[³H]GBR-12935 Binding Sites in Human Striatal Membranes: Binding Characteristics and Changes in Parkinsonians and Schizophrenics

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Abstract—The binding of the diphenyl-substituted piperazine derivative, [³H]GBR-12935, a selective dopamine uptake inhibitor, to the post-mortem human putamen was studied. Inhibition curves by dopamine uptake inhibitors suggested the existence of two populations of [³H]GBR-12935 binding sites: one is potently inhibited by mazindol and/or nomifensine, and the second binding site is benztropine- and/or GBR 12909-sensitive. In the human putamen, [³H]GBR-12935 labeled both these two distinct binding sites. The [³H]GBR-12935 binding displaced by mazindol was enriched in the mouse and rat striatum, but not in the cultured mouse neuroblastoma cell N1E-115. The mazindol-sensitive [³H]GBR-12935 binding site increased in the presence of sodium and markedly decreased in the putamen from parkinsonians (45% of controls). On the other hand, the [³H]-GBR-12935 binding displaced by benztropine showed no sodium-dependent increase and was not decreased in the putamen from parkinsonians. In the putamen from schizophrenics, the [³H]GBR-12935 binding did not significantly change in the density, while that displaced by mazindol tended to increase. It is concluded that in the human putamen, [³H]GBR-12935 binds to two distinct sites. One site is partially sodium-dependent and appears to be associated with a high-affinity dopamine uptake system on dopaminergic nerve terminals. The second binding site shows no sodium-dependency and may be associated with a nondopaminergic and/or extraneuronal DA uptake system.

The principle mechanism for termination of the actions of synaptically released monoamines such as dopamine (DA), norepinephrine (NE) and serotonin has been considered to be their uptake into the presynaptic terminal. Selective transport mechanisms exist for each amine (1), but the elucidation of the DA uptake mechanisms has been limited because of the absence of selective ligands for labeling the DA transport complex. Recent studies using radiolabeled DA uptake

blockers have described specific and saturable binding sites associated with the neuronal DA uptake system in the striatal membranes (2-7). Most of all these ligands have a poor selectivity for the DA transport complex compared with the norepinephrine (NE) uptake system. A series of diphenyl-substituted piperazine derivatives (8), 1-[2-(2-(diphenylmethoxy)ethyl)-4-(3-phenylpropyl)-piperazine (GBR 12935) and GBR 12783, were introduced as ligands for labeling the DA transport complex with high selectivity and have provided a new tool for

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the study of DA transport system (9–11).

Post-mortem human brains can provide useful information concerning human neuropharmacology and pathology. Especially, the brains of patients with Parkinson's disease (PD) have been chosen as a 'natural' nigrostriatal lesion model for studying dopaminergic transmission. The present study characterized the GBR-12935 binding sites in the post-mortem human putamen, as compared with those in the mouse and rat striatum, and the cultured mouse neuroblastoma N1E-115 cells which take up DA (12). We found that in the human putamen, two distinct GBR-12935 binding sites existed and showed different sodium-sensitivity. One is partially sodium-dependent and decreased in the putamen from parkinsonians, which is related to the neuronal DA uptake. The second binding site, which also exists in the cultured cell N1E-115, shows no sodium-dependency and no alteration in the putamen from parkinsonians. Furthermore, we studied the alteration of the binding levels in the putamen from schizophrenics in which abnormalities of the central dopaminergic transmission have been indicated.

Materials and Methods

Chemicals: [^3H]GBR-12935 (38.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Mazindol was supplied by Sandoz (Tokyo, Japan); nomifensine by Hoechst-Japan (Kawagoe, Japan), benztropine by Nihon Merck-Banyu (Tokyo, Japan) and GBR 12909 by Research Biochemicals Inc. (U.S.A.).

Tissue preparation: Brains were obtained at autopsy from 8 patients with idiopathic Parkinson's disease (PD) (6 men, 2 women, age 71.9 ± 2.3 years), 15 patients with schizophrenia (10 men, 5 women, age 65.2 ± 3.8 years) and 20 control subjects with no known psycho-neurological diseases (16 men, 4 women, age 60.6 ± 3.6 years). The time between death and autopsy was 4.8 ± 0.5 hr for patients with PD, 13.3 ± 2.5 hr for those with schizophrenia and 8.6 ± 1.6 hr for the controls. The diagnostic criteria for idiopathic PD included the clinical record (stage V according to Hoehn and Yahr (13)) and histopathological evidence of depletion of

pigmented nigral cells and the presence of Lewy bodies. The diagnosis for schizophrenia was based on DSM-III categories. All brain dissection was performed as described (14). In some cases, for statistical analysis, groups have been matched for age and post-mortem delay.

