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Regional, Cellular, and Subcellular Variations in the Distribution of D_1 and D_5 Dopamine Receptors in Primate Brain

Clare Bergson,[1,a] Ladislav Mrzljak,[2,a] John F. Smiley,[2] Mariamma Pappy,[2] Robert Levenson,[1] and Patricia S. Goldman-Rakic[2]

[1]Department of Pharmacology, Pennsylvania State College of Medicine, and [2]Section of Neurobiology, Yale University School of Medicine, New Haven, Connecticut 06510

The pathways governing signal transduction in the mesocortical and nigrostriatal dopamine systems of the brain are of central importance in a variety of drug actions and neurological diseases. We have analyzed the regional, cellular, and subcellular distribution of the closely related D_1 and D_5 subtypes of dopamine receptors in the cerebral cortex and selected subcortical structures of rhesus monkey using subtype specific antibodies. The distribution of D_1 and D_5 receptors was highly differentiated in subcortical structures. In the neostriatum, both D_1 and to a lesser extent D_5 antibodies labeled medium spiny neurons, while only D_5 antibodies labeled the large aspiny neurons typical of cholinergic interneurons. In the caudate nucleus, D_1 labeling was concentrated in the spines and shafts of projection neurons, whereas D_5 antibodies predominantly labeled the shafts, and less commonly, the spines of these cells. The D_1 receptor was abundantly expressed in the neuropil of the substantia nigra pars reticulata while the D_5 antibodies labeled only a few scattered cell bodies in this structure. Conversely, D_5 antibodies labeled cholinergic neurons in the basal forebrain more intensely than D_1 antibodies. Within the cerebral cortex and hippocampus, D_1 and D_5 antibody labeling was prominent in pyramidal cells. Double-label experiments revealed that the two receptors were frequently coexpressed in neurons of both structures. Ultrastructurally, D_1 receptors were especially prominent in dendritic spines whereas dendritic shafts were more prominently labeled by the D_5 receptor. The anatomical segregation of the D_1 and D_5 receptors at the subcellular level in cerebral cortex and at the cellular level in subcortical areas suggest that these closely related receptors may be preferentially associated with different circuit elements and may play distinct regulatory roles in synaptic transmission.

[Key words: antibody, immunofluorescence, electron microscopy, Western blotting, prefrontal cortex, working memory]

Insight into the pathways governing dopaminergic signaling in the brain has significant pharmacological and clinical importance. Schizophrenia is a prime example of a disorder that appears to involve imbalances in the mesocortical dopaminergic pathway (Seeman, 1987; Davis et al., 1991). Parkinson's disease, Tourette's syndrome, and addictive behavior represent neurological and affective disorders that also manifest significant dopaminergic involvement (Sen and Lee, 1988a,b). Knowledge of the cellular and subcellular distribution of the different dopamine (DA) receptor subtypes is therefore essential for understanding the role of the dopaminergic system in mental illness and other neuropathologies.

The physiological effects of DA neurotransmission in brain are mediated by a family of G-(guanine nucleotide-binding) protein coupled receptors (Dohlman et al., 1991). Molecular cloning has revealed the existence of five mammalian DA receptor genes (D_1–D_5), each encoding a pharmacologically distinct DA receptor subtype (reviewed in Civelli et al., 1993; Gingrich and Caron, 1993) which have been grouped into the D_1- (D_1 and D_5) and D_2- (D_2, D_3, and D_4) subfamilies based on sequence homologies and pharmacological profiles. The present study focuses on the D_1 and D_5 DA receptors.

The pharmacological and physiological properties of the D_1 and D_5 receptors have been studied primarily in transfected cell lines. In general, these studies suggest that the D_1 and D_5 receptor polypeptides display similar affinities for D_1-specific ligands, but that the D_5 receptor displays a somewhat higher affinity for DA (Grandy et al., 1991; Sunahara et al., 1991, Tiberi et al., 1991; Tiberi and Caron, 1994). While it is known that the D_1 and D_5 receptors can stimulate cAMP, presumably through a G_s-type of G-protein, D_5 transfected cells exhibit higher basal, but lower agonist stimulated adenylyl cyclase activity than do cells expressing the D_1 receptor (Tiberi and Caron, 1994). It is possible that the D_1 and D_5 receptors might also couple to other second messenger systems via different G-proteins (Kimura et al., 1995). Although not exhaustive, these studies provide hints that functional differences between the two receptors may be cell type dependent.

