BINDING EXPERIMENTS OF MUSCARINIC ACETYLCHOLINE AND DOPAMINE RECEPTORS IN HUMAN BRAINS WITH EMPHASIS ON A CASE OF STRIATONIGRAL DEGENERATION

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Abstract—In the regional distribution of \( ^{3} \text{H}-\text{quinuclidinyl benzilate} \) \((^{3} \text{H-QNB})\) binding in human brains of neurologically unaffected cases, it was highest in the caudate nucleus which was followed by the putamen, amygdala, cerebral corteces and olfactory bulb and lowest in the substantia nigra. As to the regional distribution of \( ^{3} \text{H-spiroperidol} \) binding in human control brains, it was highest in the caudate nucleus and was followed by the cerebral corteces, amygdala and lowest in the cerebellar cortex. In a case of striatonigral degeneration \( \text{(SND)} \), \( ^{3} \text{H-QNB} \) binding in the putamen and thalamus was lowered and \( ^{3} \text{H-spiroperidol} \) binding was decreased in the putamen, frontal and parietal corteces, Ammon’s horn, amygdala and substantia nigra as compared to human brains of control cases. These results were noteworthy since no pathological changes were observed in the thalamus, cerebral corteces, amygdala and Ammon’s horn in this case. The \( ^{3} \text{H-spiroperidol} \) binding was increased by injection of 6-hydroxy-dopamine \( \text{(6-OHDA)} \) into the substantia nigra of rat brains by 17% as compared to the contralateral intact side. Conversely, \( ^{3} \text{H-spiroperidol} \) binding was decreased by injection of kainic acid \( \text{(KA)} \) into the striatum of rat brains by 43% as compared to the contralateral intact side. This meant that dopamine receptors labelled by \( ^{3} \text{H-spiroperidol} \) were at least partially localized at the postsynaptic site of the nigrostriatal dopamine neuron.

Recently, biochemical studies of the neurotransmitter receptor have progressed rapidly through use of radioreceptor assay techniques. Such basic studies have been applied mainly to the neuropsychopharmacological fields \((1, 2)\) and contributed much to the elucidation of underlying pathophysiology and therapy of extrapyramidal diseases such as Parkinson disease \((3, 4)\) and Huntington chorea \((5–7)\). We measured muscarinic cholinergic \((\text{mACh})\) and dopamine \((\text{DA})\) receptor binding capacities in various regions of the rat brain and the human autopsied brain and made a comparative investigation between neurologically unaffected cases and a case of \( \text{SND} \). \( \text{SND} \) is clinically characterized by Parkinsonism with neuropathological changes occurring in the putamen and substantia nigra where there is neuronal loss with reactive gliosis. Striatal membranes prepared from rats after 6-OHDA and KA local injection are considered to be adequate materials for studying the properties of pre- and postsynaptic DA receptors.
respectively. Through these experiments, we made studies on the underlying pathogenesis of extrapyramidal diseases from the standpoint of neurotransmitter receptors.

MATERIALS AND METHODS

Human materials: Postmortem human brains including 11 neurologically unaffected cases and one case of SND were collected. The brains were fractionatedly dissected immediately after removal and the samples were kept frozen at −20°C until analysis.

Animals: Wistar strain rats (180–200 g) were decapitated and their brains were rapidly removed.

KA and 6-OHDA lesions in the striatum and nigro-striatal pathway: Some of the rats were injected unilaterally with 2 μl of a solution containing 2 μg of KA into the right striatum before the binding experiments. Coordinates were A 7.8, L 2.5, V 5.5, according to the atlas of König and Klippel (8).

In addition, the ascending DA pathway was degenerated unilaterally by injection of 8 μg of 6-OHDA in 2 μl saline containing 0.1% ascorbic acid into the right substantia nigra. Coordinates were A 2.4, L 2.0, V 8.0 (8).

Tissue preparation for 3H-QNB binding: The materials were homogenized in 0.05M Na-K phosphate buffer (pH 7.4) using the Polytron PT-10 (setting 7 for 30 sec).

3H-QNB binding assay: Aliquots of the tissue preparations (0.3 mg protein) were incubated in 10 ml of phosphate buffer (pH 7.4) containing 10.4 Ci/m mole of 3H-QNB (New England Nuclear) with or without 1 μM atropine. After 60 min incubation at 25°C, the incubation mixture was filtered rapidly under vacuum through glass fiber filters (Whatman GF/B) and washed 3 times with 5 ml of ice-cold phosphate buffer. The filters were placed in vials containing 8 ml of Triton-toluene phosphor liquid scintillation cocktail. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry.

