Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8560–8564, November 1989
Neurobiology

Localization of the mRNA for the dopamine D_2 receptor in the rat brain by *in situ* hybridization histochemistry

(oligonucleotide/pituitary/caudate-putamen/substantia nigra)

GUADALUPE MENGOD*, M. INOCENCIA MARTINEZ-MIR†, M. TERESA VILARÓ, AND JOSÉ M. PALACIOS

Preclinical Research, Sandoz Ltd., Basel 4002, Switzerland

Communicated by Sanford L. Palay, July 24, 1989

ABSTRACT **^{32}P-labeled oligonucleotides derived from the coding region of rat dopamine D_2 receptor cDNA were used as probes to localize cells in the rat brain that contain the mRNA coding for this receptor by using *in situ* hybridization histochemistry. The highest level of hybridization was found in the intermediate lobe of the pituitary gland. High mRNA content was observed in the anterior lobe of the pituitary gland, the nuclei caudate-putamen and accumbens, and the olfactory tubercle. Lower levels were seen in the substantia nigra pars compacta and the ventral tegmental area, as well as in the lateral mammillary body. In these areas the distribution was comparable to that of the dopamine D_2 receptor binding sites as visualized by autoradiography using [^{3}H]SDZ 205-502 as a ligand. However, in some areas such as the olfactory bulb, neocortex, hippocampus, superior colliculus, and cerebellum, D_2 receptors have been visualized but no significant hybridization signal could be detected. The mRNA coding for these receptors in these areas could be contained in cells outside those brain regions, be different from the one recognized by our probes, or be present at levels below the detection limits of our procedure. The possibility of visualizing and quantifying the mRNA coding for dopamine D_2 receptor at the microscopic level will yield more information about the *in vivo* regulation of the synthesis of these receptors and their alteration following selective lesions or drug treatments.**

Two types of dopamine receptors mediate the effects of this neurotransmitter in the central nervous system and in the periphery, the so-called D_1 and D_2 receptor subtypes (1). Dopamine D_2 receptors are the target for drugs used in the treatment of schizophrenia and Parkinson disease and for regulation of prolactin secretion (2). Their distribution in the mammalian brain, including human, has been extensively examined by using receptor binding autoradiography (3). Dopamine D_2 receptors have been directly visualized in living humans by positron-emission tomography (PET) (4). Binding and PET studies have suggested that dopamine D_2 receptor levels may be elevated in schizophrenics (refs. 5, 6; see, however, ref. 7). Although dopamine receptors can be visualized at the light microscopic level by means of receptor binding autoradiography, the cellular localization of these receptors cannot be determined with this technique. The use of neurotoxin or mechanical brain lesions combined with binding studies has resulted in contradictory results (8–10). In order to establish the exact cellular localization of dopamine receptors, tools with higher anatomical resolution are necessary. The cloning and sequencing of the gene coding for the rat dopamine D_2 receptor has been reported recently (11), thus opening the way to the development of these tools. On the basis of this sequence we have synthesized oligonucleotide probes and used them to visualize the regional distribution of the cells containing mRNA coding for the dopamine D_2 receptor in the rat brain and pituitary gland by *in situ* hybridization histochemistry.

MATERIALS AND METHODS

Animals. Sprague–Dawley rats (male, 200–300 g, Iffa Credo, Lyon, France) were killed by decapitation and the brains and pituitary glands were quickly removed, frozen in isopentane at −40°C, and stored at −70°C until sectioned.

Probes. The oligodeoxyribonucleotides were complementary to the base sequence encoding the first 14 amino acids (oligomer DAI) of the rat dopamine D_2 mRNA (11) and to the last 11 amino acids of the carboxyl terminus (oligomer DAII). They were made on a 380B Applied Biosystems DNA synthesizer and purified on an 8% acrylamide/8 M urea preparative sequencing gel. The oligomers (2 pmol) were labeled at their 3′ end with 25 units of the enzyme terminal deoxynucleotidyltransferase (Boehringer Mannheim) and 16 pmol of [^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) in 100 mM sodium cacodylate, pH 7.2/2 mM $CoCl_2$/0.2 mM dithiothreitol to specific activities of 1–4 × 10^4 Ci/mmol. The reactions were carried out at 37°C for 2 hr and stopped by heating for 5 min at 65°C. Labeled probes were purified by chromatography through a NACS PREPAC column (BRL) according to the manufacturer's instructions.

