Distribution of Putative D4 Dopamine Receptors in Postmortem Striatum from Patients with Schizophrenia

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The identification of five dopamine receptor subtypes has given the dopamine hypothesis of schizophrenia new life. The D4 receptor is particularly intriguing because it binds clozapine with high affinity. Putative D4 receptors were labeled in postmortem human brain by subtracting the binding of a saturating concentration of ^{3}H-raclopride (6 nM, which labels D2 and D3 receptors) from that labeled by a saturating concentration of [^{3}H]YM 09151-2 (1–1.3 nM, which labels D2, D3, and D4 receptors). In the control brain, putative D4 receptors show a homogenous distribution in striatum and nucleus accumbens. This is also true in schizophrenic brains, although the levels are significantly higher (twofold). These data are inconsistent with mRNA studies that have shown negligible amounts in striatum and accumbens, with modest amounts reported in most of cerebral cortex. These findings suggest that the putative D4 receptors are not synthesized in this region, but are presynaptically localized on striatal afferent terminals. Our findings confirm and extend the report of Seeman et al. (1993). Extension of these findings into the nucleus accumbens is important because of its extensive connections to the limbic system while the putamen is exclusively "motor" striatum.

[Key words: D4, dopamine receptors, schizophrenia, autoradiography, postmortem, brain]

The notion that excess dopaminergic activity leads to the psychotic symptoms of schizophrenia is based on pharmacological findings. Agonists can cause or exacerbate these symptoms (Connell, 1958; Griffith et al., 1972) while dopamine D2 receptor blockade correlates with antipsychotic efficacy across a wide range of neuroleptics (Creese et al., 1976; Seeman et al., 1976). Direct evidence to support the dopamine hypothesis from postmortem schizophrenic brains (for review, see, Kleinman and Nawroz, 1994) and in vivo neuroimaging studies has been inconclusive (Wong et al., 1986; Farde et al., 1987; Martinot et al., 1990; Hietala et al., 1994).

DA receptors were originally divided into two major types, D1 and D2, based on pharmacological and biochemical criteria. As a result of recent advances in molecular biology, five distinct neuronal DA receptor genes have been identified (D1–D5; Gran-

dy et al., 1989; Sokoloff et al., 1990; Sunahara et al., 1990, 1991; Van Tol et al., 1991). Genes encoding receptors belonging to the "D1-like" family include D1 and D5 receptors, while the "D2-like" family includes D2, D3, and D4 receptors. The receptors are distinguished on the basis of their molecular structure, mRNA coding and anatomical distribution, chromosomal location, and biochemical and pharmacological profile (Sibley and Monsma, 1992, for review). The discovery of three new dopamine receptors has given the dopamine hypothesis new life.

Typical neuroleptics are both D2 and D3 blockers, although the pharmacological profile for the atypical neuroleptic clozapine suggests that D2 or D3 receptor antagonism is not the locus of its mechanism of action (Sokoloff et al., 1990). Clozapine has a 10-fold higher affinity for D4 than that for D2 sites (Van Tol et al., 1991). The neuroanatomical locus for antipsychotic efficacy appears to be in the striatum/nucleus accumbens for both D2 receptors (Seeman, 1992) and D4 receptors (Seeman et al., 1993a). There appears, however, to be a mismatch for mRNA localization for D4 receptors insofar as there are relatively negligible concentration of D4 mRNA in the striatum/nucleus accumbens (Meador-Woodruff et al., 1994a,b).

In a recent study, Seeman (1993a) identified putative D4 receptors by subtracting the binding of ^{3}H-raclopride + Gpp(NH)p (which labels D2 and D3 receptors) from that of ^{3}H-YM 09151-2, which, from pharmacological studies has been shown to label D2, D3, and D4 receptors (Seeman et al., 1993b). Using homogenate binding on postmortem human putamen, Seeman et al. reported a two- to sixfold increase in D4 binding in a schizophrenic population compared to the control group (Seeman et al., 1993a,b).

