Binding of [³H]PD 128907, a Putatively Selective Ligand for the D₃ Dopamine Receptor, in Rat Brain: A Receptor Binding and Quantitative Autoradiographic Study

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[³H]PD 128907 has been proposed as a selective ligand for the D₃ dopamine receptor. This study characterizes the binding of this radioligand in rat brain using in vitro radioligand binding and autoradiographic methods. In radioligand binding studies, [³H]PD 128907 exhibited 0.3 nmol/L affinity for a single, low density site in ventral striatal membranes. The pharmacological profile for [³H]PD 128907 was similar to that of [³H](+)-7-OH-DPAT with the rank order of potency for dopamine agonists being PD 128907 ≈ 7-OH-DPAT ≈ quinpirole ≥ dopamine; for antagonists, spiperone > (+)-butaclamol ≈ domperidone ≥ haloperidol > SCH 23390. Guanyl nucleotides had no effect on the binding of either ligand. These observations indicate labeling of a dopaminergic site with characteristics consistent with the D₃ receptor. In autoradiographic studies, highest densities of [³H]PD 128907–labeled sites were observed in islands of Calleja followed by the nucleus accumbens, nucleus of the horizontal limb of the diagonal band, the molecular layer of cerebellar lobule X, and the ventral caudate/putamen. **[Neuropsychopharmacology 18:305–316, 1998]** © 1998 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

[³H]PD 128907 has been identified as a putatively selective ligand for the D₃ dopamine receptor (Akunne et al. 1995). A member of the D₂ receptor family, the D₃ site is of particular interest because its mRNA is expressed preferentially in brain regions such as the nucleus accumbens, olfactory tubercle, and islands of Calleja (Bouthenet et al. 1991; Sokoloff et al. 1990). These brain regions are terminal fields of the mesolimbic dopamine projection that is hypothesized to be involved in producing psychotic symptoms in schizophrenia (Stevens 1973). Furthermore, unlike mRNA for the D₂ receptor, relatively little D₃ mRNA is expressed in the caudate/putamen (Bouthenet et al. 1991; Sokoloff et al. 1990). This suggests that the D₃ site may be a target for novel antipsychotic drugs that might be free of extrapyramidal effects. It has also been suggested that the D₃ receptor may play a role in the reinforcing properties of cocaine (Caine and Koob 1993) and might thus represent a potential target in the treatment of drug abuse.

Since the cloning of the D₃ receptor, several radioligands thought to be selective for this site have been synthesized including [³H]PD 128907 (R-(+)-*trans*-3,4,4a, 10b-tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano[3,4-b]-

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1,4-oxazin-9-ol]) (Akunne et al. 1995) and [³H]7-OH-DPAT (7-hydroxy-diphenylaminotetralin) (Lévesque et al. 1992). However, there is some controversy over the selectivity of [³H]7-OH-DPAT for the D_3 receptors over the D_2 (Gonzales and Sibley 1995). Interaction of the ligand with the sigma site has also been reported (Wallace and Booze 1995). Although confusion over the selectivity of [³H]7-OH-DPAT may be due to the use of different assay protocols, the conflicting reports have limited the utility of this ligand as a tool for the study of D_3 sites in brain. On the other hand, PD 128907 produced behavioral and neurochemical effects in brain suggestive of activity at the D_3 site (Pugsley et al. 1995). Likewise, [³H]PD 128907 exhibited significant selectivity in transfected cell lines (Akunne et al. 1995). However, the utility of this ligand in rat brain has not yet been demonstrated.

Because comprehensive study of a novel receptor system in brain requires the ability to selectively visualize the receptor protein, as well as receptor mRNA, the utility of [³H]PD 128907 as a D_3-selective agent was evaluated. The pharmacological profile, guanyl nucleotide regulation, and regional distribution of [³H]PD 128907–labeled sites in rat brain are described and assessed using receptor binding and quantitative autoradiographic methods.

MATERIALS AND METHODS

[³H]PD 128907 Binding Assays

The [³H]PD 128907 binding assays were performed according to a modification of the methods of Akunne et al. (1995). Discrete brain regions were isolated by free-hand dissection on ice from the brains of adult male Sprague-Dawley rats (Harlan Bioproducts, Indianapolis, IN). Brain tissue was homogenized with a PRO250 Homogenizer (setting 4 of 6) for 10 s in 20 volumes of assay buffer (50 mmol/L Tris, 1 mmol/L EDTA; pH 7.4). The crude homogenate was centrifuged twice at 48,000 × g for 15 min, resuspending the pellet in 20 volumes of assay buffer each time. The final pellet from membrane preparation was resuspended in buffer to yield a final concentration of 10 mg original wet weight (o.w.w.)/ml. Binding assays were performed in duplicate in disposable polystyrene tubes. The final assay volume was 0.5 ml. For binding site saturation studies, 8–10 concentrations of (+)-[N-propyl-2,3-³H]PD 128907 (116 Ci/mmol; Amersham, Arlington Heights, IL) were used. For all other studies, the final concentration of [³H]PD 128907 was ~0.3 nmol/L. Unless otherwise noted, ventral striatal (nucleus accumbens and olfactory tubercle) membranes were incubated with [³H]PD 128907 and various concentrations of competing drugs (RBI, Natick, MA) or 5′-guanylyl-imidodiphosphate (Gpp(NH)p) (Sigma, St. Louis, MO). Binding was initiated by the ad-

dition of membrane homogenate. Nonspecific binding was defined by 10 μmol/L quinpirole. Preliminary experiments indicated the optimal incubation time at 23°C to be 3 h. The reaction was terminated by rapid filtration through Whatman GF/B filters pretreated with 0.5% polyethyleneimine using a Brandel cell harvester. Filters were washed 3 times with 3 ml ice-cold buffer (50 mmol/L Tris; pH 7.4 at 23°C), and placed in scintillation vials. After the addition of Beckman Ready Protein+ scintillation cocktail, vials were shaken, allowed to equilibrate for 2 h, and radioactivity quantitated using a Beckman 6500 scintillation counter. Protein concentrations were determined using the BCA method (Pierce, Rockford, IL). Specific binding of [³H]PD 128907 is expressed as fmol/mg protein. Data from saturation and competition experiments were analyzed using the nonlinear least-squares curve-fitting program LIGAND. Results are expressed as the mean ± SE.