Northern Analysis. Total RNA was isolated from dissected rat striatum and cerebellum according to Chomczynski and Sacchi (12), denatured with glyoxal (13), run on 1% agarose gels, and blotted onto nylon membranes (Hybond N, Amersham). The blots were hybridized with labeled oligoprobe for 18 hr at 42°C in 50% formamide/0.6 M NaCl/0.02 M Tris·HCl, pH 7.5/ 0.001 M EDTA/0.1% sodium pyrophosphate/0.2% SDS/0.25 mg of heparin per ml. Filters were washed twice in 2× SSC (1× SSC: 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS at room temperature for 5 min and twice in 0.2× SSC/0.1% SDS at 42°C for 15 min. The blots were exposed to an x-ray film for 2 days at −70°C with an intensifying screen.

***In Situ* Hybridization Histochemistry.** Sections (20 μm thick) were cut with a microtome cryostat (Leitz 1720), thaw-mounted onto gelatin-coated slides, and kept at −20°C until use. The frozen tissue sections were thawed to room temperature, air dried, and fixed by immersion for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS: 2.6 mM KCl/1.4 mM KH_2PO_4/136 mM NaCl/8 mM Na_2HPO_4), washed once in 3× PBS and twice in 1× PBS, 5 min each, and dehydrated in a graded series of ethanol (60, 80, 95, and 100%). They were hybridized with the corresponding labeled oligonucleotide probe at a final concentration of 0.4–0.8 pmol/ml in 40% formamide/600 mM NaCl/10 mM Tris·HCl,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed at: Sandoz AG, 386/228, Basel 4002, Switzerland.
†Permanent address: Departemento Farmacologia i Farmacotècnia, Universitat de València, Valencia, Spain.

pH 7.5/1 mM EDTA/0.1% Ficoll/0.1% bovine serum albumin/0.1% polyvinylpyrrolidone/500 μg of tRNA per ml. Hybridizations were carried out overnight under nescofilm coverslips in a humid chamber at 42°C. The sections were then washed with four 1-hr rinses in 600 mM NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA at 42°C and dehydrated with ethanol containing 0.3 M ammonium acetate (pH 7.0). The hybridized slides were apposed to βmax film (Amersham). Films were developed after 3 weeks. Duplicates of the hybridized sections were dipped into Kodak NTB-3 nuclear track emulsion diluted 1:1 with 0.6 M ammonium acetate. The emulsion was developed after 3 weeks in Kodak D19 and tissues were stained with Giemsa. Sections were examined using light- and dark-field microscopy (Leitz, Orthoplan).

Receptor Autoradiography. Receptor binding was visualized by autoradiography with the selective dopamine D_2 agonist [^{3}H]SDZ 205-502, as described (14). The rat brain sections were preincubated for 30 min in 170 mM Tris·HCl (pH 7.5) at room temperature and then incubated for 90 min at room temperature in the same buffer with 1 nM [^{3}H]SDZ 205-502 (specific activity, 107 Ci/mmol; synthesized by R. Voges, Biopharmaceutical Department, Sandoz). At the end of the incubation the slides were washed twice (1 min each) in the same buffer at 4°C. Nonspecific binding was determined by incubating alternate sections in the same conditions and buffers but with the addition of 1 μM (+)-butaclamol to the incubation buffer. The slides were then dried with cold air, and autoradiograms were generated by apposing the labeled tissue to [^{3}H]Ultrofilm (LKB). Films were analyzed with the aid of a computerized image-analysis system (MCID, Imaging Research, Saint Catharines, ON).