The following study employed quantitative receptor autoradiography (1) to visualize putative D4 receptors in human striatum, (2) to determine the anatomical distribution of putative D4 receptors in human striatum, and (3) to compare the amount and distribution in our schizophrenic population to a number of control groups and to test the hypothesis that elevation in "D2-like" receptors in schizophrenia was a consequence of previous exposure to neuroleptics. Putative D4 receptor numbers were compared between schizophrenics and three control populations: neurological controls, neuroleptic controls, and nonschizophrenic suicides. This study tests the notion of the specificity of the findings with respect to psychiatric diagnosis and neuroanatomy.

Materials and Methods

Materials. ^{3}H-YM 09151-2 (81.4 Ci/mmol) and ^{3}H-raclopride (79.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA). (+)Butaclamol was purchased from Research Biochemicals Internation-

Table 1. Patient demographics

Diagnosis	N	Age (range) ± SEM	Sex	PMI ± SEM
Schizophrenia	7	59.14 (34–80) ± 6.1	4F/3M	19.36 ± 3.8
Control	7	55.71 (33–87) ± 6.7	3F/4M	28.04 ± 5.3
Neuroleptic control	8	51.75 (28–81) ± 7.2	1F/7M	33.1 ± 7.12
Suicide	7	50.57 (23–86) ± 8.5	1F/6M	18.3 ± 2.9

al (Natick, MA). Gpp(NH)p and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of brain tissue quantitative receptor autoradiography. Brain specimens in the NIMH brain collection were obtained from the Washington, DC, Medical Examiner's Office. Blood and urine samples were collected for toxicological analysis and for neuroleptic level determination. None of the subjects had measurable serum levels at the time of death. Psychiatric diagnosis was determined by independent review of medical records by at least two psychiatrists. Table 1 lists demographic information on all subjects included in this study.

As listed above, three different control groups were compared to the schizophrenic patient group. The first was normal controls. The second was a neuroleptic control group, described in Table 2, consisting of a mixture of diagnostic categories, united only in their previous exposure to neuroleptics. This group was included to ascertain if receptor changes in the schizophrenic population resulted from previous exposure to neuroleptics.

The third control group consisted of nonschizophrenic suicides, included for comparison, since many schizophrenics commit suicide.

After collection from autopsy, the brain tissue was dissected into 1 cm coronal slabs that were individually frozen in isopentane cooled on dry ice ($-40°C$). Tissue blocks were stored at $-70°C$ until processed. Coronal sections (14 μm) were cut from tissue blocks at $-20°C$ using a cryostat (Jung Frigocut 2800, Nussloch, Germany) and subsequently heat mounted onto gelatin-coated slides. Slide-mounted sections were stored with desiccant inside plastic containers at $-70°C$ until used in assays.

^{3}-H-YM 09151-2 assay (D2, D3, and D4 receptors). Slide-mounted sections were preincubated in buffer (50 mM Tris-HCl, pH 7.4 at room temperature, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl^2, 4 mM MgCl$_2$, 120 mM NaCl) for 10 min. Sections were transferred to buffer containing a saturating concentration (1–1.3 nM) of ^{3}H-YM 09151-2 for 180 min. Nonspecific binding was determined in the presence of 10 μM (+)butaclamol. The reaction was terminated by two 5 min rinses in ice-cold buffer followed by a dip in ice-cold double distilled water. Sections were dried under a stream of cold air and stored in desiccant overnight at room temperature before being placed in cassettes. Sections and tritiated microscales (Amersham Corp., Arlington Heights, IL) were apposed against ^{3}H-ultrafilm (LKB Instruments, Gaithersburg, MD) for 6 d at 4°C. Assay was carried out twice on adjacent sections and the results averaged.

^{3}H-Raclopride assay (D2 and D3 receptors). Slide-mounted sections were preincubated for 1 hr at room temperature in buffer containing

200 μM Gpp(NH)p to remove endogenous dopamine that is known to compete with ^{3}H-raclopride for binding to receptors (Seeman et al., 1989). Sections were transferred to buffer containing a saturating concentration (6 nM) of ^{3}H-raclopride for 120 min. Nonspecific binding was determined as before, in the presence of 10 μM (+)butaclamol. The reaction was terminated and slide-mounted sections were dried as before. Sections were apposed against ^{3}H-ultrafilm for 12 d.