Autoradiographic studies in rhesus monkey brain have shown that binding sites for the D_1-specific ligand, SCH 23390, are highly concentrated in the cerebral cortex relative to D_2 binding sites (Goldman-Rakic et al., 1990; Lidow et al., 1991). Previous work from this laboratory has demonstrated that both D_1 and D_5 receptors localize to cortical pyramidal cells (Smiley et al., 1994; Bergson et al., 1995) where the D_1 receptor is preferentially localized in pyramidal cell dendrites and spines (Smiley et al.,

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Correspondence should be addressed to Patricia S. Goldman-Rakic, Section of Neurobiology, Yale University School of Medicine, P.O. Box 208001, New Haven, CT 06510.

[a] Contributed equally to this work.

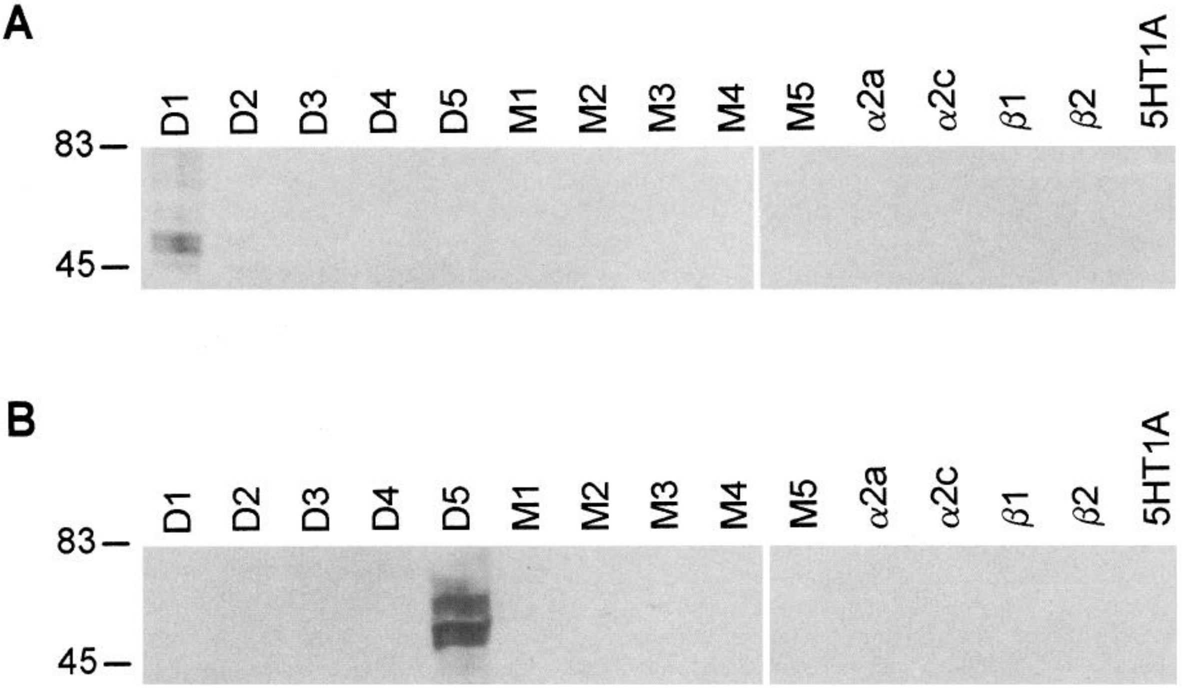

Figure 1. Subtype specificity of D_1 and D_5 antibodies. D_1 (*A*) or D_5 (*B*) antibodies reacted with immunoblots of crude membrane fractions (5 μg/lane) prepared from recombinant *Sf*9 cells expressing human D_1 D_2, D_{3rat}, $D_{4.2}$, and D_5 dopaminergic; M1, M2, M3, M4, and M5 muscarinic, α2a, α2c, β1 and β2 adrenergic; and 5HT1A serotonergic receptors. Proteins were fractionated on SDS-containing 10% polyacrylamide gels, and transferred to poly(vinylidene difluoride) filters. The positions of molecular mass markers in kDa are indicated on the left.