[³H](+)-7-OH-DPAT Binding Assays

Assays were performed as described for [³H]PD 128907 (see above) with the following exceptions. Membranes were prepared in assay buffer (50 mmol/L HEPES, 1 mmol/L EDTA; pH 7.4, at 4°C) to yield a final concentration of 7.5 mg o.w.w./ml. The concentration of [³H](+)-7-OH-DPAT (139 Ci/mmol; Amersham, Arlington Heights, IL) was ~0.2 nmol/L for single point assays. Nonspecific binding was defined by 10 μmol/L quinpirole. Preliminary experiments indicated the optimal incubation time at 23°C to be 1 h. Bound ligand was separated from free by rapid filtration through untreated Whatman GF/B filters. The wash buffer was 50 mmol/L Tris, 120 mmol/L NaCl; pH 7.4 at 23°C.

Receptor Autoradiography with [³H]PD 128907

Adult male Sprague-Dawley (200–300 g; Charles River Laboratories, Wilmington, DE) were killed by decapitation, brains rapidly removed, frozen in isopentane, and stored at −70°C until sectioning. Sagittal and coronal brain sections (20 μm) were cut on a cryostat, thaw-mounted onto chrome-alum/gelatin-coated slides and stored at −70°C until use.

[³H]PD 128907 autoradiography was performed according to a modification of methods previously described (Levant et al. 1993). Optimal assay conditions were determined in preliminary wash-out and association experiments. Slide-mounted brain sections were brought to room temperature and allowed to dry thoroughly. Duplicate sections from the same animal were used for each data point. Slides were incubated with ~0.7 nmol/L [³H]PD 128907 in assay buffer (50 mmol/L Tris, 1 mmol/L EDTA, pH 7.4 at 23°C) for 2 h at 23°C. Nonspecific binding was defined in the presence of 1 μmol/L spiperone. After incubation, slides were dipped

in ice-cold assay buffer, washed for two consecutive 2-min periods in ice-cold assay buffer, dipped in ice-cold deionized H_2O, and dried under a cool air stream. Radiolabeled sections were subsequently apposed to ³H-Hyperfilm (Amersham, Arlington Heights, IL) with 20 μm [³H]methylmethacrylate autoradiographic standards (Amersham, Arlington Heights, IL) for a period of 12 weeks. ³H-Hyperfilm was developed according to the manufacturer's instructions. Brain sections were then stained with cresyl violet.

Autoradiographic images were digitized and quantified using the Macintosh-based video densitometry program NIH "Image" version 1.4. Best-fit curves of optical density generated by the [³H]methylmethacrylate autoradiographic standards resulted when a Rodbard plot was used to describe the relationship between optical density and radioactivity. Brain regions were identified according to the atlas of Paxinos and Watson (1986). Autoradiograms of coronal sections were sampled bilaterally. Measurements represent average pixel optical density by volume analysis. Results, expressed as fmol/mg tissue equivalent were not corrected for differential quenching by white and gray matter. Digitized images of autoradiograms from Image were used to generate the figures presented in this article.

RESULTS

Radioligand-Binding Studies

Preliminary Studies of [³H]PD 128907 Binding in Ventral Striatal Membranes. Preliminary studies were performed to determine the optimal tissue concentration

and length of incubation (data not shown). When used at a concentration equal to the K_D value (~0.3 nmol/L), [³H]PD 128907 binding reached steady state within 3 h at 23°C. At a final tissue concentration of 10 mg o.w.w./ml, less than 3% of the total [³H]PD 128907 was bound at steady state. Specific binding represented 60% of the total [³H]PD 128907 bound.

Saturation Analysis of [³H]PD 128907 and [³H](+)-7-OH-DPAT Binding. Binding site saturation analyses were performed with [³H]PD 128907 and [³H](+)-7-OH-DPAT in ventral striatal (nucleus accumbens and olfactory tubercle) membranes. Analysis by LIGAND for [³H]PD 128907 (0.01–3 nmol/L) revealed a single binding site with a K_D value of 0.30 ± 0.02 nmol/L and a B_{max} value of 26 ± 2.1 fmol/mg of protein (Figure 1). Binding of [³H](+)-7-OH-DPAT (0.01–3 nmol/L) was consistent with interactions at a single site with a K_D value of 0.18 ± 0.01 nmol/L and a B_{max} value of 36 ± 2.1 fmol/mg of protein (Table 1).

To evaluate the D_2/D_3 selectivity of [³H]PD 128907 and [³H](+)-7-OH-DPAT, saturation studies were performed in caudate/putamen, a brain area that expresses the D_2 receptor predominantly over the other D_2-related subtypes (for review, see Levant 1996). Saturation analysis of [³H]PD 128907 binding in caudate/putamen was performed using significantly higher concentrations of radioligand (0.5–75 nmol/L) than those used to assess putative D_3 binding. These studies revealed no specific binding of [³H]PD 128907 in caudate/putamen above that attributable to the low levels of putative D_3 binding observed in that tissue.

Saturation analysis of [³H](+)-7-OH-DPAT in caudate/putamen was also performed using significantly
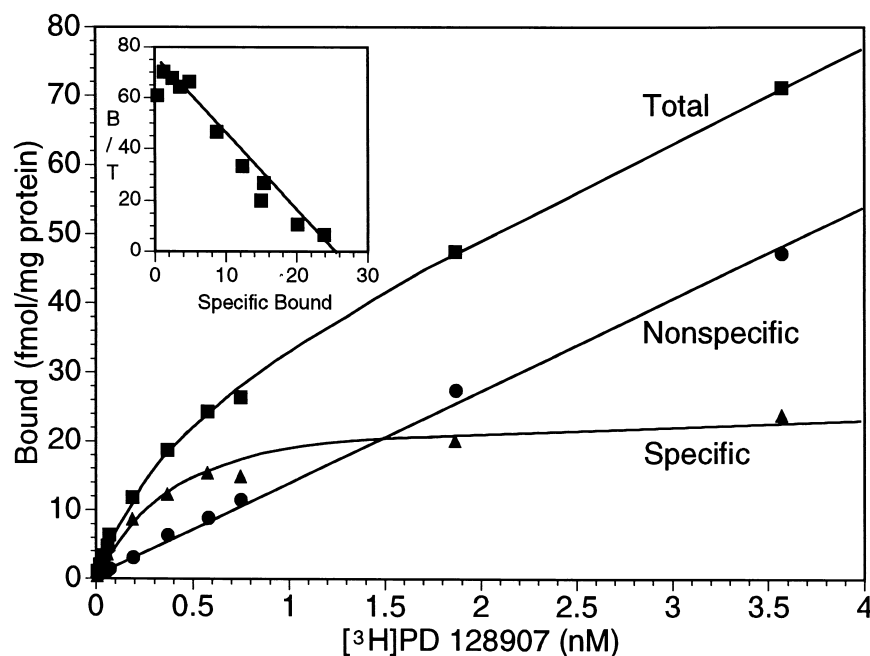

Figure 1. Saturation isotherms of [³H]PD 128907 binding in rat ventral striatal membranes. Membranes were incubated with 11 concentrations of [³H]PD 128907 (0.01–3 nmol/L) as described in Materials and Methods. Nonspecific binding was defined in the presence of 10 μmol/L quinpirole. Three independent determinations yielded a K_D value of 0.30 ± 0.02 nmol/L and a B_{max} value of 26 ± 2.1 fmol/mg protein as estimated by the nonlinear least-squares curve fitting program LIGAND. Data from all saturation analyses were consistent with the interaction of the radioligand with a single binding site. Representative results are shown.