Sections were analyzed using a Macintosh computer-assisted image analysis system (NIH image 1.52, public domain). Units are expressed in fmol/mg protein. The striatum was divided into dorsal-caudate, ventral-caudate, nucleus accumbens, dorsal-putamen, and ventral-putamen for separate regional analysis. Specific binding was determined by subtracting nonspecific from total binding. For ^{3}H-YM 09151-2, nonspecific binding represented 30% total binding at a saturating concentration. For ^{3}H-raclopride, it represented 14%. Specific ^{3}H-raclopride binding represented approximately 50% of specific ^{3}H-YM 09151-2 binding. D4 receptor number was determined by subtracting the specific binding of ^{3}H-raclopride (which binds to D2 and D3 receptors) from the specific binding of ^{3}H-YM 09151-2 (which labels D2, D3, and D4 receptors), according to the strategy originally proposed by Seeman et al. (1993a).

Statistical comparisons between groups were carried out using one-way ANOVA for each individual subregion followed by post hoc Fisher PLSD comparison.

Results

Specific ^{3}H-YM 09151-2 binding

Table 3 displays binding of various radioligands across patient groups. Specific ^{3}HYM 09151-2 binding differed across the groups [5.75(29), $p = 0.0037$ ANOVA], in the dorsal caudate. Schizophrenics had increased binding compared to all other groups, $p < 0.05$, by Fisher PLSD. A similar increase in binding was observed in dorsal putamen [6.115(29) $p = 0.0027$ ANOVA], ($p < 0.05$, compared to control, neuroleptic control, and suicide, Fisher PLSD), ventral caudate [3.397(29) $p = 0.0327$, $p < 0.05$, compared to control, neuroleptic control, and suicide, Fisher PLSD], and ventral putamen [3.884(29) $p = 0.0203$ ANOVA, $p < 0.05$, compared to control, neuroleptic control, and suicide, Fisher PLSD]. No significant increases in binding were observed in the nucleus accumbens.

Specific ^{3}H-raclopride binding

No significant differences in binding were observed between groups for any striatal region for ^{3}H-raclopride binding. Table 3 shows specific binding values for ^{3}H-raclopride in the various groups. Binding shows slight but nonsignificant elevations in the schizophrenic group and slight but nonsignificant reductions in the neuroleptic control group.

Putative D4 binding

Putative D4 receptors show a fairly homogenous distribution in control striatum. Highest values were seen in ventral caudate (49.58 ± 5.5 fmol/mg protein) and nucleus accumbens (46.34 ± 5.8 fmol/mg protein). Dorsal caudate revealed 44.98 ± 4.9 fmol/mg protein, dorsal putamen 43.39 ± 6.5 fmol/mg protein, and ventral putamen (42.7 ± 5.35 fmol/mg protein) putative D4

Table 2. Neuroleptic control group diagnosis

Case	Diagnosis
1	Bipolar disorder
2	Bipolar disorder, alcohol abuse
3	Major depressive disorder
4	Acute encephalopathy, systemic lupus erythematosus, subacute bacterial endocarditis, right middle cerebral artery infarction
5	Antisocial personality disorder, phencyclidine and amphetamine abuse
6	Psychotic disorder, not otherwise specified
7	Dementia associated with alcoholism
8	Dementia associated with alcoholism

