ^{125}I-NT. In two control SN the autoradiograms showed, as previously reported (10), high levels of NT binding sites in SN, pars compacta (Fig. 2, A and C). In contrast, there was a marked decrease in staining with two parkinsonian SN (Fig. 2, B and D), confirming the data obtained with test tube binding. The degree of disappearance of NT binding sites was much more pronounced for one patient (Fig. 2B) than for the other (Fig. 2D). Moreover,

Table 2 : Thermodynamics parameters for the binding of ^{125}I-NT to pooled homogenates of control and parkinsonian SN (number of experiments in parenthesis)

	High affinity		Low affinity		Total binding capacity
	Kd_1 [a]	Bm_1 [b]	Kd_2 [a]	Bm_2 [b]	B_T [b]
Control (n=3)	0.22 ± 0.04	26.73 ± 4.33	3.68 ± 0.11	61.25 ± 7.54	88.08 ± 0.94
Parkinsonian (n=3)	0.036 ± 0.016*	1.39 ± 0.29*	3.75 ± 0.57	65.74 ± 4.59	67.19 ± 4.38**
% receptor Loss		95 %			24 %

a. association constants are expressed in nM
b. binding capacities are expressed in fmol/mg of protein in the homogenates
*. significantly different from control ($p < 0.01$)
**. significantly different from control ($p < 0.05$)

this binding loss seemed to be related to the degree of depigmentation of these two parkinsonian SN.

DISCUSSION

The major finding of the present studies was the important "loss of binding" in parkinsonian SN either assayed individually or in pools by radioreceptor assay, or observed in individual SN analyzed by autoradiographic technique.

One major difficulty in studying autopsy material is the possibility that alterations observed are the results of post mortem changes. Our data clearly demonstrated that, at least in the rat St, neither freezing, nor time from death to freezing of the brain region had an influence on the stability of NT specific binding sites. In individually assayed control human subjects, we were unable to find significant correlations between age, sex, pre-mortem conditions and post mortem delays ; furthermore, variability from one SN to another was in the same range for control and parkinsonian subjects (see Table 1). For all these reasons, it appears very unlikely that the "loss of binding" observed in parkinsonian SN reflected other factors than the degenerative disease itself.

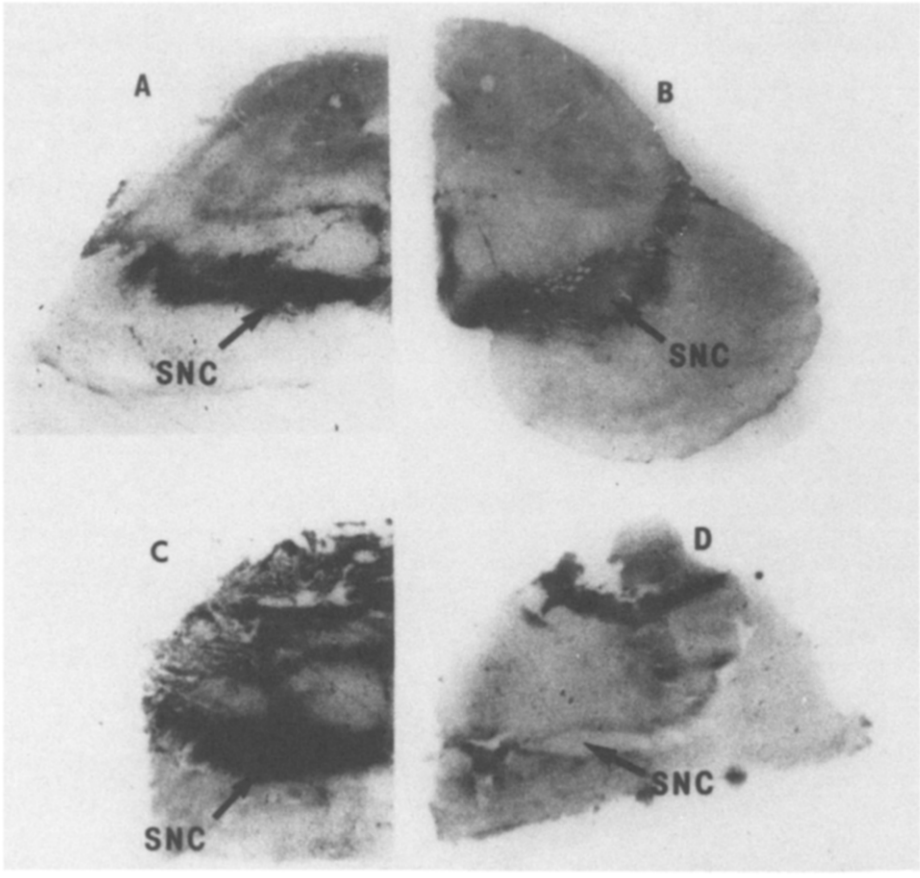

Figure 2 : Autoradiograms of ^{125}I-NT binding to human SN section from 2 control subjects (A and C) and 2 parkinsonian subjects (B and D). The sections were incubated with ^{125}I-NT and processed as described previously (10). SNC : substantia nigra, pars compacta .

A striking result was the gradual decrease in "binding loss" with increasing concentration of ligand in individually assayed SN. Scatchard plot analysis of experiments conducted with homogenates from pooled SN showed that there was a considerable loss of high affinity state receptors (95 %) in parkinsonian SN. Accordingly, from 0.018 nM to 1 nM labelled ligand, the "loss of binding" fluctuated between 63 and 72 % of control subjects. Such values are in good agreement with autoradiographic studies performed at 4 nM (^{3}H)-NT (13) . With higher concentrations of ^{125}I-NT, the difference between control and parkinsonian gradually decreased to reach a minimal of 31 %, a value different from that of 55 % observed at 100 nM by Uhl et al. (13). Interpretation of the loss of high affinity state receptors in parkinsonian SN remains unclear, since

the biochemical and physiological relevance of these two populations of binding sites are unknown despite the fact that they only differ in their association rate constant (11) and that photoaffinity labelling of NT receptor with high and low specific radioactivity photoreactive NT analogs disclosed a unique pattern of specifically labelled protein (18). Whatever the interpretation of Scatchard plots, our data clearly demonstrated an important loss of binding in parkinsonian SN, that was confirmed by autoradiographic techniques (see Fig. 2).

A major question, is whether the alterations observed could be due either to DA cell losses, or to other neurotransmitter neurons or processes loss, or both ? Taking into account the loss of NT receptors in rats whose SN was lesioned (7, 19) and the similar range of binding loss observed with others neurotransmitters in parkinsonian SN (see (20) for review), it appears very likely that, at least, the major portion of the observed receptor changes are accounted for by DA cell losses. Thus, Uhl's report (13) and our present data strongly support the view that in human SN, as in rat SN (7), NT receptors occur on DA cell bodies and/or dendrites.

Whether this NT receptor changes represent a primary dysfunction or secondary changes in response to altered DA function remains to be determined, as well as the role of this NT deficiency in the symptomatology of parkinsonism.

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