Table 1. Affinity and Density of Sites Labeled by [³H]PD 128907 and [³H](+)-7-OH-DPAT in Rat Ventral Striatum and Caudate/Putamen

Radioligand	K_D (nmol/L)	B_{max} (fmol/mg protein)
Ventral striatum		
[³H]PD 128907	0.30 ± 0.02	26 ± 2.1
[³H](+)-7-OH-DPAT	0.18 ± 0.01	36 ± 2.1
Caudate/putamen		
[³H]PD 128907	ND	ND
[³H](+)-7-OH-DPAT	23 ± 1.5 nM	218 ± 5.7

Membranes were incubated with 9–11 concentrations of [³H]PD 128907 or [³H](+)-7-OH-DPAT (0.01–3 nmol/L in ventral striatum, 0.5–75 nmol/L in caudate/putamen) as described in Materials and Methods. Nonspecific binding was defined in the presence of 10 μmol/L quinpirole. Data represent the mean ± SEM of three independent determinations as estimated by the nonlinear least-squares curve fitting program LIGAND. Data from all saturation analyses were consistent with the interaction of the radioligand with a single binding site. ND = not detected.

Table 2. Pharmacological Profile of Sites Labeled by [³H]PD 128907 and [³H](+)-7-OH-DPAT

| | K_i (nmol/L) | |
Drug	[³H]PD 128907	[³H](+)-7-OH-DPAT
Dopamine agonists		
(+)-PD 128907	0.53 ± 0.11	0.73 ± 0.17
(±)-7-OH-DPAT	0.42 ± 0.06	1.1 ± 0.38
Quinpirole	0.74 ± 0.07	2.0 ± 0.16
Dopamine	20 ± 2	5.5 ± 1.0
Dopamine antagonists		
Spiperone	1.0 ± 0.11	0.59 ± 0.04
(+)-Butaclamol	14 ± 2.0	5.5 ± 1.2
Domperidone	34 ± 5.7	9.6 ± 1.1
Haloperidol	24 ± 3.1	36 ± 4.0
SCH 23390	610 ± 129	303 ± 54
Other		
(+)-N-allylnormetazocine	>100,000	>100,000

Membrane homogenates were incubated with [³H]PD 128907 (∼0.3 nmol/L) or [³H](+)-7-OH-DPAT (∼0.2 nmol/L) and up 11 concentrations of competing drug (10^{-11} to 10^{-4} mol/L) as described in Materials and Methods. [³H]PD 128907 and [³H](+)-7-OH-DPAT binding assays were performed using ventral striatum and nonspecific binding was defined using 10 μmol/L quinpirole. Data were analyzed using the nonlinear, least-squares curve fitting program LIGAND and represent the mean ± SEM of three to four independent determinations. Data from all competition studies were consistent with the interaction of each ligand with a single binding site.

higher concentrations of radioligand (0.5–75 nmol/L) than those used to assess putative D_3 binding. In addition to the very low levels of binding attributable to the putative D_3 site, these studies indicated the labeling of a single site with a K_D value of 23 ± 1.5 nmol/L and a B_{max} value of 218 ± 5.7 fmol/mg protein.

Pharmacological Profile of [³H]PD 128907 and [³H](+)-7-OH-DPAT Binding Sites.   The pharmacological profiles of [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites were determined in ventral striatal membranes using a variety of dopaminergic drugs including those shown to possess significant D_2/D_3 selectivity in previous studies (Sokoloff et al. 1990; Lévesque et al. 1992; Levant and De Souza 1993; Akunne et al. 1995) (Table 2). Dopamine agonists exhibited the following rank order of potency in competition with [³H]PD 128907: (±)-7-OH-DPAT ≈ (+)-PD 128907 ≈ quinpirole > dopamine. A similar rank order of potency was observed in competition with [³H](+)-7-OH-DPAT: (+)-PD 128907 ≥ (±)-7-OH-DPAT ≈ quinpirole ≥ dopamine. In competition with [³H]PD 128907, dopamine antagonists inhibited binding with the following rank order of potency: spiperone > (+)-butaclamol ≥ haloperidol ≈ domperidone > SCH 23390 (Figure 2). Antagonists inhibited binding of [³H](+)-7-OH-DPAT with a similar rank order: spiperone > (+)-butaclamol ≥ domperidone > haloperidol > SCH 23390. Overall, dopamine agonists and antagonists exhibited highly similar rank order of potencies in competition with [³H]PD 128907 and [³H](+)-7-OH-DPAT and had a correlation coefficient of 0.707 (Table 2).

Because previous studies have suggested an interaction of [³H]7-OH-DPAT with the sigma site (Wallace and Booze 1995), the affinity of $sigma_1$ ligand N-allyl-normetazocine for [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites was determined. N-allylnormetazocine had negligible affinity in competition for either putatively D_3-selective ligand.

In all instances, competition data were consistent with interactions at a single binding site as analyzed by LIGAND. Because the density of [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites in other brain regions was quite low (see below), pharmacological characterization of sites in other brain areas was not undertaken.

Guanyl Nucleotide Regulation of [³H]PD 128907 and [³H](+)-7-OH-DPAT Binding.   In the presence of the nonhydrolyzable GTP analog, Gpp(NH)p (0.1 mmol/L), 99 ± 6% of total specific [³H]PD 128907 binding was observed in ventral striatal membranes. Similarly, 96 ± 4% of total specific [³H](+)-7-OH-DPAT binding was observed in the presence of 0.1 mmol/L Gpp(NH)p. Because the density of [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites in other brain regions was quite low (see below), assessment of the guanyl nucleotide regulation of sites in other brain areas was not undertaken.