RESULTS AND DISCUSSION

Specific hybridization with the ^{32}P-labeled oligonucleotide probes was obtained in the rat pituitary and coronal brain sections at various rostrocaudal levels. Marked differences in the degree of specific hybridization were observed in various regions, providing good evidence that dopamine D_2 receptor mRNA can be detected and its regional and cellular distribution can be mapped out in brain sections by means of the *in situ* hybridization histochemistry technique. This distribution was compared with that of the receptor visualized by binding, and is illustrated in Figs. 1–3.

To assess the specificity of our probe, we performed Northern analysis on total RNA from rat cerebellum and striatum as described under *Materials and Methods*. After the hybridization and washes, a single band of about 3000 nucleotides (data not shown) was seen in the striatum but not in the cerebellum, in agreement with the published report (11). To measure the presence of a possible nonspecific hybridization of the probe to the tissue the following controls were performed. A second oligomer, DAII, complementary to another region of the dopamine D_2 receptor mRNA was used as hybridization probe and showed the same pattern of hybridization as the DAI oligonucleotide (not shown). All of the different hybridization signals could be blocked by competition of the ^{32}P-labeled DAI probe with a 20-fold excess of the unlabeled probe (not shown). Melting curve analysis showed that the melting temperature of the hybrids was close to the predicted value (data not shown). Oligomers for other receptors such as the muscarinic acetylcholine receptor subtypes M1, M2, M3, M4, and M5, as well as probes for the serotonin 1C and serotonin 2 receptors, were used on sections adjacent to those studied with the dopamine D_2 receptor probe and showed a completely different pattern of hybridization (data not shown).

In the majority of the brain areas examined and in the pituitary gland there was a qualitatively good anatomical correlation between the distribution of dopamine D_2 receptor mRNA hybridization signal and the binding of [^{3}H]SDZ 205-502. In the pituitary gland high levels of hybridization (Fig. 1 *A* and *D*) and binding (Fig. 1 *C* and *E*) were seen in the intermediate lobe, moderate hybridization and binding signals were seen in the anterior lobe, and only background levels for both signals were seen in the posterior lobe. These results are in agreement with abundant binding and functional data on the presence of D_2 receptors in the pituitary gland (15) and with high levels of dopamine D_2 receptor mRNA found by Northern blot analysis (11). Our results provide direct evidence for the presence of dopamine D_2 receptor mRNA in the melanocytes of the intermediate lobe and a widespread distribution in the cells of the anterior lobe.

Very good agreement between the distribution of the hybridization signal and receptor binding was also seen in the nuclei caudate-putamen and accumbens (Fig. 2). In the nucleus accumbens, the distribution of dopamine D_2 receptor mRNA was relatively homogeneous and with a lower density than that seen in the caudate-putamen. In the nucleus caudate-putamen, clear antero-posterior and latero-medial decreasing gradients of hybridization signal were observed (Fig. 2 *C* and *E*). These gradients overlapped with similar gradients of receptor binding observed in these studies (Fig. 2 *D* and *F*)