Cell culture: Mouse neuroblastoma clone N1E-115 cells were grown in Dulbecco's modified eagle medium (GIBCO) supplemented with 10% newborn calf serum. The cells were incubated at 37°C in an atmosphere consisting of 10% CO_2 and 90% humidified air. The medium was changed at 2 day intervals. Cells were grown to confluency prior to use.

Membrane preparation: The striatum from mouse and rat and the putamen from human brain were homogenized in 20 vol. of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) with a Brinkmann Polytron (setting at 7 for 15 sec). The homogenates were then centrifuged (for 20 min at $38,000 \times g$) three times with resuspension of the intermediate pellets in fresh buffer. Cultured cells were harvested mechanically with a rubber policeman, pelleted at $600 \times g$ for 10 min, in the above buffer, homogenated and centrifuged as described above. The final pellets were stored at -80°C until assayed.

Binding assay of [^3H]GBR-12935: Each incubation mixture consisted of 2 ml of buffer (50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl and 0.01% bovine serum albumin) containing 0.1–0.3 mg of membrane proteins, 0.5 nM or 1 nM concentrations of [^3H]GBR-12935, and when indicated, various other drugs. The mixtures were incubated for 45 min at 25°C , and the binding process was terminated by rapid filtration under vacuum through a Whatman GF/B filter. Each of the filters was presoaked in 0.3% polyethyleneimine for at least 60 min before use to reduce the nonspecific absorption of free radioligand to glass filters. Nonspecific binding was defined as the binding in the presence of 5 μM mazindol or 20 μM benztropine. Radioactivity on the filters was counted in a liquid scintillation spectrometer at 45% efficiency. Protein assay was performed by the method of Lowry et al. (15).

Student's *t*-test (two-tailed) was used for statistical analysis.

Results

Drug inhibition of [³H]GBR-12935 binding: Inhibition curves of [³H]GBR-12935 (1 nM) binding by four potent DA uptake inhibitors are shown in Fig. 1. In the mouse and rat striatum, the rank order of potencies was: GBR 12909 > mazindol > nomifensine > bntropine. In the human putamen, the binding patterns of mazindol and nomifensine were different from those in the mouse and rat striatum, i.e., only 40–50% of the total [³H]GBR-12935 binding was inhibited at maximal concentration (0.1 mM) of mazindol and nomifensine, whereas these drugs inhibited the binding more effectively than

bntropine at low concentration (1–10 nM). In contrast, GBR12909 and bntropine inhibited [³H]GBR-12935 binding with similar potencies in all tissues. The mazindol- and/or nomifensine-insensitive site was displaced by GBR 12909 and bntropine, and was enriched in the human putamen. This binding site also existed in the cultured cell N1E-115 derived from mouse neuroblastoma which possesses a DA uptake system (12).

Sodium dependency of [³H]GBR binding in human putamen: Previous reports have shown that in the rat, [³H]GBR-12935 binding associated with a DA uptake system is dependent upon sodium, whereas that in some brain regions and tissues was inhibited by sodium (11). The effect of sodium on [³H]GBR-12935 binding in the human putamen is shown in Fig. 2. When the [³H]-GBR-12935 (1 nM) binding displaced by 5 μ M mazindol was measured as a function of NaCl concentration, sodium-dependent increase of the binding was observed; the mazindol-sensitive specific binding was increased by about 30% at the sodium concentration of 150 mM. This sodium-dependency of the binding in the human putamen was not so marked as that of rat striatum (11). In contrast, the specific bind-

Fig. 1. Inhibition of [³H]GBR-12935 binding (1 nM) to the mouse striatal (A), rat striatal (B), human putamen (C) and N1E-115 cell membranes (D) by mazindol (□), nomifensine (●), bntropine (■) and GBR-12909 (△). Data are expressed as a percent of the total binding measured in the absence of each drug (mouse striatum, 3.1 pmol/mg protein; rat striatum, 3.5 pmol/mg protein; human putamen, 1.2 pmol/mg protein; N1E-115 cells, 2.1 pmol/mg protein), and represent the mean of at least five experiments performed in triplicate, in which similar results were obtained.

