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D_1 dopamine receptor immunoreactivity in human and monkey cerebral cortex: Predominant and extrasynaptic localization in dendritic spines

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ABSTRACT **Antibodies to the D_1 dopamine receptor were used to localize this protein in several areas of human and monkey cerebral cortex with light and electron microscopy. In addition to cell body labeling in monkeys, all areas of humans and monkeys had a neuropil label with a laminar distribution predicted by previous D_1 receptor autoradiography studies. Using electron microscopy, this neuropil label was seen in numerous dendritic spines, in dendritic shafts, and in occasional axon terminals. While labeled spines were common, they represented only a subset of all cortical spines. Serial sectioning through labeled spines showed that the diaminobenzidine reaction product was usually not at postsynaptic densities but instead was displaced to the side of the large asymmetric (presumed glutamatergic) synapse. Furthermore, most labeled spines did not receive synapses with dopaminergic features, suggesting that many D_1 receptors are at extrasynaptic sites, possibly receiving dopamine via diffusion in the neuropil. Similarly, double labeling failed to reveal D_1 labeling at synapses of tyrosine hydroxylase immunoreactive axons. Localization to numerous dendritic spines suggests that a primary role of D_1 receptors is modulation of glutamatergic input to cortical pyramidal cells.**

Dopamine receptors in the cerebral cortex are a likely site at which antipsychotics and other pharmacological agents affect cognition. A description of the cortical circuitry of dopamine might lead to improved pharmacological therapies and perhaps to insights into psychiatric disorders. Although dopamine synapses and their cellular targets in the cortex have been described in both primates (1–3) and rats (4–7), it is not known which of the family of dopamine receptors are utilized by these synapses. Molecular approaches have identified five dopamine receptors to date (8), and it is plausible that these are differentially localized at subsets of dopamine synapses, or alternatively that some of them are colocalized. Furthermore, there is evidence that some dopamine receptors are present at sites other than conventional synapses. For example, neurotransmitter release studies in the basal ganglia indicate that axons of glutamate, acetylcholine, and possibly γ-aminobutyric acid (GABA) cells have functional dopamine receptors (9, 10), even though they do not receive dopamine synapses (11, 12). Nonsynaptic interactions could occur by close contact with dopamine axons (12) or by local diffusion of dopamine in the neuropil (13, 14).

The recent production of receptor subtype-specific antibodies (15–17, 47) provides tools to identify the cell types and neural processes that contain dopamine receptors. Initial studies in rats have demonstrated the expected high density of D_1 and D_2 immunoreactivity in the basal ganglia (15–17, 47). In the primate cerebral cortex, mRNA expression studies (18–22) and ligand binding studies (23, 24, 48) predict that D_1, D_4, and D_5 receptors are comparatively dense, with less D_2 receptors and perhaps no D_3 receptors. Here we demonstrate the distribution of D_1 dopamine receptor immunoreactivity in the human and monkey cerebral cortex. Aside from the potential clinical relevance, primate cortex is advantageous for these studies because it has a high density of dopamine innervation compared to rodent cortex (25).

MATERIALS AND METHODS

Two adult and one juvenile (14 months) macaque monkeys (*Macaca mulatta*) were perfused with 4% paraformaldehyde/0.08% glutaraldehyde/0.2% picric acid in phosphate buffer (PB) (pH 7.4) and processed as described (1), and tissue was selected from the frontal, parietal, and occipital cortices. Monkeys were used with regard to the National Institutes of Health Guidelines for Animal Research. Human cortex from two patients was obtained during surgery for intractable epilepsy. Tissue from the ventrolateral parietal cortex of a 26-year-old female, and occipital cortex including primary visual cortex of a 22-year-old female, was processed and immersion fixed in 4% paraformaldehyde/0.1 M PB, pH 7.4, as described (2). Human tissue was obtained with the informed consent of patients and with approval of the Yale Human Investigations Committee.

Two primary antibodies were used, a rat monoclonal and a rabbit polyclonal antibody, both directed to the C-terminal 97 amino acids of the human D_1 dopamine receptor fused to a polypeptide fragment of glutathione *S*-transferase (GST) (17). Immunolocalization was done with ABC-Elite kits from Vector Laboratories visualized by exposure to 0.03% diaminobenzidine, 0.01% H_2O_2 or with the diaminobenzidine glucose oxidase reaction (26). For double labeling, tissue already labeled for dopamine receptors was treated with anti-tyrosine hydroxylase (TH) antibody, which was visualized with silver-enhanced 1 nM gold-conjugated secondary antibodies (Amersham) as described (27). Rat anti-D_1 receptor antibody was visualized in the same tissue as rabbit anti-TH antibody (Pel-Freez Biologicals) and rabbit anti-D_1 antibody was combined with mouse anti-TH antibody (Chemicon).