Table 3. Binding results of radioligands in the various groups

	Dorsal caudate	Ventral caudate	Nucleus accumbens	Dorsal putamen	Ventral putamen
³H-YM 09151-2					
Schizophrenic	122.3 ± 9.9*ᴧ	105.8 ± 6.4*ᵠᴧ	110.2 ± 15.7	133.4 ± 12.0*ᵠᴧ	124.0 ± 7.2*ᵠᴧ
Control	82.6 ± 5.7	79.8 ± 6.7	94.3 ± 7.8	87.8 ± 5.6	91.2 ± 7.6
Neuroleptic control	88.3 ± 9.5	83.6 ± 7.1	90.9 ± 7.3	85.4 ± 8.6	92.2 ± 8.9
Suicide	85.4 ± 5.2	81.9 ± 5.4	93.3 ± 7.9	94.2 ± 8.7	94.2 ± 7.6
F; p	5.75; 0.0037	3.397; 0032	0.723; 0.55	6.12; 0.00275	4.01; 0.0185
³H-Raclopride					
Schizophrenic	43.2 ± 11.25	33.84 ± 7.3	50.15 ± 12.3	47.14 ± 15	50.86 ± 15.5
Control	34.5 ± 7.9	28.2 ± 7.9	47.2 ± 7.7	40.3 ± 6.9	45.29 ± 7.5
Neuroleptic control	27.5 ± 5.02	27.8 ± 4.9	35.8 ± 5.9	29.04 ± 6.2	33.43 ± 8.0
Suicide	42.2 ± 3.9	33.9 ± 5.6	46.5 ± 4.3	44.55 ± 2.3	46.9 ± 1.9
F; p	0.939; 0.44	0.274; 0.84	0.592; 0.63	0.8; 0.5	0.621; 0.6
³H-YM 09151-2-³H-Raclopride					
Schizophrenic	79.1 ± 10.9*ᵠ	71.9 ± 7.4*ᵠ	74.34 ± 9.5*ᵠ	86.3 ± 13.7*ᵠᴧ	73.2 ± 11.9*ᵠ
Control	44.9 ± 4.9	49.6 ± 5.5	46.3 ± 5.8	43.4 ± 6.5	42.7 ± 5.3
Neuroleptic control	60.3 ± 6.42	54.9 ± 5.8	60.2 ± 4.2	57.6 ± 4.3	59.0 ± 4.12
Suicide	41.7 ± 6.7	46.0 ± 6.8	45.44 ± 8.9	56.2 ± 6.5	46.4 ± 7.6
F; p	5.3; 0.0055	3.13; 0.04	3.38; 0.033	4.64; 0.01	3.16; 0.04

Values are expressed as means ± SEM. *$p < 0.05$, schizophrenic versus control; ᵠ$p < 0.05$, schizophrenic versus suicide; ᴧ$p < 0.05$, schizophrenic versus neuron. Units are expressed in fmol/mg protein.

receptors. Figure 1 shows the distribution of D4 receptors in striatum and nucleus accumbens relative to D2 and D3 receptors.

Putative D4 binding in the various groups is displayed in Table 3 (³H-YM 09151-2 − ³H-raclopride). Putative D4 binding was significantly increased (in schizophrenics compared to controls, suicides, and neuroleptic controls, $p < 0.05$, Fisher PLSD) in dorsal putamen [4.644(28) $p = 0.013$, ANOVA]. Levels of putative D4 receptors were significantly higher in schizophrenics compared to normal controls and suicides but were not elevated compared to neuroleptic controls ($p < 0.05$, Fisher PLSD) in dorsal caudate [5.3(29) $p = 0.0055$], ventral caudate [3.13(29), $p = 0.043$], nucleus accumbens [3.37(27), $p = 0.035$], and ventral putamen [3.17(29), $p = 0.412$]. Figure 2 graphically represents this data.

Discussion

Using a radioligand subtraction method (Seeman et al., 1993a) putative D4 receptors are increased in schizophrenics relative to controls. This is in agreement with the previous findings of Seeman et al. (1993a), although it extends the findings demonstrating increases in putative D4 receptors in all regions of striatum and nucleus accumbens, where there is a homogenous distribution. Absolute values are lower than those in the Seeman study, possibly because the homogenate binding technique may reveal higher binding than slide-mounted sections. Extension of these findings into the nucleus accumbens is important because of its extensive connections to the limbic system while the putamen is exclusively "motor" striatum (Oades and Halliday, 1987; Alexander et al., 1990).