Specific 3H-QNB binding was defined as the total binding minus the binding in the presence of 1 μM atropine.

Tissue preparation for 3H-spiroperidol binding: The materials were homogenized in ice-cold 15 mM Tris-HCl buffer (pH 7.4, 5 mM Na2-EDTA, 1.1 mM ascorbic acid) using a Teflon glass homogenizer (500 rpm, 15 up-down strokes for 30 sec). These homogenates were incubated at 37°C for 1 hr and stored frozen at −20°C. Before use, the crude homogenates were thawed and centrifuged (15 min at 40,000×g) at 4°C. The homogenates were homogenized further using the Polytron PT-10 (setting 7 for 20 sec).

3H-spiroperidol binding assay: The specific binding of 3H-spiroperidol was assayed in glass test tubes in which the aliquots of the following were placed: 0.8 ml Tris-HCl buffer with or without non-radioactive spiroperidol (final concentration 1 μM), 0.2 ml of 3H-spiroperidol, 35.9 Ci/m mole (New England Nuclear) and 0.2 ml of tissue homogenates (0.3 mg protein). After incubation for 30 min at 22°C, an aliquot was removed and filtered through a Whatman GF/B filter. Radioactivity was measured by liquid scintillation spectrometry in 8 ml of Formula 947 cocktail (New England Nuclear). Specific binding of 3H-spiroperidol was defined as the total amount bound minus that bound in the presence of 1 μM non-radioactive spiroperidol. All samples were assayed in triplicate. Proteins were determined by the method of Lowry et al. (9).

Drugs: The drugs used were atropine, pilocarpine (Wako Pure Chemicals), (+)-butaclamol, (−)-butaclamol (Ayerst Laboratory), chlorpromazine (Shionogi), dopamine (Nakarai Chemicals), fluphenazine, trifluoperazine (Yoshitomi), haloperidol (Dainippon), methysergide, thioridazine (Sandoz),...
6-OHDA, KA (Sigma Chemicals), pimozide (Fujisawa), QNB, trifluperidol (Roche), serotonin (Merck) and spiroperidol (Janssen Pharmaceutical).

RESULTS

Saturation of specific $^3$H-QNB binding in the rat striatum and human caudate: A single binding site was observed in the Scatchard analysis of the saturation isotherms of specific $^3$H-QNB binding in both rat striatum and human caudate nucleus membranes. The $K_D$ value of the rat striatum membranes was 0.205 nM, and the Bmax was 1600 f mol/mg protein. The $K_D$ value of human caudate nucleus membranes was 0.206 nM and Bmax was 1200 f mol/mg protein (Fig. 1).

Association and dissociation of $^3$H-QNB binding in the rat striatum: The time course of specific $^3$H-QNB binding in the rat striatum is shown by Fig. 2. Specific $^3$H-QNB binding to the rat striatum membrane plateaued by 90 min (Fig. 2). The rate constant of association ($K_1$) was 0.0632/nM/min.

The rate of dissociation of the $^3$H-QNB-receptor complex was studied by labelling striatum preparations with $^3$H-QNB at 25°C and then measuring the decline of bound $^3$H-QNB after further incubation at 25°C with 1 μM atropine. The half-life for dissociation at 25°C was about 90 min. The rate constant for dissociation at 25°C ($K_2$) was 0.0077/min. The $K_D$ value determined by the ratio of $K_2$ to $K_1$ was 0.122 nM which was a little lower than the $K_D$ value determined in Scatchard experiments.

Inhibition of $^3$H-QNB binding to the muscarinic receptor in the rat striatum by muscarinic agonists and antagonists: Muscarinic antagonists effectively inhibited $^3$H-QNB binding in rat striatum membranes. QNB and atropine inhibited specific $^3$H-QNB binding by 50% at 0.5–0.9 nM. Pilocarpine, an agonist, was approximately 6,000–10,000 fold less potent as an inhibitor of $^3$H-QNB binding than those antanogists were (Fig. 3).

Regional distribution of specific $^3$H-QNB binding in the rat brain: Specific $^3$H-QNB bindings were measured in 14 discrete regions of 3 rat brains. The examinations revealed the highest level of specific $^3$H-QNB binding in the striatum followed by the olfactory tubercle, motor cortex, sensory cortex, frontal cortex and hippocampus, and the lowest in the cerebellum (Table 1).