Fig. 2. Effect of NaCl on the specific binding of [³H]GBR-12935 (1 nM) in the human putamen. Specific [³H]GBR-12935 binding was measured in the presence of increasing concentrations of NaCl at a ligand concentration of 1 nM. Nonspecific binding was determined with 20 μ M bntropine (●) or 5 μ M mazindol (□). Data are expressed as a percent of the specific binding measured in the absence of NaCl (bntropine-displacable site, 920 fmol/mg protein; mazindol-displacable site, 210 fmol/mg protein) and represent the mean of at least three experiments performed in triplicate, in which similar results were obtained.

Fig. 3. Density of specific [^3H]GBR-12935 binding sites in the putamen from controls ($\square$), parkinsonians ($\blacksquare$) and schizophrenics ($\bullet$). Specific [^3H]GBR-12935 binding was estimated at a ligand concentration of 0.5 nM. Nonspecific binding was determined with 5 μM mazindol (A) or 20 μM benztropine (B). Each point represents an individual subject. The horizontal bars represent the mean (fmol/mg protein). Mean values $\pm$ S.E.M. (fmol/mg protein) are as follows: the 5 μM mazindol-displacable site: controls, 90.4 ± 8.9 ($n=15$); parkinsonians, 40.3 ± 8.1 ($n=6$), * $P<0.005$; schizophrenics, 98.7 ± 5.5 ($n=15$); the 20 μM benztropine-displacable site: controls, 206.4 ± 14.2 ($n=15$); parkinsonians, 230.1 ± 26.4 ($n=6$); schizophrenics, 212.3 ± 13.6 ($n=10$).

ing of [^3H]GBR-12935 to the human putamen was inhibited by increasing concentrations of sodium when 20 μM benztropine was used for determining the nonspecific binding. The specific [^3H]GBR-12935 binding displaced by 20 μM benztropine also existed in N1E-115 cell membranes and was inhibited by increasing concentrations of sodium (data not shown).

The [^3H]GBR-12935 binding in the putamen from patients with Parkinson's disease and schizophrenia: In the putamen from parkinsonians, the specific [^3H]GBR-12935 (0.5 nM) binding displaced by 5 μM mazindol was significantly lower (about 45% of control) than that in the putamen from controls ($P<0.005$) (Fig. 3). The binding displaced by 5 μM mazindol in the putamen from schizophrenics tended to increase, although the difference between the means was not statistically significant. When 20 μM benztropine was employed to determine nonspecific binding, no significant difference was seen between the controls, parkinsonians and schizophrenics. In schizophrenics, no significant changes of the binding levels was

observed regardless of whether or not the patients had been on neuroleptics. The possible influences on the binding levels by age, the time from death to autopsy and the preservation time were neglected since no correlation was observed between the binding levels and these factors (data not shown). Furthermore, no significant changes in binding were found when the subjects were well matched for age, the time from death to autopsy and the preservation time.

Discussion

The [^3H]GBR-12935 binding to the post-mortem human putamen was shown to have some distinct properties from that to the mouse and rat striatum. Mazindol and nomifensine were potent inhibitors of the [^3H]GBR-12935 binding in the striatal membranes of mouse and rat, in agreement with the previous report (11). In the human putamen the inhibition of [^3H]GBR-12935 binding by mazindol and nomifensine was not as potent as in the mouse and rat striatum. The inhibition was 40–50% of the total binding at the maximal concentrations of