Receptor labeling was processed simultaneously with the following controls: (*i*) Primary antibodies were omitted. (*ii*) Primary antibodies were preadsorbed with the D_1–GST fusion protein (0.5 mg/ml) conjugated to Affi-Gel beads (Bio-Rad) (17). (*iii*) Primary antibodies were similarly preadsorbed only with the GST fusion protein (0.5 mg/ml). With both antibodies, labeling seen with light microscopy was eliminated by omission of the primary antibody and preadsorption with the D_1–GST fusion protein but not by GST alone. For electron microscopy preadsorption control tissue processed

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Abbreviations: TH, tyrosine hydroxylase; GST, glutathione *S*-transferase.

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with the rat anti-D_1 antibody was sampled from layers II and V of all areas and was devoid of label except for rare diaminobenzidine precipitates on some mitochondrial membranes, which was easily distinguished from specific label.

RESULTS

Light Microscopy. Cerebral cortex was labeled with two antibodies to the C terminus of the human D_1 dopamine receptor (17). While both antibodies gave identical laminar distribution of label (Fig. 1), the rat monoclonal antibody gave a stronger signal than the rabbit polyclonal antibody and could also be seen in the perinuclear cytoplasm of numerous cells. While labeling in cell perikarya was not distinct in human cortex, it was present in all areas of monkey cortex, seen in numerous cells distributed in layers II–VI, most of which were clearly pyramidal in shape (Fig. 1 *B* and *C*). Electron microscopy showed this perikaryal labeling to be restricted to the Golgi apparatus (Fig. 1*F*).

Both antibodies labeled gray matter neuropil with a diffuse, granular appearing label (Fig. 1). In contrast to the perikaryal label, the neuropil label had a distinct laminar distribution, which was very similar to that of D_1 receptor ligand binding (23, 24). In the postcentral gyrus and in frontal, parietal, cingulate, and occipital association cortices, this labeling was heaviest in layers Ib–II and V–VI, lighter in layers Ia and III, and very light in layer IV, resulting in a bilaminar labeling pattern. In primary motor cortex this bilaminar pattern was less distinct, with comparatively dense labeling throughout the gray matter (Fig. 1*D*). Human and monkey primary visual cortex had a trilaminar pattern due to an additional band of label, localized to upper layer IVb in monkey (Fig. 1*G*). In human cortex, this middle band was light, had a patchy or discontinuous appearance, and was localized to layer IVa (Fig. 1*H*).