Regional distribution of specific $^3$H-QNB
Fig. 2. Association and dissociation of specific $^3$H-QNB binding. For the measurement of association, membranes of rat striatum were incubated at 25°C with 0.25 nM $^3$H-QNB for increasing periods before filtration (A). To determine dissociation, specifically bound $^3$H-QNB was measured at increasing periods after addition of 0.1 $\mu$M atropine to tubes already incubated for 90 min at 25°C as in the association experiments (B). Results are the means of 3 experiments.

Table 1. Specific $^3$H-QNB binding in various regions of human brain

| Region of brain                | Specific $^3$H-QNB binding (fmole/mg protein) ± S.E.M. |
|--------------------------------|-------------------------------------------------------|
| Striatum                       | 1064 ± 105                                            |
| Olfactory tubercle              | 929 ± 18                                              |
| Motor cortex                   | 920 ± 20                                              |
| Sensory cortex                 | 904 ± 13                                              |
| Visual cortex                  | 891 ± 16                                              |
| Frontal cortex                 | 875 ± 16                                              |
| Auditory cortex                | 833 ± 12                                              |
| Hippocampus                    | 768 ± 21                                              |
| Olfactory bulb                 | 632 ± 16                                              |
| Septal area                    | 543 ± 41                                              |
| Midbrain                       | 342 ± 17                                              |
| Pons & Medulla oblongata       | 273 ± 6                                               |
| Hypothalamus                   | 269 ± 27                                              |
| Cerebellum                     | 95 ± 3                                                |

Specific $^3$H-QNB binding values are given in fmole/mg protein and are the means of 3 samples ± S.E.M. In specific $^3$H-QNB binding assay, aliquots of the tissue preparation containing 0.3 mg protein were incubated in 10 ml of 0.05 M Na-K phosphate buffer containing 0.25 nM $^3$H-QNB with or without 1 $\mu$M atropine.

Saturation of specific $^3$H-spiroperidol binding in the rat striatum and human caudate: When the range of $^3$H-spiroperidol concentrations was 0.03 to 1 nM, a single binding site was revealed in Scatchard analysis of the saturation data of specific $^3$H-
spiroperidol binding in both rat striatum and human caudate nucleus membranes. The $K_D$ value of the rat striatum membranes was 0.45 nM, and $B_{max}$ was 365 fmole/mg protein.

As for the human caudate nucleus mem-

| Region of brain                  | Neurologically indifferent case (n=4) | SND |
|----------------------------------|--------------------------------------|-----|
| Caudate nucleus                  | 609± 69                              | 500 |
| Putamen                          | 488±155                              | 78  |
| Amygdaloid nucleus               | 446±109                              | 443 |
| Cerebral cortex (frontal lobe)   | 291±136                              | 454 |
| Olfactory bulb                   | 243±141                              | 276 |
| Cerebral cortex (parietal lobe)  | 229±74                               | 301 |
| Ammon's horn                     | 172±63                               | 179 |
| Thalamus                         | 145±30                               | 64  |
| Pons                             | 120±29                               | 71  |
| Pallidum                         | 73±44                                | 46  |
| Cerebellar cortex                | 27±11                                | 23  |
| Cerebral white matter            | 27±8                                 | 44  |
| Substantia nigra                 | 26±12                                | 81  |

Specific $^3$H-QNB binding was assayed as described in Table 1.

Fig. 4. Saturation of specific $^3$H-spiroperidol binding. Increasing concentrations of $^3$H-spiroperidol (0.03–1 nM) were incubated with membranes of rat striatum (A) and human caudate (B). Specific binding was defined as the total binding minus binding in the presence of 1 nM non-radioactive spiroperidol. Results are the means of 3 experiments. Ordinate: specifically bound $^3$H-spiroperidol (fmol/mg protein). The inset gives a Scatchard plot of the same data.
branes, the $K_D$ value was $0.32 \text{ nM}$, and $B_{\text{max}}$ was $265 \text{ fmol/mg protein}$ (Fig. 4).

Association and dissociation of $^{3}\text{H}$-spiropiperidol binding in the rat striatum: The time course of specific $^{3}\text{H}$-spiropiperidol binding in the rat striatum is shown by Fig. 5. The association of $^{3}\text{H}$-spiropiperidol binding to rat striatum membranes reached an equilibrium in 28 min at 22°C. The association rate constant was $0.188/\text{nM/min}$. The dissociation of $^{3}\text{H}$-spiropiperidol in the rat striatum was observed by addition of $1 \mu\text{M}$ non-radioactive spiropiperidol. The half-life for dissociation at 22°C was 9 min.

The $K_D$ value determined by the ratio of the dissociation constant to the association constant was $0.4 \text{ nM}$ which agreed with the $K_D$ value determined in the saturation experiment (Fig. 5).