Regional Distribution of [³H]PD 128907 and [³H](+)-7-OH-DPAT Binding.   Preliminary studies of [³H]PD 128907 and [³H](+)-7-OH-DPAT binding in caudate/putamen and vestibulocerebellum revealed interactions
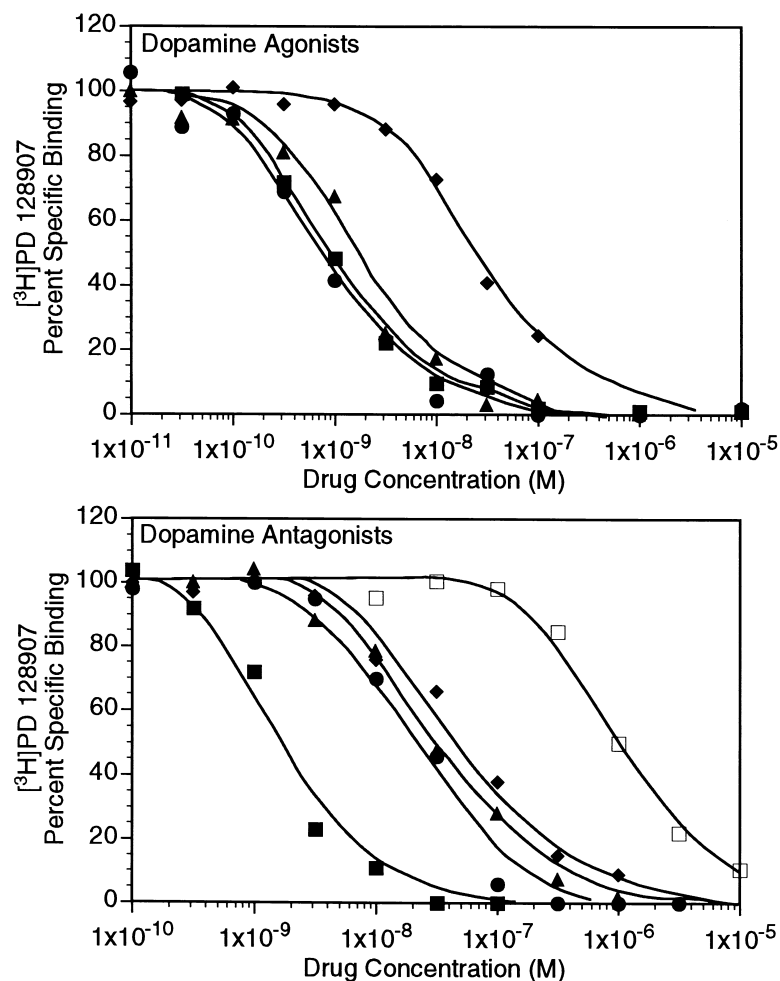
Figure 2. Competition of [³H]PD 128907 binding by dopamine agonists and antagonists. Rat ventral striatal membranes were incubated with ~0.3 nmol/L [³H]PD 128907 and up to 11 concentrations of competing drug (10^{-11} to 10^{-5} mol/L) as described in Materials and Methods. Data were analyzed and competition curves generated using the nonlinear least-squares curve fitting program LIGAND. Representative results are shown. The quantitative data from these studies are summarized in Table 1. Dopamine agonists: ▲, PD 128907; ■, 7-OH-DPAT; ●, quinpirole; ◆, dopamine. Dopamine antagonists: ■, spiperone; ●, (+)butaclamol; ▲, domperidone; ◆, haloperidol; □, SCH 23390.

with a single binding site of similar K_D value to those observed in ventral striatum and of relative density similar to that described below (data not shown). Accordingly, single-point binding analysis was used to examine the regional distribution of the binding sites for these radioligands.

Sites labeled by [³H]PD 128907 and [³H](+)-7-OH-DPAT exhibited similar regional distributions in rat brain (Table 3). Binding of both radioligands was highest in ventral striatum (nucleus accumbens and olfactory tubercles), followed by the vestibulocerebellum. Moderate levels of [³H]PD 128907 and [³H](+)-7-OH-DPAT were observed in the caudate/putamen, hypothalamus, and substantia nigra/ventral tegmental area. Moderately low to low densities of sites were detected in frontal cortex, amygdala, hippocampus, and dorsal vermis. Overall, the regional distributions of sites labeled by these radioligands was highly correlated ($r^2 = 0.900$).

The regional distribution of [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites was compared with sites labeled by the D_2/D_3 agonist ligand [³H]quinpirole. The density of [³H]quinpirole-labeled sites in

brain areas such as the caudate/putamen, ventral striatum, and substantia/nigra/ventral tegmental area was significantly higher than those labeled by either [³H]PD 128907 or [³H](+)-7-OH-DPAT. The correlation coefficients for the regional distribution of [³H]quinpirole–labeled sites with those labeled by [³H]PD 128907 and [³H]7-OH-DPAT were 0.138 and 0.292, respectively.