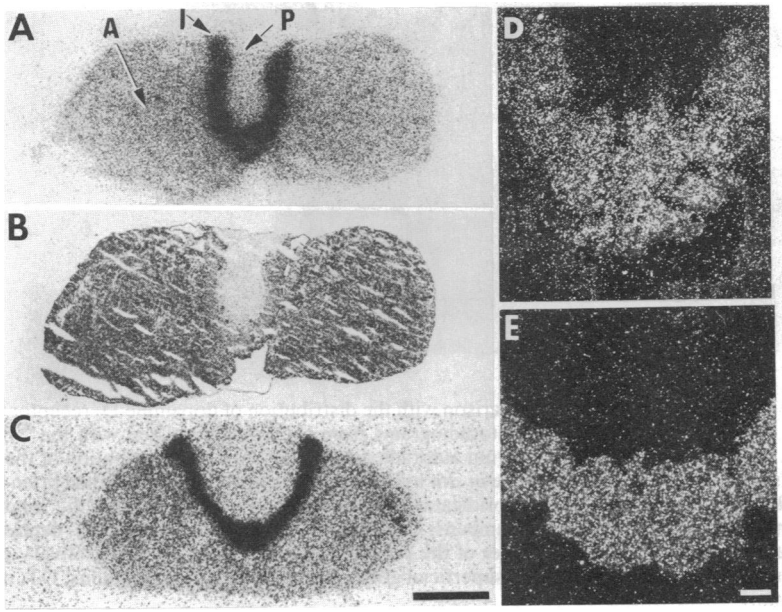

FIG. 1. Dopamine D_2 receptor mRNA localization in the rat pituitary gland. (*A*) Autoradiogram obtained from a tissue section hybridized with the ^{32}P-labeled oligonucleotide probe (DAI). The autoradiographic localization of receptor binding sites is shown in *C*. (*B*) Tissue section stained with cresyl violet to illustrate the distribution of the anterior (A), intermediate (I), and posterior (P) lobes of the rat pituitary gland. (*D*) Dark-field photomicrograph showing the localization of autoradiographic grains in the intermediate lobe of a rat pituitary gland hybridized as in *A*. The localization of receptor binding is illustrated at the same level of resolution in *E*. (Bars = 1 mm in *A–C* and 0.1 mm in *D* and *E*.)