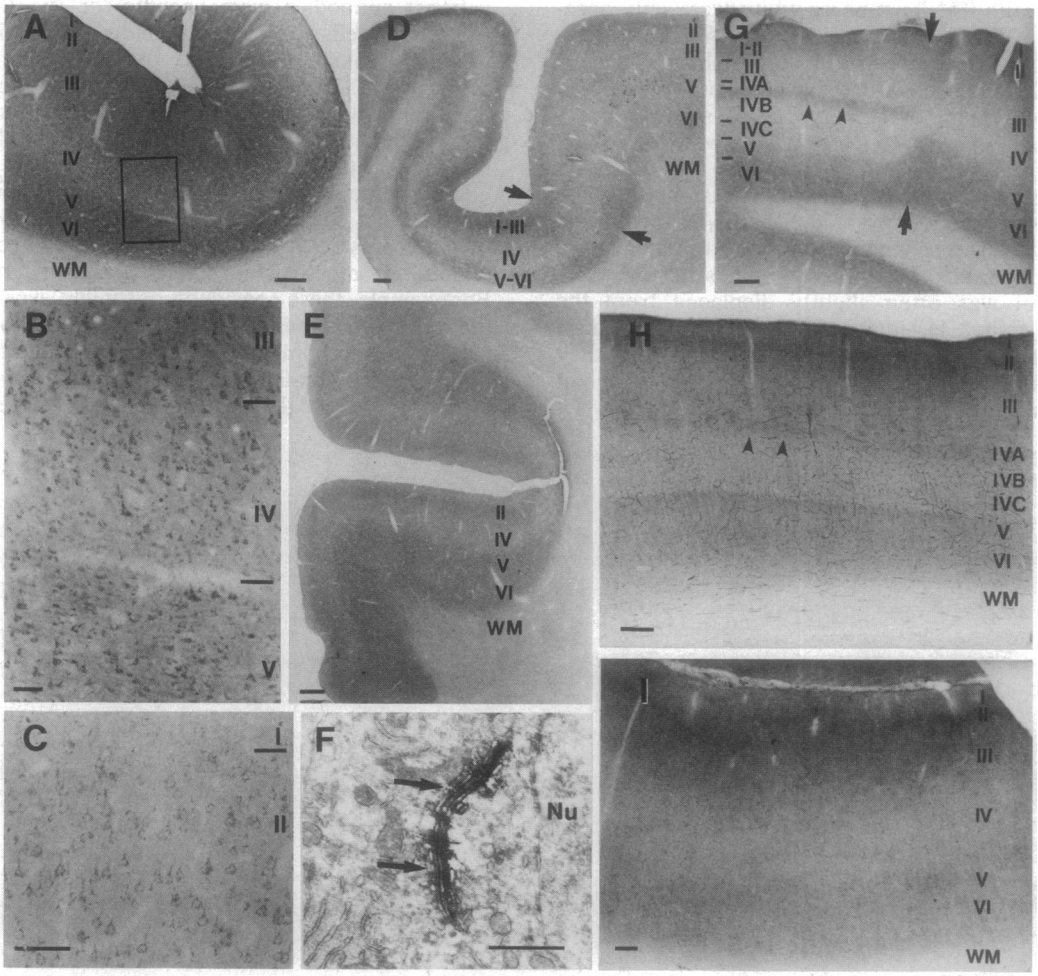

FIG. 1. Light microscopic appearance of D_1 immunoreactivity. Label is seen both as a diffuse neuropil label with a distinct laminar distribution and as a perikaryal labeling in cells of layers II–VI. Cortical layers were identified on Nissl-stained immunolabeled sections. The neuropil label had a laminar distribution predicted by receptor autoradiography studies (23, 24, 48), except for the localization in upper layer IVb instead of IVa in monkey visual cortex. This discrepancy might be explained by the higher resolution of immunocytochemistry compared to autoradiography. (*A*) Monkey prefrontal cortex (principal sulcus). Neuropil labeling is comparatively heavy in layers I–II and V–VI, slightly less in III, and sparse in IV, resulting in a bilaminar labeling pattern. (*B*) Enlargement of *A* (*Inset*), showing that bilaminar label distribution is due to diffuse neuropil label, distinct from immunoreactivity in cell perikarya. (*C*) Perikaryal labeling in layer II of monkey motor cortex. Numerous pyramidal-shaped cells were clearly visualized in layers II–VI of all areas of monkey cortex. (*D*) Monkey central sulcus. The distinct bilaminar pattern in the postcentral gyrus (left) gives way to a more homogeneous laminar distribution of label at the border with primary motor cortex (arrows). (*E*) Cortex on the banks and fundus of the monkey anterior cingulate sulcus displays a bilaminar neuropil label due to a comparative lack of label in layer IV. (*F*) Perikaryal labeling was due to D_1 immunoreactivity in the Golgi apparatus (arrows), seen in this low-power electron micrograph (Nu, cell nucleus). (*G*) Monkey primary visual cortex had a trilaminar labeling pattern, with label in layers I–II, upper IVb (arrowheads), and V–VI. At the border of areas 17 and 18 (arrows), this pattern gives way to a bilaminar pattern typical of most cortical areas. (*H*) Human primary visual cortex also had a trilaminar labeling pattern due to label in layers I–II, IVa (arrowheads), and V–VI. Blood vessels are visible in this sample due to endogenous peroxidases. (*I*) Human parietal cortex had a bilaminar pattern of label similar to monkey association cortex. WM, white matter. (*A*, *H*, and *I*, bars = 250 μm; *B* and *C*, bars = 50 μm; *D* and *E*, bars = 500 μm; *F*, bar = 1 μm.)

Electron Microscopy. Electron microscopy of samples from layers II and V of all areas showed a striking consistency of D_1 immunoreactivity that was similar with both antibodies. An abundance of dendritic spines constituted the most common labeled structure, with somewhat less label in dendritic shafts and uncommon label in axon terminals. Although labeled spines were plentiful, they represented only a fraction of all spines, suggesting a selectivity of D_1 receptor effects.