Inhibition of $^{3}\text{H}$-spiropiperidol binding of various chemical agents in the rat striatum and frontal cortex membranes: Butyrophenones and other neuroleptics were the most potent inhibitors in the striatum. Serotonergic drugs such as serotonin and methysergide inhibited $^{3}\text{H}$-spiropiperidol binding 11–14 times more potently in the frontal cortex than in the striatum (Table 3).

Regional distribution of specific $^{3}\text{H}$-spiropiperidol binding in the human brains: Binding in the human brains of neurologically unaffected cases revealed the highest value in the caudate, cerebral cortices, amygdala and olfactory bulb, and the lowest value was in the cerebellar cortex. In the SND case, $^{3}\text{H}$-spiropiperidol binding was decreased in the putamen, frontal and parietal cortices, Ammon's horn, amygdala and substantia nigra (Table 4).

Effect of KA lesion on striatal DA receptors: After microinjection of KA into the striatum, $^{3}\text{H}$-spiropiperidol binding densities were decreased by 43% on the 20th day as compared to the contralateral intact side, while the $K_D$ value remained unchanged (Table 5).

Effect of 6-OHDA lesion on striatal DA receptors: $^{3}\text{H}$-spiropiperidol binding was increased by injection of 6-OHDA into the
Table 3. Drug competition for $^3$H-spiroperidol binding to rat striatum and frontal cortex membranes

| Drugs            | Striatum IC50 (nM) | Frontal cortex IC50 (nM) |
|------------------|--------------------|-------------------------|
| (Neuroleptics)   |                    |                         |
| Spiroperidol     | 0.3                | 1                       |
| Haloperidol      | 15                 | 150                     |
| (+)—Butaclamol   | 6                  | 25                      |
| (—)—Butaclamol   | 13,000             | 52,000                  |
| Pimozide         | 110                | 230                     |
| Trifluoperidol   | 35                 | 105                     |
| Chlorpromazine   | 100                | 750                     |
| Thioridazine     | 350                | 850                     |
| Fluphenazine     | 40                 | 60                      |
| Trifluoperazine  | 230                | 820                     |
| (Others)         |                    |                         |
| Dopamine         | 14,000             | 112,000                 |
| Serotonin        | 25,000             | 2,200                   |
| Methysergide     | 2,500              | 175                     |

IC50 values (drug concentrations which inhibited specific binding of $^3$H-spiroperidol by 50%) were obtained from inhibition experiment on at least 3 separate brains. Specific binding of 0.5 nM $^3$H-spiroperidol was defined as that inhibited by 1 μM non-radioactive spiroperidol.

Table 4. Specific $^3$H-spiroperidol binding in various regions of human brain

| Region           | Neurologically indifferent case | n   | SND |
|------------------|--------------------------------|-----|-----|
| Caudate nucleus  | 96±10                          | 3   | 95  |
| Frontal cortex   | 91±20                          | 7   | 60  |
| Parietal cortex  | 90±17                          | 7   | 51  |
| Amygdaloid nucleus | 87±4                          | 4   | 70  |
| Olfactory bulb   | 67±10                          | 5   |     |
| Putamen          | 63±20                          | 8   | 31  |
| Ammon’s horn     | 49±14                          | 5   | 16  |
| Substantia nigra | 46±8                           | 4   | 25  |
| Pallidum         | 45±19                          | 6   | 46  |
| Thalamus         | 45±19                          | 7   | 30  |
| Pons             | 33±4                           | 3   | 31  |
| Olivary nucleus  | 27±3                           | 3   |     |
| Cerebellar cortex| 11±2                           | 5   | 8   |

Specific $^3$H-spiroperidol binding values are given in f mole/mg protein and are the mean ±S.E.M. In specific $^3$H-spiroperidol binding assay, aliquots of the tissue preparation containing 0.3 mg protein were incubated in 1 ml of 0.015 M Tris-HCl buffer containing 0.5 nM $^3$H-spiroperidol with or without 1 μM non-radioactive spiroperidol.

substantia nigra by 17% on the 20th day as compared to the contralateral intact side, while the $K_D$ value remained unchanged (Table 6).