Significant controversy exists over the expression of D4 receptors in the brain. Previous studies using Northern blot analysis has shown levels of D4 mRNA in rat striatum and hippocampus to be present, but two orders of magnitude lower than levels of D2 mRNA (Van Tol et al., 1991). A distribution profile by Northern blot was carried out in several monkey brain areas, indicating relatively high levels in frontal cortex, midbrain, medulla, and amygdala (Van Tol et al., 1991). Lower levels were

detected in striatum and hippocampus with no signal in the cerebellum. This distribution is more consistent with a limbic rather than motor distribution. In a more detailed study using *in situ* hybridization histochemistry, modest amounts of D4 mRNA have been reported in the hypothalamus, amygdala, hippocampus, nucleus accumbens, and much of the cerebral cortex with relatively negligible amounts in the striatum (Meador-Woodruff et al., 1994a,b). Thus, there is a disproportionate number of putative D4 receptors in the striatum, far in excess of what the relative D4 mRNA levels would suggest. These findings imply that D4 receptors are not synthesized in the basal ganglia, but rather have a presynaptic localization on afferent terminals in the striatum. This issue is currently under further investigation in our laboratory. D4 receptors may exist on terminals of corticostriatal neurons and may allow for dopaminergic regulation of glutamatergic neurotransmission. Increases in putative D4 autoreceptors to compensate for increased glutamatergic activity in striatum is an interesting hypothesis to explain their elevation in schizophrenia.

A second major issue to be addressed with regard to these findings is whether or not neuroleptics are responsible for these results. As in Seeman's study, a neuroleptic control group was not found to be increased statistically relative to the normal subjects. Moreover, the toxicological screens in the schizophrenic group were negative, suggesting that they were drug free at the time of death. Although the duration of this drug-free period is unclear, it was sufficiently long that D2 and D3 receptors measured by ³H-raclopride binding were not increased relative to controls. This is in agreement with previous studies done in our group (Knable et al., 1994). None of these findings, however, definitely rule out a neuroleptic effect causing an increase in putative D4 receptors, but they certainly make it less likely.

Of the previous studies that observed increases in D2 receptors in schizophrenic basal ganglia, the radioligands used were those that have an affinity for D2, D3, and D4 receptors: ³H-spiperone (Lee et al., 1978; Owen et al., 1978; Lee and Seeman, 1980; Mackay et al., 1982; Mita et al., 1986; Mjorndal and Win-
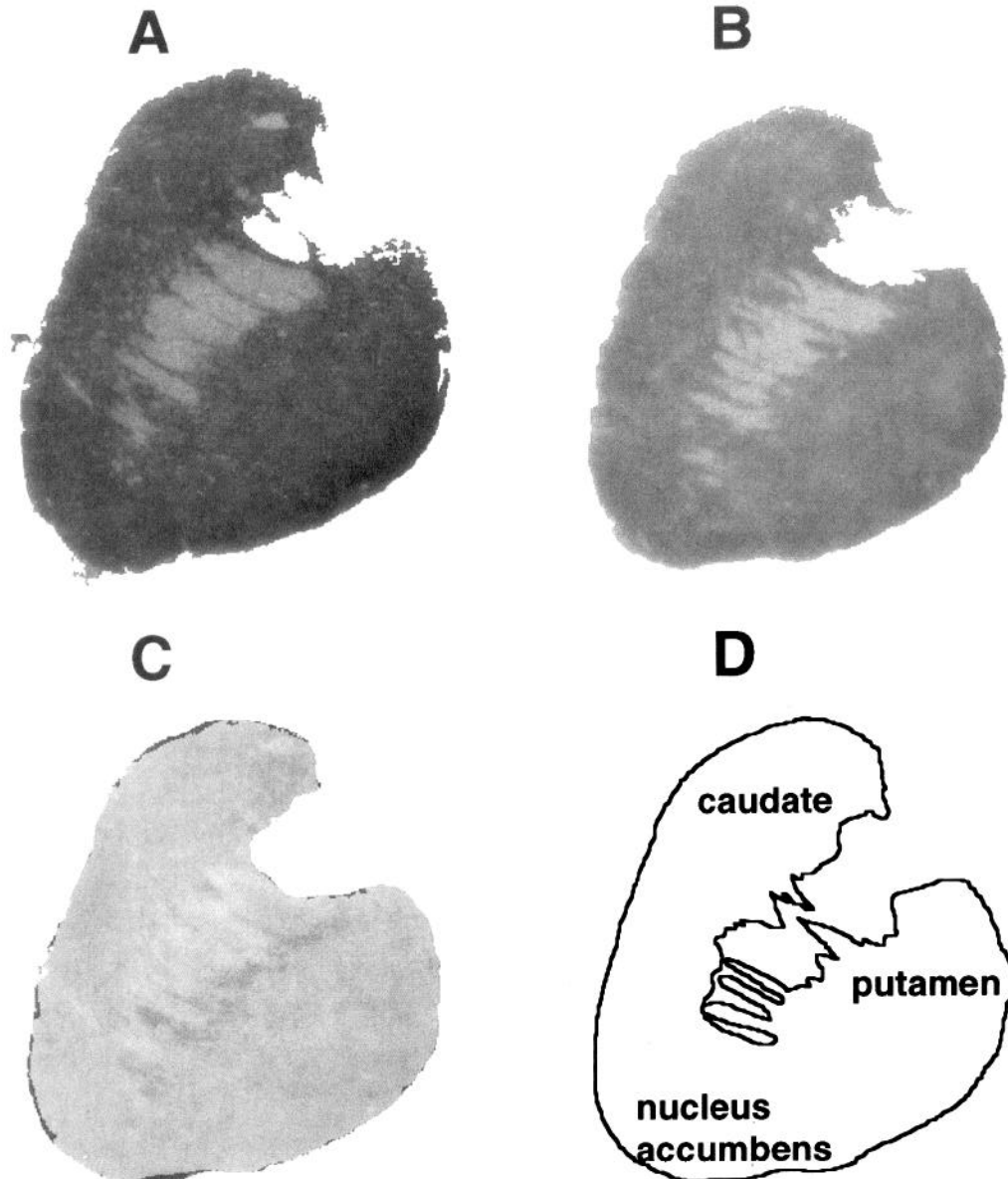
Figure 1. Photomicrographs from autoradiographs for binding of ³H-YM 09151-2 to D2, D3, and D4 receptors, (*A,*) ³H-raclopride to D2 and D3 receptors (*B*). *C* depicts putative D4 binding through computer-assisted subtraction of ³H-raclopride from ³H-YM 09151-2. *D* shows a drawing delineating the structures from a control brain.