D_1 immunoreactivity sometimes filled spines but more often was seen as a patch of label in the spine neck or head (Fig. 2). In the spine head, it was often displaced to the side of the postsynaptic density of the large asymmetric synapse. Serial sections through labeled spines typically demonstrated only a single synaptic input, which was invariably large and asymmetric, characteristic of glutamate but not dopamine synapses (3). The absence of conventional dopamine synapses on D_1 immunoreactive spines was further supported by double labeling in layers I–II of monkey prefrontal cortex. Axons labeled with TH immunoreactivity were only occasionally seen in direct contact with D_1 immunoreactivity, even though they were often in close proximity. Furthermore, none of 21 synapses formed by TH axons was seen to be labeled with D_1 immunoreactivity (Fig. 3), suggesting that some dopamine synapses lack or have low levels of D_1 receptors.

In dendritic shafts, D_1 immunoreactivity also was usually seen as patches of reaction product, either in the cytoplasm or associated with the postsynaptic density of asymmetric synapses (data not shown). Similar to spines this synapse-associated label was often slightly displaced from the postsynaptic density. D_1 immunoreactive axon terminals (data not shown) did not appear to be dopaminergic; they usually made large asymmetric synapses uncharacteristic of dopamine axons, and in double-labeling experiments D_1 immunoreactivity was not seen in TH immunoreactive axons. In some cases D_1 immunoreactive axons were seen to form asymmetric synapses on D_1 immunoreactive spines.

DISCUSSION

The following arguments support the specificity of the D_1 immunoreactivity described. First, preadsorption controls were devoid of label. Second, although the rat monoclonal antibody gave more intense label, the labeling was very similar with both antibodies. Third, the laminar distribution of labeling was in good agreement with ligand binding studies, which identified the D_1 class of receptors (23, 24, 48). Fourth, these same antibodies labeled postsynaptic densities of some TH immunoreactive synapses in the monkey caudate (unpublished observation). Finally, these affinity-purified antibodies were previously shown to label D_1-rich areas of the rat brain, and both the rabbit (17) and rat (unpublished observations) antibodies were specific for the D_1 receptor in transfected cells and in Western blots of striatal tissue. Taken together, these data argue that the immunoreactivity achieved with these antibodies reflects the cortical distribution of the D_1 receptor. However, as is common with immunocytochemistry, we cannot exclude the possibility of cross-reactivity with some uncharacterized protein with high sequence homology to the D_1 dopamine receptor. Nor can we exclude that some D_1 receptors were overlooked because they are present at low concentrations or because they are structurally modified during cell transport and processing. For example, these considerations might especially apply to the perikaryal labeling in monkey cortex, which was not clearly seen in human or in rat (unpublished observation).

Cortical Distribution of D_1 Immunoreactivity. Electron microscopy revealed that the diffuse neuropil labeling seen with