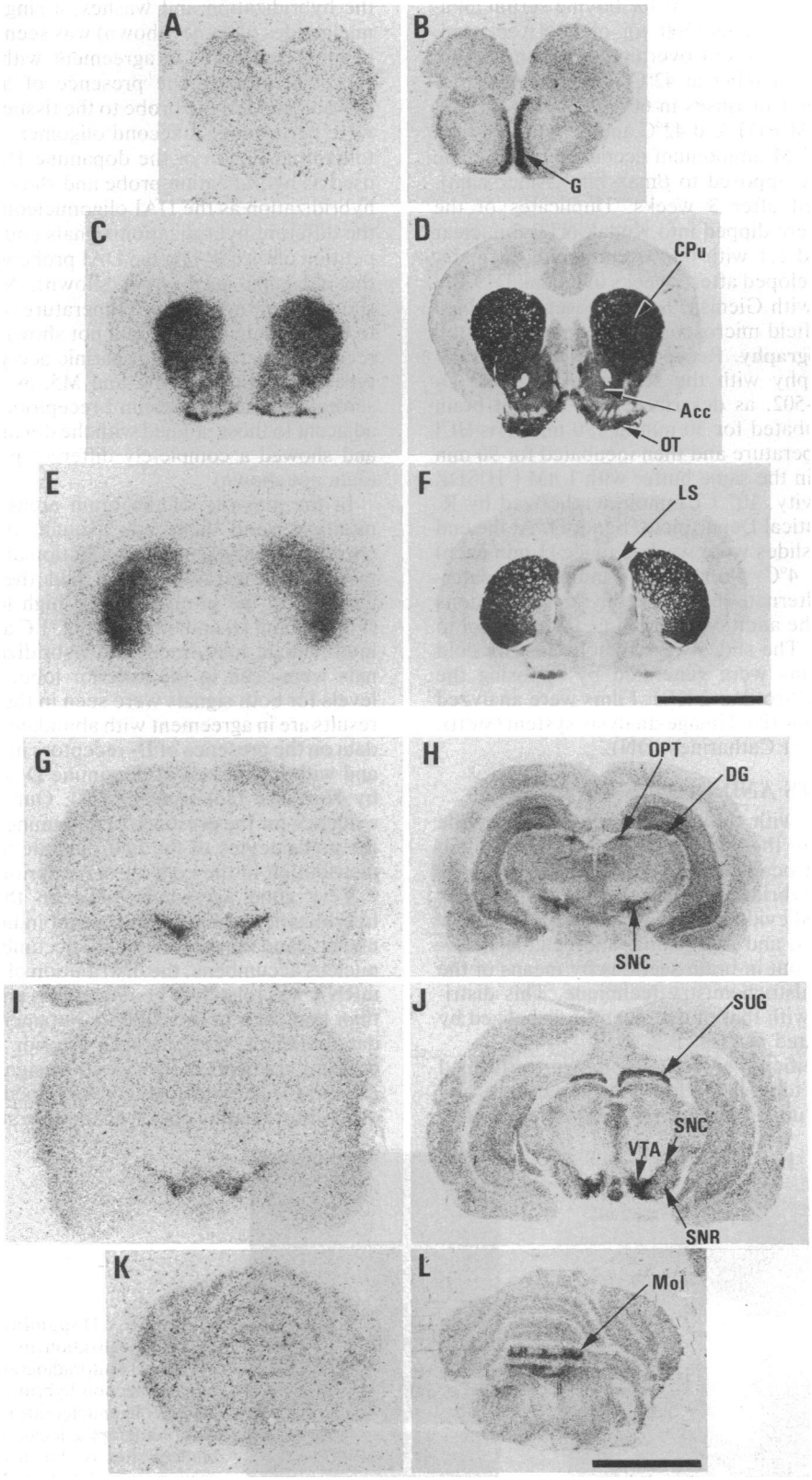

FIG. 2. Distribution of the dopamine D_2 receptor mRNA in the rat brain. Comparison with the distribution of dopamine D_2 receptor binding. Pictures on the left column (*A*, *C*, *E*, *G*, *I*, and *K*) are photographs from film autoradiograms from sections hybridized with the ^{32}P-labeled oligomer (DAI). On the right column (*B*, *D*, *F*, *H*, *J*, and *L*) the photographs are from autoradiograms and show the distribution of the binding of [^{3}H]SDZ 205-502 to dopamine D_2 receptor at levels similar to those shown for the *in situ* hybridization histochemistry. Coronal brain sections are presented in a rostro-caudal progression. Dark areas are those rich in hybridization or binding signal. The nonspecific binding was homogeneous and comparable to that seen in white matter areas. Acc, nucleus accumbens; CPu, nucleus caudatus-putamen; DG, dentate gyrus; G, glomerular layer of the olfactory bulb; LS, lateral septum; Mol, molecular layer of the cerebellar lobules 9 and 10; OPT, olivary pretectal nucleus; SNC, substantia nigra compacta; SNR, substantia nigra reticulata; SUG, superficial gray layer of the superior colliculus; OT, olfactory tubercle; VTA, ventral tegmental area. (Bars = 5 mm.)

and previously reported with [^{3}H]SDZ 205–502 and other D_2 ligands (3, 14, 16). Higher-resolution autoradiograms obtained by dipping the hybridized sections in liquid nuclear track emulsion revealed a widespread labeling of neurons throughout the nucleus caudate-putamen (Fig. 3 *A* and *B*). One of the highest densities of hybridization signal in the rat brain was observed in the densely packed pyramidal layer of the olfactory tubercle. Liquid emulsion autoradiograms showed the silver grains concentrated over the cell bodies of this layer, whereas the polymorph and plexiform layers presented only background levels of labeling (not shown). This distribution contrasts with that of the binding sites, which are also enriched in the plexiform layer.

At more caudal levels, the hybridization signal was seen in the lateral nucleus of the mammillary bodies (not shown), in agreement with the presence of binding sites in this nucleus (3, 14).

The dopaminergic cell bodies of the ventral mesencephalon also contained important densities of dopamine D_2 receptor mRNA transcripts (Fig. 2 *G* and *I*). The density was higher in the substantia nigra pars compacta than in the ventral tegmental area, whereas no signal above background was seen in the substantia nigra pars reticulata. With the liquid emulsion, silver grains were seen concentrated in the large neuronal cell bodies characteristic of the dopaminergic cells of these areas (Fig. 3 *C* and *D*). Further confirmation was obtained by unilateral lesion of these neurons by stereotaxic injection of 6-hydroxydopamine. The degeneration of the dopaminergic cells was followed by a loss of the hybridization signal with the dopamine receptor probe (data not shown) and also with an oligonucleotide complementary to the tyrosine hydroxylase mRNA (17). The presence of the dopamine D_2 receptor mRNA in the substantia nigra pars compacta and ventral tegmental area is in good agreement with the presence of dopamine D_2 receptor binding in these areas and with receptor losses following 6-hydroxydopamine lesion (3, 16). These results suggest, in addition, that dopaminergic neurons express dopamine D_2 receptors, which are probably transported later to their dendrites and axonal terminals (18).