Autoradiographic Studies

Substrate Specificity of [³H]PD 128907 Binding in Slide-Mounted Brain Sections. The relative potencies of several compounds at [³H]PD 128907 binding sites in sagittal sections of rat brain are depicted in Figure 3. These data are quantified in Figure 4. Overall, comparable substrate specificity of [³H]PD 128907 binding was obtained in autoradiographic studies in nucleus accumbens and the molecular layer of cerebellar lobule X (Figure 4). (±)-7-OH-DPAT (10 μmol/L), spiperone (1 μmol/L), and (+)-butaclamol (1 μmol/L) each inhibited [³H]PD 128907 to identical baseline levels in both brain areas examined. The potencies of (±)-7-OH-DPAT and SCH 23390 were similar to that observed in radioligand binding studies with IC_{50} values of roughly
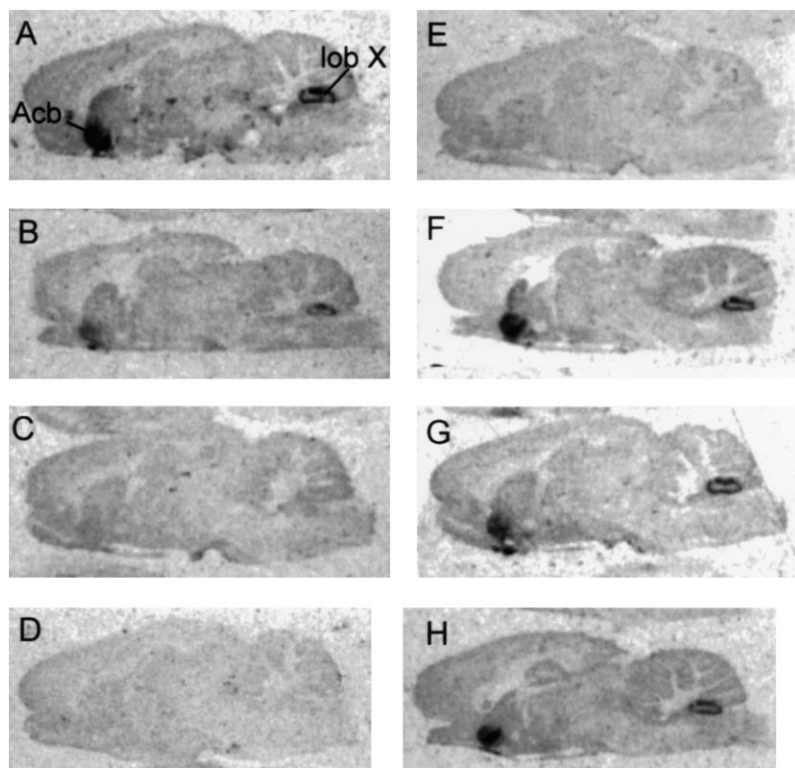
Figure 3. Substrate specificity of [³H]PD 128907–labeled sites in rat brain. Slide-mounted sagittal sections (lateral 1.20–1.40 mm) were incubated with 0.7 nmol/L [³H]PD 128907 in the presence of absence of various compounds as indicated. **(A)** total binding, **(B)** (±)-7-OH-DPAT (0.5 nmol/L), **(C)** (±)-7-OH-DPAT (10 nmol/L), **(D)** spiperone (1 μmol/L), **(E)** (+)-butaclamol (1 μmol/L), **(F)** (−)-butaclamol (1 μmol/L), **(G)** SCH 23390 (1 μmol/L), **(H)** Gpp(NH)p (1 mmol/L). Acb = nucleus accumbens, lob X = cerebellar lobule X. Quantified data from these autoradiograms is shown in Figure 4.

0.5 nmol/L and 1 μmol/L, respectively. (−)-Butaclamol (1 μmol/L) and Gpp(NH)p (1 mmol/L) failed to inhibit [³H]PD 128907 in either nucleus accumbens or cerebellar lobule X.

Autoradiographic Localization of [³H]PD 128907–Labeled Sites.   The distribution of [³H]PD 128907–labeled sites in rat brain is shown in Figure 5. Quantitative analy-

sis of regional [³H]PD 128907 binding appears in Table 4. Specific [³H]PD 128907 binding was heterogeneously distributed throughout the brain with highest densities (>10 fmol/mg tissue equiv.) observed in the islands of Calleja, followed by the nucleus accumbens. Within the nucleus accumbens, somewhat more [³H]PD 128907 binding was observed in the shell than in the core. Dense binding was also observed in the nucleus of the horizon-

Table 3. Regional Distribution of [³H]PD 128907 and [³H](+)-7-OH-DPAT Binding in Rat Brain Membranes: Comparison with the Distribution of [³H] Quinpirole–Labeled Sites

Brain Region	Specific Binding (fmol/mg protein)		
	[³H]PD 128907	[³H]7-OH-DPAT	[³H]Quinpirole[a]
Ventral striatum	12 ± 1.0	9.6 ± 1.7	42
Vestibulocerebellum	5.0 ± 1.1	6.5 ± 0.96	—
Hypothalamus	3.4 ± 0.46	3.6 ± 0.87	7
Caudate/putamen	2.3 ± 0.40	4.4 ± 0.39	85
Substantia nigra/VTA	2.0 ± 0.41	3.7 ± 0.29	12
Amygdala	1.8 ± 0.32	2.7 ± 0.19	4
Frontal cortex	1.4 ± 0.30	1.8 ± 0.13	4
Dorsal vermis	0.71 ± 0.28	2.1 ± 0.32	2
Hippocampus	0.25 ± 0.17	1.4 ± 0.32	3

Membranes prepared from specific rat brain regions were assayed for [³H]PD 128907 or [³H]7-OH-DPAT as described in Materials and Methods. The concentrations of [³H]PD 128907 and [³H]7-OH-DPAT ∼0.3 nmol/L and ∼0.2 nmol/L, respectively. Nonspecific binding was defined by 10 μmol/L quinpirole. Data shown represent the mean ± SEM of three independent determinations. Ventral striatum = nucleus accumbens and olfactory tubercle. VTA = ventral tegmental area.

[a]Data represent the mean to two independent determinations performed with identical results as reported in Levant et al. 1992.
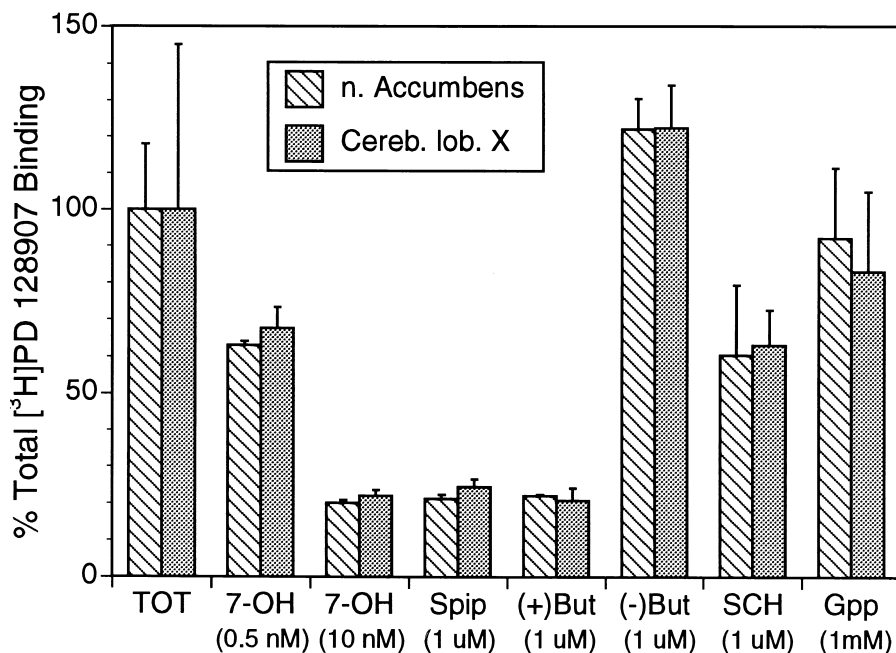
Figure 4. Quantification of [³H]PD 128907–labeled sites in specific brain regions in the presence of various compounds. Film images in Figure 3 were digitized and quantified as described in Materials and Methods. Each data point represents the mean ± SEM obtained from four slides (two sections per slide). TOT = total binding; 7-OH = (±)-7-OH-DPAT; Spip = spiperone; (+)But = (+)-butaclamol; (−)But = (−)-butaclamol; SCH = SCH 23390; Gpp = Gpp(NH)p.