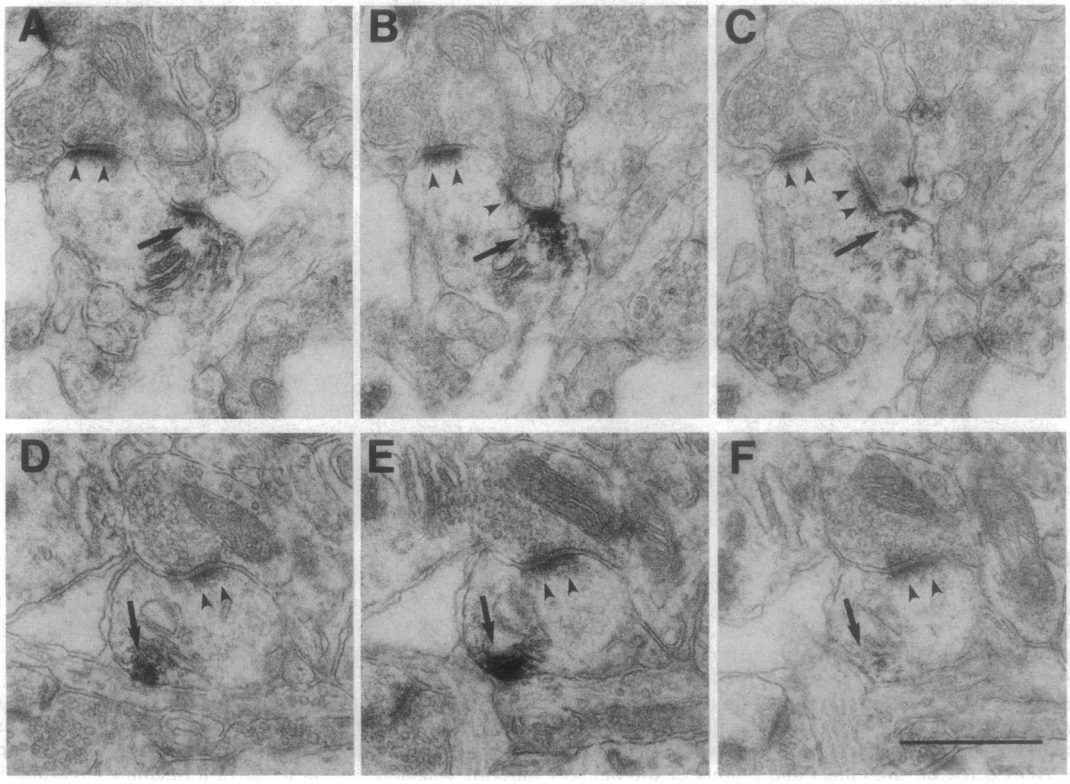

FIG. 2. With electron microscopy, D_1 immunoreactivity was most commonly found in dendritic spines. Label was usually seen as a patch of membrane-associated reaction product, which was not directly apposed to a synaptic specialization, even though it was often near the asymmetric (presumed glutamatergic) synaptic density. (*A–C*) Serial sections showing D_1 immunoreactivity (arrows) to the side of an asymmetric synapse (arrowheads) in a spine head. (*D–F*) Serial sections showing D_1 immunoreactivity across from an asymmetric synapse onto a spine head. Label was not present in subsequent sections on either of these spines, and no other synapses were present. (Bar = 0.5 μm.)

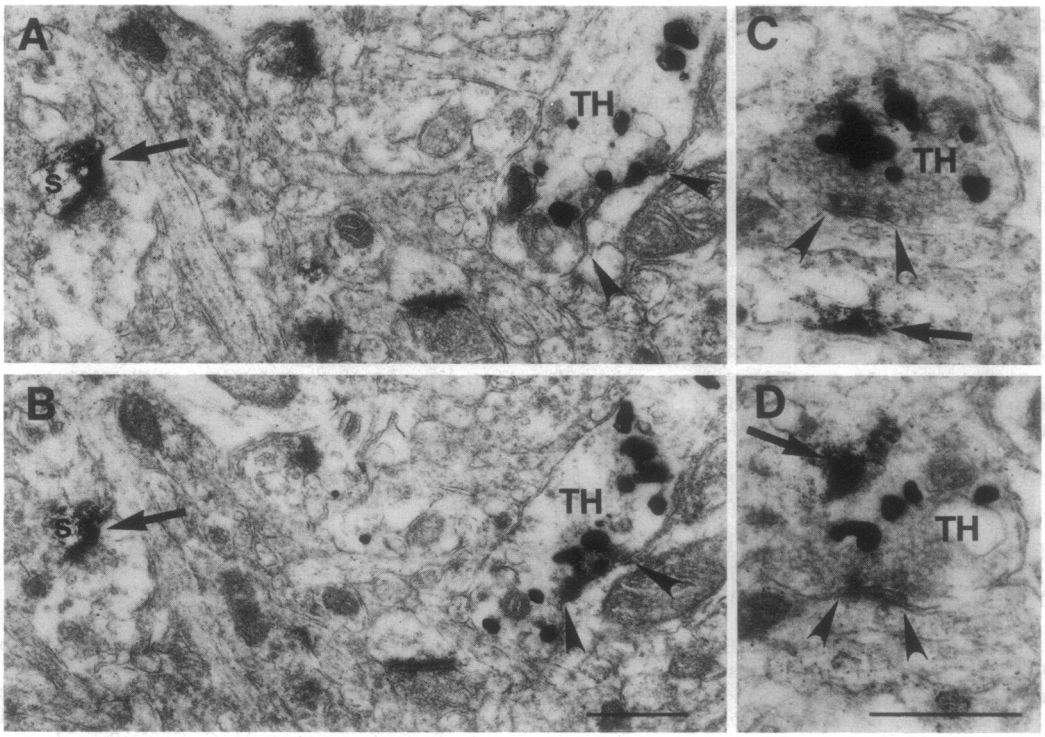

FIG. 3. Double labeling with TH and D_1 immunoreactivity failed to demonstrate D_1 labeling at synapses of TH axons. (*A* and *B*) Serial sections showing a TH immunoreactive axon (TH) labeled with silver-enhanced colloidal gold forming a synapse (arrowheads), which is not D_1 immunoreactive. A D_1 immunoreactive spine (S) is seen nearby (arrows). (*C* and *D*) Serial sections showing a TH immunoreactive axon forming a synapse that is not D_1 immunoreactive. D_1 immunoreactivity is seen in two immediately adjacent dendrites (arrows). In *D*, the D_1 immunoreactivity is seen abutting the TH axon distal from the synapse. Such direct contact between TH axons and D_1 immunoreactivity was uncommon. (Bars = 0.5 μm.)