The presence of dopamine D_2 receptors localized in dopaminergic terminals in the striatum is well documented by functional studies, but direct anatomical evidence was still missing. Our results indicate that in the striatum, dopamine D_2 receptors are expressed by cells intrinsic to this nucleus and also localized to presynaptic dopaminergic terminals. The absence of detectable levels of hybridization in the cortex agrees well with recent autoradiographic experiments showing no effect of decortication on dopamine D_2 binding in the striatum (ref. 8; also see ref. 10).

Dopamine D_2 receptor binding is, however, also found in other brain areas where no hybridization signal was detected

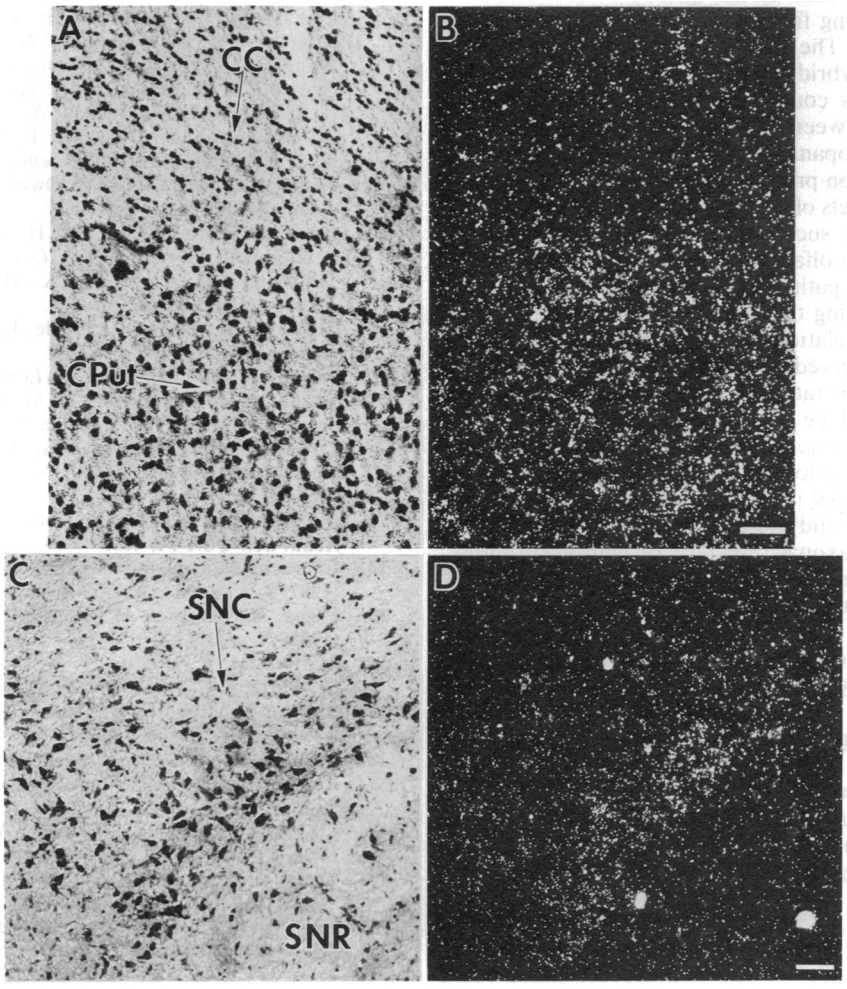

FIG. 3. Cellular localization of the dopamine D_2 receptor mRNA in the rat caudate-putamen nucleus (CPut) (*A* and *B*) and substantia nigra (*C* and *D*). Pictures are photomicrographs from Giemsa-stained tissue sections (*A* and *C*) or the autoradiograms (*B* and *D*) from the same sections that have been hybridized with the probe, dipped in nuclear track emulsion, and photographed under dark-field illumination. Autoradiographic grains are seen as bright points and show the enrichment of grains over cells in the nucleus caudatus or over the dopaminergic cells in the substantia nigra pars compacta (SNC) but not in the corpus callosum (CC) or the substantia nigra pars reticulata (SNR). (Bars = 0.1 mm.)