DISCUSSION

It has been reported that $^3$H-QNB is a potent mAch antagonist and binds specifically
to mAch receptors in brain homogenates (10). In this study, we confirmed the biochemical characteristics of \(^3\)H-QNB binding to mAch receptors and also investigated the regional distribution of \(^3\)H-QNB binding in rat and human brains. High binding sites were noticed in the striatum, cerebral cortex and limbic areas such as the hippocampus, amygdala, olfactory bulb and olfactory tubercle both in rat and human brains; and their tendencies were much the same. This result was nearly consistent with the hitherto reported regional distribution of \(^3\)H-QNB binding in rat (11), monkey (12) and human brains (6, 7). Recently, multiplicity of DA receptors has been proposed by several authors. Cools and Van Rossum suggested the classification of DA receptors into two pharmacological distinct types: the excitation-mediating (DAe) receptors and the inhibition-mediating (DAi) receptors (13). Carlsson provided an idea for the presynaptic DA receptor and called it an “autoreceptor” (14). Kebabian and Calne suggested that DA receptors might be divided by biochemical and pharmacological criteria into two classes: \(D_1\) receptors which were linked to DA sensitive adenylate cyclase and \(D_2\) receptors which were not linked (15). List and his colleagues also recently reported on a high affinity binding site for \(^3\)H-DA which they termed a \(D_3\) receptor (16).

DA receptors have been measured using various DA antagonists and agonists. \(^3\)H-haloperidol (17), \(^3\)H-spiroperidol (18) and \(^3\)H-domperidone (19) are antagonist ligands of DA receptors; and \(^3\)H-DA (20), \(^3\)H-apomorphine (21), \(^3\)H-N-n-propylnorapomorphine (22) and \(^3\)H-2-amino-6,7-dihydroxytetralin (\(^3\)H-ADTN) (23) are agonist ligands of DA receptors.

In \(^3\)H-spiroperidol binding in the striatum membrane of the rat whose postsynaptic nerve cell body was destroyed by intrastriatal injection of KA, the binding sites were markedly decreased. In \(^3\)H-spiroperidol binding in the striatum membrane of the rat whose presynaptic DA fibers were degenerated by injection of 6-OHDA into the nigrostriatal pathway, the binding sites were increased. These results suggested that DA receptors labelled by \(^3\)H-spiroperidol were at least partially localized at the postsynaptic site of the nigrostriatal DA neuron.

In the inhibition experiment of \(^3\)H-spiroperidol binding in the rat striatum and frontal cortex, DA antagonists showed more potent inhibition than DA agonists. Serotonergic drugs such as serotonin and methysergide inhibited the binding 11–14.
times more potently in the frontal cortex than in the striatum, and this fact suggested the possibility that $^3$H-spiroperidol also labelled serotonergic components in the frontal cortex.

In the regional distribution of $^3$H-spiroperidol binding in the human control brain, the number of binding sites was the largest in the caudate, followed by the cerebral cortices, amygdala and olfactory bulb. This result was nearly consistent with the DA distribution pattern in the brain, and it also agreed with the regional distribution of $^3$H-spiroperidol binding in the rat brain reported by Laduron et al. (24). The high binding sites in the cerebral cortices were considered to partly reflect serotonin receptors. In the regional distribution of $^3$H-QNB binding of a case of SND, the number of binding sites was decreased in the putamen and thalamus in comparison to those of the control brain. Decrease in $^3$H-QNB binding in the putamen of the SND case was considered to correspond to neuronal loss at this region, but it was noteworthy that $^3$H-QNB binding decreased in the thalamus where no pathohistological abnormalities were observed. In the regional distribution of $^3$H-spiroperidol binding of SND, the number of binding sites was decreased in the putamen, frontal and parietal cortices, amygdala, Ammon's horn and substantia nigra in comparison to those of the control brain. Decrease of $^3$H-spiroperidol binding in the substantia nigra and putamen of SND was considered to correspond to neuronal loss; but the decrease was also noticed in the pathohistologically intact frontal and parietal cortices, amygdala and Ammon's horn. Mesocortical DA pathways are distributed in the frontal cortex, amygdala and Ammon's horn (25); and decrease of DA receptors in these areas suggested biochemical changes in mesocortical DA pathways. Thus, it might be considered that in SND, DA pathways including not only nigrostriatal DA pathways but also mesocortical DA pathways were widely changed biochemically. On the other hand as previously described, $^3$H-spiroperidol binding sites in the frontal cortex included serotonin receptors to a considerable extent; therefore, this decrease of binding in the frontal cortex in the case of SND does not necessarily mean exclusive involvement of the DA receptors, i.e., serotonin-containing neuronal structures might be affected by biochemically pathologic processes.

This is the first reported study on the regional distribution of neurotransmitter receptors in SND brain. Further accumulation of data, and the comparative investigation with those in olivopontocerebellar atrophy and Shy-Drager syndrome which are considered allied to SND are expected.

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