light microscopy was due to patches of label found predominantly in dendritic spines but also in dendritic shafts and occasional axon terminals. This ultrastructural distribution is similar to that described in the rat caudate (15, 17), suggesting a parallel organization of D_1-mediated effects in the cortex and basal ganglia, even though their input is from different dopamine cells (28).

Although the abundance of D_1 immunoreactive spines is consistent with our previous descriptions of dopamine synapses (1–3), the present results indicate that not all and perhaps not even most D_1 receptors are directly apposed to dopamine synapses. First, serial sectioning through labeled spines typically did not reveal dopamine-like synapses but only the large asymmetric synapses characteristic of glutamate axons. Second, double labeling showed only infrequent contacts between TH axons and D_1 immunoreactivity, even though the two were often in close proximity. Third, we were unable to demonstrate D_1 immunoreactivity at synapses formed by TH immunoreactive axons. The latter result is not unambiguous, because it is possible that a subpopulation of D_1 immunoreactive synapses was overlooked, or that the D_1 receptors at these synapses were below the level of detection with the labeling method used. Nevertheless, the findings suggest that some or all cortical dopamine synapses do not utilize D_1 receptors and that a substantial portion of D_1 effects occur at sites other than synaptic specializations. Combined with our previous demonstrations of frequent dopamine synapses to dendritic spines and shafts (1, 3, 5), these results indicate a coexistence of synaptic and nonsynaptic mechanisms of dopamine neurotransmission.

Because immunocytochemistry for electron microscopy does not reliably label all antigenic processes, we cannot exclude the presence of fine dopamine (i.e., TH immunoreactive) processes directly apposed to D_1 immunoreactive sites. However, several lines of evidence have indicated neurotransmitter effects via diffusion in the neuropil (see ref. 14), and similar extrasynaptic localizations have been reported for glycinergic (29), muscarinic (30), and glutamatergic (31) receptors. Furthermore, electrochemical measurements indicate that physiologically relevant dopamine concentrations can diffuse from the synaptic cleft (13). The present finding that D_1 immunoreactivity is not frequently in contact with identified TH axons provides anatomical support for diffusion as a mechanism of dopamine transmission.

In addition to the neuropil labeling, our findings in monkey cortex indicate that D_1 receptors are produced by numerous cells in layers II–VI and that most of these cells have the appearance of pyramidal cells. This cellular distribution is in agreement with *in situ* hybridization studies in monkey and human motor cortex (21). Rat neocortex appears to have substantially less D_1 receptor immunoreactivity (17), in agreement with *in situ* hybridization studies, which found D_1 receptor mRNA mainly in cells of the deep cortical layers (32–35). While one published account of D_1 immunoreactivity in rat neocortex found a strong bilaminar cell distribution (15), it has been pointed out that the antibody used might detect another protein in addition to the D_1 receptor (17).

Physiological Implications. The presence of D_1 immunoreactivity in many cells and abundant spines is consistent with physiological studies, which have shown dopamine responses in most if not all cortical neurons (36–39). Localization in spines suggests a D_1 modulation of glutamatergic synapses onto pyramidal cells, given that almost all cortical spines belong to pyramidal cells (40, 41) and that spines are the major site of glutamate input to pyramidal cells (42). Physiological studies have often proposed a glutamate modulating role of dopamine (38, 39, 43–45), which is supported by recent studies in human and rat cortex demonstrating differential effects of dopamine on responses to *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors

(38, 39). Similar effects have also been seen in rat caudate (39), and it seems possible that glutamate modulation is a common theme of dopamine actions in the central nervous system. In the caudate, dopamine synapses are found on only a subset of dendritic spines, suggesting a selective modulation of a subpopulation of glutamatergic synapses (46). The presence of D_1 receptors in a subset of cortical spines might indicate a similar selectivity in the cerebral cortex.

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