tal limb of the diagonal band. Moderate densities of [³H]PD 128907 binding (5–10 fmol/mg tissue equiv.) were observed in the molecular layer of cerebellar lobule X, olfactory tubercle, ventral pallidum, substantia nigra pars compacta, and caudate/putamen. [³H]PD 128907 binding in the caudate/putamen, however, was not uniform with greater binding observed in the anteroventral and dorsomedial portions of the nucleus with relatively little binding in the dorsal portion. Low to very low densities of [³H]PD 128907 binding (<5 fmol/mg tissue equiv.) were observed in the other brain region examined such as the hypothalamus, thalamus, amygdala, cortical areas excluding piriform, cerebellum excluding the molecular layer of lobule X, olfactory bulb, and hippocampus. No differential labeling of cell layers of the hippocampus and dentate gyrus was observed.

DISCUSSION

Because of the potential therapeutic importance of the dopamine D_3 site (Sokoloff et al. 1990; Caine and Koob 1993), considerable effort has been directed toward understanding its neurobiology. To this end, several radioligands purported to be D_3 selective have been identified including [³H]PD 128907 and [³H]7-OH-DPAT. Before extensive study of this novel receptor in brain may be undertaken, however, the ability of radioligands to distinguish between closely related dopamine receptor subtypes, as well as other receptors, must be demonstrated. The present study assesses the D_3 selectivity of [³H]PD

128907 in rat brain and uses this radioligand to characterize the pharmacological profile, guanyl nucleotide regulation, and regional distribution of putative D_3 sites.

Receptor binding analysis revealed that, under the in vitro assay conditions used in this study, [³H]PD 128907 exhibited a binding profile in rat brain consistent with interactions at a single binding site. The pharmacological profile of [³H]PD 128907–labeled sites labeled was similar to that of [³H](+)-7-OH-DPAT, suggesting that these ligands label a common D_2-like binding site. Whereas the pharmacology of these sites was similar to that of the classic D_2 receptor (for review, see Seeman and Grigoriadis 1987; Sibley and Monsma 1992), sites labeled by the putative D_3-selective ligands differ from the D_2 receptor in that [³H]PD 128907– and [³H](+)-7-OH-DPAT–labeled sites are present in lower density, display a different regional distribution, and are insensitive to guanyl nucleotides. Thus, neither ligand appears to interact significantly with the D_2 receptor under the assay conditions used in this study.

Instead, sites labeled by [³H]PD 128907 and [³H](+)-7-OH-DPAT display characteristics similar to those reported for the cloned D_3 receptor in expression systems in terms of pharmacological profile and apparent insensitivity to guanyl nucleotides (Boundy et al. 1993; Freedman et al. 1994; MacKenzie et al. 1994; Sokoloff et al. 1990, 1992). Furthermore, the densities and regional distributions of [³H]7-OH-DPAT– and [³H]PD 128907–labeled sites in rat brain are generally consistent with the distribution of D_3 receptor mRNA (Bouthenet et al. 1991) and the distribution of D_3 receptor binding estimated from experiments using nonselective radioli-
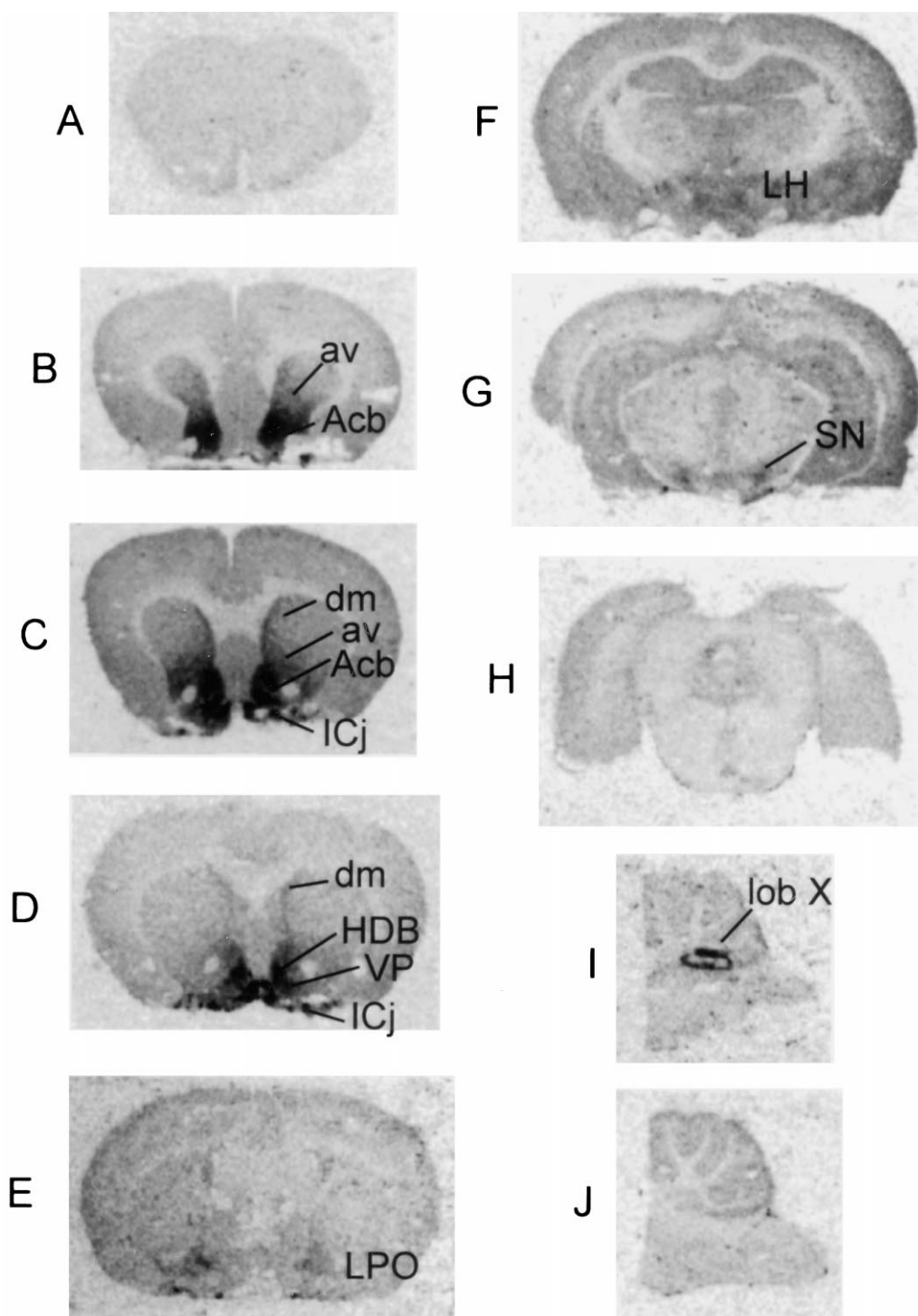
Figure 5. Autoradiographic localization of [³H]PD 128907–labeled sites in rat brain. Data shown represent total binding. Specific autoradiograms are representative of those obtained from each of four individual animals (two sections per slide). The darker areas of the autoradiograms indicate regions of higher receptor density. Slide-mounted brain sections (20 μm) were incubated with ~0.7 nmol/L [³H]PD 128907 as described in Materials and Methods. Nonspecific binding, as defined by 1 μm spiperone, 1 μm (+)-butaclamol, or 10 μm (±)-7-OH-DPAT is shown in Figure 3. Quantified data from these autoradiograms are summarized in Table 4. Stereotaxic coordinates for each level: **(A)** Bregma 4.20 mm, **(B)** Bregma 2.20 mm, **(C)** Bregma 1.30 mm, **(D)** Bregma 0.48, **(E)** −0.30 mm, **(F)** Bregma −3.60 mm, **(G)** Bregma −4.80 mm, **(H)** Bregma −7.64 mm, **(I)** Lateral 0.40 mm, **(J)** Lateral 2.40 mm. Abbreviations: Acb = nucleus accumbens, av = anteroventral caudate/putamen, dm = dorsomedial caudate/putamen, ICj = islands of Calleja, HDB = nucleus of the horizontal limb of the diagonal band, VP = ventral pallidum, LPO = lateral preoptic area, LH = lateral hypothalamus, SN = substantia nigra, lob X = cerebellar lobule X.