blad, 1986; Hess et al., 1987; Seeman et al., 1987), ³H-apomorphine (Lee et al., 1978; Lee and Seeman, 1980), and ³H-flupenthixol (Cross et al., 1981). Increases previously attributed to "D2" receptor density elevation may, instead, have actually reflected increases in D4 receptor density (Seeman, 1993a). Alternatively, these increases may have resulted from previous neuroleptic exposure (Mackay et al., 1982), Kornhuber at al., 1989). However, in studies where no change was detected, ³H-spiperone was also used as a radioligand (MacKay et al., 1978). As an added complication, under some conditions ³H-YM 09151-2 and ³H-raclopride appear to label many more "receptors" than ³H-spiperone. Seeman (1993a) has offered the explanation that ³H-spiperone binds to dimers of dopamine receptors, which could account for the difference. Clearly, the pharmacological characteristics of these ligands need additional investigation.

This study has significant methodological limitations. The first is the complicated approach to the labeling of putative D4 receptors in that the specificity of the findings is dependent on subtracting the specific binding of two radioligands. Such an approach can lead to increased variability, but in the current absence of specific D4 radioligands, this is the only available approach. Another approach would be to perform ³H-YM 09151-2 binding in the presence of a cold saturating concentration of raclopride. This was attempted by Seeman (1993a) using ³H-YM 09151-2 in the presence of 200 nM raclopride, and was unsuccessful because it eliminated the binding of ³H-YM 09151-2 to the control tissue, making comparisons with the schizophrenic tissue difficult (Seeman et al., 1993a).

Despite these reservations, there is a twofold increase in some receptor population, possibly D4, in the schizophrenic group relative to the control groups. In order to confirm that these