with our probes. These areas could be grouped in several anatomically different systems. First, in the olfactory bulb important densities of dopamine D_2 receptor binding have been visualized in the glomerular layer with many different ligands and after *in vivo* (19) and *in vitro* labeling (3, 14, 16). The density of binding sites is comparable, when SDZ 205-502 is used as ligand (14, 19), to that seen in the intermediate lobe of the pituitary. However, we did not detect any significant hybridization signal in the olfactory bulb (Fig. 2 *A* and *B*). A second system where the presence of dopamine D_2 receptor binding is also well documented is the visual system. In the rat brain, significant densities of dopamine D_2 receptor binding can be observed in the superficial gray layer of the superior colliculus and in the olivary pretectal nucleus, components of the visual pathway (14). We did not detect hybridization in these areas (Fig. 2 *I* and *J*). A third group of brain areas where dopamine D_2 receptor binding has been repeatedly shown comprises the lateral septum, the hippocampus, the entorhinal cortex, and other cortical areas. No detectable hybridization signal with our probes was observed in these regions. Finally, dopamine D_2 receptor ligands such as [^{125}I]iodosulpride (16) or [^{3}H]SDZ 205-502 (14) have shown that the rat cerebellum contains some binding sites with properties compatible with dopamine D_2 receptor binding. These binding sites are particularly enriched in the molecular layer of lobules 9 and 10 of the cerebellum and because of their columnar organization in this area, an association of these sites with climbing fibers from the inferior olive has been postulated (14). The cerebellum, however, did not present a detectable hybridization signal (Fig. 2 *K* and *L*).

Several explanations could be proposed to account for these discrepancies between the distribution of dopamine D_2 receptor binding and dopamine D_2 receptor mRNA. It could be that our hybridization probes are not sensitive enough to detect the very low levels of expression in those areas where receptor binding is low, such as the neocortex. Other areas, however, including the olfactory bulb, hippocampus, components of the visual pathways, and cerebellum, contain levels of receptor binding that are as high as those seen in areas where good correlation between binding and hybridization signal was observed. Alternatively, it could be postulated that the turnover rate is different and, consequently, mRNA transcripts will be below our detection level. We think, however, that for some areas, a simpler explanation could be that these receptors visualized by binding autoradiography are synthesized in cell bodies located outside the areas examined in this study. There is also evidence for the distant synthesis and axonal transport of neurotransmitter receptors in many brain areas and peripheral systems. Receptors in the olfactory and visual systems are probably synthesized by cells in, for example, the nasal epithelium and retina, respectively, and transported to the brain where they may be presynaptically located. This hypothesis does not account, however, for the lack of correlation between hybridization and binding in neocortex, hippocampal formation, and cerebellum, where the intrinsic and extrinsic sources of innervation were examined. No hybridization could be observed in any cell population in cortex, septum, thalamus, hypothalamus, cerebellar cortex, or inferior olive, to mention some of the possible sources. The only alternative left, excluding the lack of sensitivity, is that these receptors are encoded by mRNAs that are not recognized by our probes. The possible existence of multiple subtypes of dopamine D_2 receptor binding sites has been a matter of controversy (20) and at the present time is still unsettled.