Table 4. Autoradiographic Distribution of [³H]PD 128907–Labeled Sites Rat Brain

Brain Region	[³H]PD 128907 Specific Binding fmol/mg Tissue Equivalent
Cerebral cortex	
Frontal	0.32 ± 0.32
Cingulate	0.90 ± 0.60
Parietal	1.3 ± 0.65
Piriform	2.9 ± 0.90
Temporal	0.60 ± 0.38
Entorhinal	0.85 ± 0.60
Olfactory tubercles	8.1 ± 2.1
Islands of Calleja	60 ± 14
Nucleus accumbens	
Core	16 ± 3.2
Shell	23 ± 4.0
Caudate/Putamen	
Dorsomedial	5.1 ± 0.38
Anterovental	7.3 ± 1.9
Dorsal	1.6 ± 1.2
Ventral pallidum	7.3 ± 2.5
Globus pallidus	1.5 ± 0.75
Septal area	1.4 ± 0.60
Nucleus of the vertical limb of the diagonal band	1.7 ± 0.80
Nucleus of the horizontal limb of the diagonal band	11 ± 4.2
Bed nucleus of the stria terminalis	3.5 ± 1.3
Medial preoptic area	3.8 ± 2.9
Magnocellular preoptic nucleus	3.5 ± 1.6
Thalamus	
Mediodorsal	1.9 ± 1.0
Ventrolateral	1.2 ± 0.54
Hypothalamus	
Arcuate nucleus	1.2 ± 0.54
Ventromedial nucleus	0.75 ± 0.32
Anterior area	2.3 ± 1.3
Lateral area	2.4 ± 0.99
Medial mammallary nucleus	3.4 ± 0.49
Medial and lateral geniculate nuclei	0.95 ± 0.49
Amygdala	1.5 ± 0.85
Hippocampus	
CA1	0.95 ± 0.38
CA2	0.80 ± 0.38
CA3	1.0 ± 0.44
Dentate gyrus	0.90 ± 0.44
Subiculum	1.09 ± 0.99
Substantia nigra	
Pars compacta	5.2 ± 0.08
Pars reticulata	3.5 ± 0.27
Ventral tegmental area	2.1 ± 0.99
Periaqueductal gray	0.85 ± 1.2
Superior colliculus	0.80 ± 0.54
Raphe nucleus	0.21 ± 0.15
Medial lemniscus	1.42 ± 0.85
Ventral lateral lemniscus	ND
Pontine nucleus	0.27 ± 0.21
Cerebellum	
cortex, lateral lobe	0.32 ± 0.27
cortex, vermis	0.60 ± 0.21
Lobule X - molecular layer	9.6 ± 4.8
Brain stem	0.32 ± 0.21

Data were derived from quantification of autoradiograms produced from four animals and are expressed at the mean ± SEM. Coronal sections (two per slide) were incubated with ∼0.7 nmol/L [³H]PD 128907. Nonspecific binding was defined in the presence of 1 μmol/L spiperone. The unit of receptor density (fmol/mg tissue equiv.) is that supplied by the manufacturer of [³H]methylmethacrylate autoradiography standards (Amersham). The results have not been corrected for differential quenching of tritium by gray and white matter and may thus underestimate the densities of binding sites in brain regions having a higher proportion of white matter. ND = not detected.

gands (Landwehrmeyer et al. 1992; Levant et al. 1993; Parsons et al. 1993; Booze and Wallace 1995). Taken together, the present data suggest the interaction of these radioligands with a common binding site with the characteristics of the D_3 dopamine receptor.