these drugs. In contrast, GBR 12909 and benztropine inhibited [³H]GBR-12935 binding with similar potencies in all tissues. Furthermore, the Scatchard plots of the benztropine-sensitive binding in the human striatal membranes were curvilinear, indicating the multiple binding sites (data not shown). Thus, it seems that at least two populations of [³H]GBR-12935 binding sites exist in the human striatal membranes: one is mazindol- and/or nomifensine-sensitive, and the second is benztropine- and/or GBR 12909-sensitive. Recently, Andersen reported that in the rat striatal membranes, [³H]GBR-12935 labeled the classical DA uptake site and a piperazine acceptor site, although Scatchard analysis indicated a single binding site (16). When 5 μ M mazindol was used to estimate non-specific binding, Janowsky et al. (17) obtained a K_d of 2.34 nM and a B_{max} of 1.36 pmol/mg protein in the human caudate nucleus, while they reported a K_d of $\sim$ 1 nM and a B_{max} of $\sim$ 5 pmol/mg protein in the rat striatal membranes (11). Bonnet et al. (10), using [³H]GBR 12783, obtained a K_d of 1.6 nM and a B_{max} of 10.3 pmol/mg protein in the rat striatal membranes. Thus, the B_{max} value of the mazindol-sensitive [³H]GBR-12935 binding site appeared to be lower in the human striatum than in the rat striatum, with a K_d value similar to that of the rat striatum.

We found that the two distinct [³H]GBR-12935 binding sites in the human putamen showed different sodium-sensitivity. It is generally accepted that the high-affinity DA uptake is sodium-dependent (1). Since the mazindol-sensitive [³H]GBR-12935 binding site showed a sodium-dependent increase, this site appears to be related to the high-affinity DA transport system. The reduction of the mazindol-sensitive [³H]GBR-12935 binding in the PD putamen and in the rat striatum lesioned with 6-hydroxydopamine supports this hypothesis (11, 17).

The degree of decreased bindings in the PD putamen agrees well with the recent report by Janowsky et al. (17). We obtained, however, rather lower binding levels as compared with those reported by Janowsky et al. (17), probably owing to the difference in membrane preparations. There is a noteworthy

concept that it is necessary to lose 80–85% of the nigral neurons and to deplete the striatum of at least 80% of its dopamine content for frank symptoms of PD to appear (18). It, therefore, appears that the degree of observed reductions in mazindol-sensitive [³H]GBR-12935 bindings is lower than that of neuronal loss. This discrepancy between the degrees of neuronal loss and the reduction of the mazindol-sensitive [³H]GBR-12935 binding may be attributable to the compensation mechanism. Alternatively, the [³H]GBR-12935 appears to label sites unrelated to the neuronal high-affinity DA uptake system, as discussed below.

The [³H]GBR-12935 binding to the human putamen using benztropine as a displacer showed no sodium-dependent increase. This sodium-independency of [³H]GBR-12935 binding to the human putamen appears not to be artifacts due to the post-mortem delay and tissue handling, since the sodium-dependency of [³H]GBR-12935 binding to the rat striatum was not altered after storage for at least up to 10 hr at 25°C (O. Shirakawa et al., unpublished data). Such a sodium-independent and benztropine-sensitive [³H]GBR-12935 binding site also exists in the cultured cells N1E-115. N1E-115 cells have a DA uptake system, yet its affinity for DA is very low ($K_m = \sim$ 10 μ M), with temperature dependency and partial sodium dependency (O. Shirakawa et al., unpublished data). In addition, mazindol and nomifensine had little effect on the [³H]GBR-12935 binding in N1E-115. Since the benztropine-sensitive [³H]GBR-12935 binding site did not decrease in the PD putamen in which the dopaminergic nigrostriatal pathway is degenerated, this binding site may be related to extraneuronal components such as the DA uptake site in glial cells. It was reported that monoamines such as DA was taken up by not only neurons but also astrocytes (19). Thus, the heterogeneity of [³H]GBR-12935 binding sites in the human putamen may reflect different compartments of the DA uptake system in the brain. We cannot, however, exclude the possibility that the differences in the binding reflects the species differences in the characteristics of the DA transporter system *per se*.

In the present study, we could not find

apparent abnormalities of [^3H]GBR-12935 binding in the putamen from schizophrenics. Although it has been widely accepted that there are abnormalities of the dopaminergic transmission in schizophrenia, our results suggest that disturbances in the DA uptake system, if any, are not primarily associated with schizophrenic etiology and symptomatology.

In conclusion, characteristics of [^3H]GBR-12935 binding sites showed species differences, and there are two distinct [^3H]GBR-12935 binding sites in the human putamen: one is partially sodium-dependent and potentially displaced by mazindol and nomifensine, and the second binding site is inhibited by sodium and benztropine- and/or is GBR 12909-sensitive. The former is associated with the high-affinity DA uptake system on the dopaminergic nerve terminals, and the latter may be related mainly to non-dopaminergic and/or extraneuronal components.

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