In summary, using synthetic oligonucleotides as probes and *in situ* hybridization histochemistry, we have visualized the distribution of the cells expressing the mRNA coding for the dopamine D_2 receptors in the rat brain and pituitary gland. *In situ* hybridization histochemistry combined with immunohistochemical techniques (21) can be used to define the precise neurochemical characteristics of the cells expressing the dopamine D_2 receptor gene. Its combination with quantitative image analysis will also permit the study of alterations in the expression of the dopamine D_2 receptor gene after selective lesions or drug treatments.

The authors are grateful to K.-H. Wiederhold for the expert photographic work. M.I.M.-M. is supported by a fellowship from the Conselleria de Cultura, Educació i Ciència de la Generalitat Valenciana.

1. Kebabian, J. W. & Calne, D. B. (1979) *Nature (London)* **277**, 93–96.
2. Creese, I., Burt, D. R. & Snyder, S. H. (1978) in *Handbook of Psychopharmacology*, eds. Iversen, L. L., Iversen, S. D. & Snyder, S. H. (Plenum, New York), Vol. 10, pp. 37–89.
3. Palacios, J. M., Camps, M., Cortés, R. & Charuchinda, C. (1988) in *Parkinson's Disease and Movement Disorders*, eds. Jankovic, J. & Tolosa, E. (Urban & Schwarzenberg, Baltimore), pp. 27–36.
4. Wagner, H. N., Jr., Burns, H. D., Dannals, R. F., Wong, D. F., Langstrom, B., Duelfer, T., Frost, J. J., Ravert, H. T., Links, J. M., Rosenbloom, S. B., Lukas, S. E., Kramer, A. V. & Kuhar, M. J. (1983) *Science* **221**, 1264–1266.
5. Wong, D. F., Wagner, H. N., Jr., Tune, L. E., Dannals, R. F., Pearlson, G. D., Links, J. M., Tamminga, C. A., Broussolle, E. P., Ravert, H. T., Wilson, A. A., Toung, J. K. T., Malat, J., Williams, J. A., O'Tuama, L. A., Snyder, S. H., Kuhar, M. J. & Gjedde, A. (1986) *Science* **234**, 1558–1563.
6. Cross, A. J., Crow, T. J. & Owen, F. (1981) *Psychopharmacology* **74**, 122–124.
7. Farde, L., Wiesel, F. A., Hall, H., Halldin, C., Stone-Elander, S. & Sedvall, G. (1987) *Arch. Gen. Psychiatry* **44**, 671–672.
8. Trugman, J. M., Geary, W. A., II, & Wooten, G. F. (1986) *Nature (London)* **323**, 267–269.
9. Schwarcz, R., Creese, I., Coyle, J. T. & Snyder, S. H. (1978) *Nature (London)* **271**, 766–768.
10. Palacios, J. M. (1986) *Nature (London)* **323**, 205.
11. Buzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A. & Civelli, O. (1988) *Nature (London)* **336**, 783–787.
12. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
13. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4835–4838.
14. Charuchinda, C., Supavilai, P., Karobath, M. & Palacios, J. M. (1987) *J. Neurosci.* **7**, 1352–1360.
15. Pazos, A., Stoeckel, M. E., Hindelang, C. & Palacios, J. M. (1985) *Neurosci. Lett.* **59**, 1–7.
16. Martres, M.-P., Bouthenet, M.-L., Salès, N., Sokoloff, P. & Schwartz, J.-C. (1985) *Science* **228**, 752–755.
17. Savasta, M., Ruberte, E., Palacios, J. M. & Mengod, G. (1989) *Neuroscience* **29**, 363–369.
18. Palacios, J. M. & Pazos, A. (1986) in *Quantitative Receptor Autoradiography*, eds. Boast, C., Snowhill, E. & Altar, C. A. (Liss, New York), pp. 173–197.
19. Closse, A., Camps, M., Wanner, A. & Palacios, J. M. (1988) *Brain Res.* **440**, 123–132.
20. Creese, I. & Snyder, S. H. (1980) *Adv. Biochem. Psychopharmacol.* **24**, 89–94.
21. Chan-Palay, V., Yasargil, G., Hamid, Q., Polak, J. M. & Palay, S. L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3213–3215.