Thus, in our hands, both $[^3H]PD$ 128907 and $[^3H](+)$-7-OH-DPAT label the putative D_3 site in rat brain. Although both ligands exhibited high affinity, they differed in their selectivity between the D_2 and D_3 sites. Saturation analysis using high concentrations of $[^3H]PD$ 128907 (0.5–75 nmol/L) in caudate/putamen, a brain area that expresses D_2 receptor predominantly over other D_2-related subtypes (for review, see Levant 1996), indicated no binding above that attributable to the low density of putative D_3 sites present in that tissue. Thus, the affinity of this radioligand for the D_2 receptor is sufficiently low to preclude determination by filtration assay (i.e., $K_D > 100$ nmol/L) (Bennett and Yamamura 1985). As such, it is concluded that the D_2/D_3 receptor selectivity of this radioligand is significantly greater than 300-fold and that labeling of D_2 receptors is negligible under the in vitro assay conditions used in this study. In contrast, similar saturation studies with $[^3H](+)$-7-OH-DPAT revealed binding to a site in caudate/putamen of appropriate density for the D_2 receptor (Levant 1995; Levant et al. 1992) with a K_D value of 23 nmol/L. Thus, $[^3H](+)$-7-OH-DPAT exhibits only about 100-fold selectivity for the putative D_3 receptor over the D_2 under the in vitro assay conditions used in this study.

Interactions of $[^3H]$7-OH-DPAT with the sigma site have also been reported (Wallace and Booze 1995). The low potency of the sigma$_1$ ligand $(+)N$-allylnormetazocine in the radioligand binding studies, however, suggests no such interaction for either $[^3H](+)$-7-OH-DPAT or $[^3H]PD$ 128907 under the in vitro conditions used in our radioligand binding studies. Likewise, autoradiographic localization of $[^3H]PD$ 128907 binding sites indicated very low density of labeling in regions noted for dense expression of sigma sites such as the cerebellar cortex (Gundlach et al. 1986).

Whereas the present data indicate that selective labeling of putative D_3 sites may be reasonably obtained using either $[^3H]PD$ 128907 and $[^3H]$7-OH-DPAT, labeling of the D_2 site by $[^3H]$7-OH-DPAT has been reported (Gonzales and Sibley 1995). One likely factor contributing to the conflicting reports of the D_2/D_3-selectivity of $[^3H]$7-OH-DPAT is the use of different in vitro assay conditions. Assay conditions, particularly buffer composition, have been shown to influence the D_2/D_3 selectivity of this ligand in vitro (Lévesque et al. 1992; Burris et al. 1995). Specifically, the greatest D_2/D_3 selectivity with $[^3H]$7-OH-DPAT was obtained in the absence of Mg^{2+} and the presence of EDTA. Buffer systems excluding Mg^{2+} and containing EDTA were also selected for $[^3H]PD$ 128907 (Akunne et al. 1995). We have found that specific binding of $[^3H]$quinpirole in striatal membranes

was nearly abolished in a Tris-EDTA buffer system (unpublished observation), in concordance with previous studies indicating that the high affinity agonist state of D_2-like receptors is not favored in the absence of Mg^{2+} (Sibley and Creese 1983). Because selective visualization of the putative D_3 receptor appears to require in vitro assay conditions that disfavor D_2 binding, these conditions may also affect the binding or functional properties of the D_3 receptor. For example, the failure of guanyl nucleotides to inhibit $[^3H]$7-OH-DPAT and $[^3H]PD$ 128907 binding could result from a lack of coupling of the receptors to G-proteins. Alternatively, although the binding of agonist ligands such as $[^3H]PD$ 128907 and $[^3H]$7-OH-DPAT is presumed to be to receptors in the high affinity agonist state, this observation might also result if the labeled receptors were actually in the low affinity agonist conformation. Accordingly, care must be taken in the interpretation of data obtained with these methods with regard to their physiological implications.

The distribution of putative D_3 sites in brain has been of particular interest and is the primary basis for the hypothesis that receptor may be a suitable target for novel antipsychotics (Sokoloff et al. 1990). The present data confirm previous studies indicating highest densities of putative D_3 sites in the islands of Calleja followed by the nucleus accumbens as assessed by either radioligand binding or autoradiographic methods. Dense labeling by $[^3H]PD$ 128907 has also been reported in the nucleus accumbens and islands of Calleja of the human brain (Hall et al. 1996). As predicted by the distribution of D_3 receptor mRNA (Bouthenet et al. 1991; Curran and Watson 1995; Diaz et al. 1995; Landwehrmeyer et al. 1993; Mengod et al. 1992; Sokoloff et al. 1990), several other brain areas, such as the molecular layer of cerebellar lobule X, ventral pallidum, substantia nigra pars compacta, bed nucleus of the stria terminalis, medial and magnocellular preoptic areas, and nucleus of the horizontal limb of the diagonal band also exhibited moderately dense labeling by $[^3H]PD$ 128907.

Whereas the density of D_3 sites in caudate/putamen were low overall, the sites are differentially distributed within the nucleus and are moderately dense in the dorsomedial and anteroventral areas. The presence of moderately dense putative D_3 binding in the "limbic" anteroventral striatum is consistent with the similar afferent input as the nucleus accumbens from the allo- meso-, and periallocortices as well as the ventral tegmental area, basolateral amygdala, hippocampus, and prefrontal cortex (Heimer et al. 1995). The presence of D_3 sites in the dorsomedial aspect of the caudate, which also receives afferent from the mesocortex (Heimer et al. 1995), was suggested by previous studies using $[^3H]$quinpirole (Levant et al. 1993).

Whereas dense $[^3H]PD$ 128907 binding was observed in brain areas such as the islands of Calleja and nucleus

accumbens where dense expression of D_3 receptor mRNA has been reported, the observed densities of putative D_3 sites and the reported densities of D_3 mRNA were not directly correlated throughout the brain. For example, very dense expression of D_3 mRNA expression was reported in areas such as cerebellar lobule X and the bed nucleus of the stria terminalis (Bouthenet et al. 1991) but only moderately dense [³H]PD 128907 binding was observed in these regions. Other notable discrepancies in the distributions of D_3 mRNA and binding include the medial geniculate nucleus, pyramidal cell layer of the hippocampus, granular layer of the dentate gyrus, and certain thalamic and amygdaloid nuclei where moderate to dense expression of D_3 mRNA were observed (Bouthenet et al. 1991), but relatively little putative D_3 binding. These discrepancies may be due to the transport of receptors to brain regions remote from cell bodies or the differential regulation of transcription and translation in different brain areas. This issue must be resolved by further study.

In summary, the present data demonstrate the binding of [³H]PD 128907 to dopaminergic binding sites in rat brain that are generally consistent with previous studies of the D_3 receptor with respect to density, pharmacological profile, guanyl nucleotide regulation. The regional distribution of [³H]PD 128907–labeled sites is similar those labeled by other putatively selective D_3 ligands with the highest density in limbic brain areas and is generally similar to the distribution of D_3 mRNA.

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