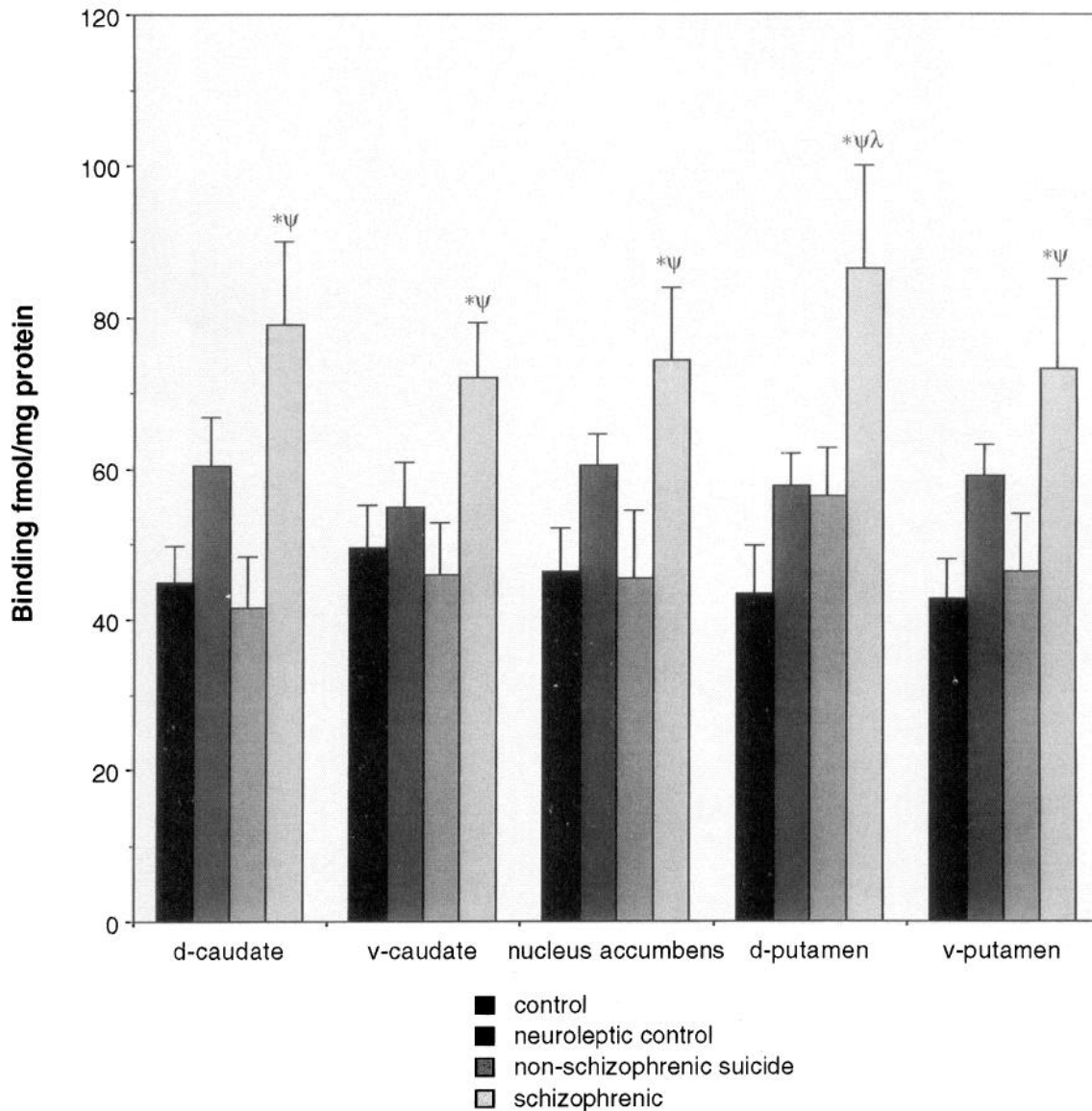# D4

[3H]YM 09151-2 - [3H]raclopride

Figure 2. Graphic representation of the mean ± SEM of D4 receptor binding in control, neuroleptic control, nonschizophrenic suicide, and schizophrenic groups. Regional binding densities are shown for dorsal caudate, ventral caudate, nucleus accumbens, dorsal putamen, and ventral putamen. $*p < 0.05$, schizophrenic versus control, $^\psi p < 0.05$, schizophrenic versus nonschizophrenic suicide; $^\lambda p < 0.05$, schizophrenic versus neuroleptic control. Units are expressed in fmol/mg protein.

changes are truly D4 receptors, it would be best to repeat such experiments using a specific D4 receptor ligand, when such a compound becomes available.

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