1994). Recent functional studies suggest a highly specific role for D_1-like receptors in modulating the excitatory inputs that establish spatial memory fields in the monkey prefrontal cortex (Williams and Goldman-Rakic, 1995). This modulation is presumably accomplished by the selective distribution of D_1 and/or D_5 receptors in prefrontal neurons. To better understand the molecular mechanisms underlying this mnemonic process, it is important to determine whether the D_5 receptor is localized to the same cellular compartments as the D_1 receptor, for example, the spines of pyramidal cells. In addition, the molecular and pharmacological similarities between the two receptors raise important questions about the organization of the dopaminergic system. Are the D_1 and D_5 receptors present in the same cell types as has been shown for members of the D_2 family of DA receptors (Surmeier et al., 1992)? Do the two subtypes subserve different functions related to the different circuit elements which contact them? To begin to address these issues, we have analyzed the distribution of the D_1 and D_5 receptor polypeptides at the cellular and subcellular level in various brain regions and neuronal cell classes of the rhesus monkey brain.

Material and Methods

Fusion protein constructs. cDNA fragments encoding the divergent C-terminal regions of the human DA D_1 (aa residues 364–448) and D_5 (aa residues 375–477) receptors were generated by PCR using human D_1 (Sunahara et al., 1990; Zhou et al., 1990) and D_5 (Grandy et al., 1991; Sunahara et al., 1991) cDNAs as template. The following synthetic oligonucleotide primer pairs were used for PCR amplification: (1) $D_1$5′: 5′-GTGGAATTCAACAATGGGGCCGCGATGTTT-3′/ $D_1$3′: 5′-TACGTCGACTCAGGTTGGGTGCTGACCGTT-3′, (2) $D_5$5′: 5′-TTGGAATTCAGCCACTTCTGCTCCCGCACG-3′/ $D_5$3′: 5′-GCGTCGACAGTTTAATGGAATCCATTCGGG-3′. PCR was carried out with *Pfu* polymerase and *Pfu* buffer #1 (Stratagene) and run for 35 cycles (1 min at 95°C, 1 min at 50°C, 1 min at 72°C) using a Coy thermocycler. The PCR products were digested with *Eco*RI and *Sal*I and inserted in-frame into a modified version of the bacterial expression vector pMalc2 (New England Biolabs) producing maltose binding protein (MBP)-dopamine receptor fusion proteins, MBP-D_1 and MBP-D_5. pMalc2 was modified by insertion of an *Nhe*I linker into the *Hind*III site of the polylinker sequence, introducing stop codons into all three reading frames. A second set of gene fusions was generated by subcloning the D_1 and D_5 PCR products into the expression vector pGEX-4T-1 (Pharmacia) to yield plasmids encoding glutathione *S*-transferase (GST)-dopamine receptor fusion proteins GST-D_1 and GST-D_5. All constructs were confirmed by dideoxynucleotide chain termination sequencing.

Preparation of fusion proteins and rabbit immunizations. MBP-D_1 and MBP-D_5 were induced in *E. coli* strain XL-1 blue by the addition of 1 mM isopropyl thiogalactoside. Soluble fusion proteins were purified using amylose resin (New England Biolabs) according to the manufacturers instructions and protein concentrations determined as described by Bradford (1976). Three New Zealand White rabbits were immunized with each fusion protein as previously described (Shyjan and Levenson, 1989). Antibodies reactive with the D_1 and D_5 portions of MBP-D_1 and MBP-D_5 were affinity purified on nitrocellulose strips containing the GST-dopamine receptor fusion proteins GST-D_1 and GST-D_5, respectively (Sambrook et al., 1989).

Membrane preparation, N-*glycosidase F digestion, and immunoblotting.* Membranes from recombinant baculovirus-infected *Sf*9 cells expressing dopaminergic, muscarinic, adrenergic and serotonergic receptors were a generous gift of Dr. Michael Dennis (Biosignal, Inc., Montreal, Canada). Two grams of prefrontal cortex and caudate were dounce-homogenized on ice in 25 ml buffer [250 mM sucrose, 10 mM Tris, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4], and centrifuged at 600 × *g* for 10 minutes. The microsomal fraction was pelleted by centrifugation of the supernatant in a Ti70 rotor at 37,500 rpm for 60 min and resuspended in 10 mM Tris, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, pH 7.4. To remove N-linked sugars, 50 mg of brain microsomes were heated to 65°C for 15 min in 0.5% SDS, 1% b-mercaptoethanol (BME). The denatured proteins were incubated at 37°C with 500 U *N*- glycosidase F (PNGaseF, New England Biolabs) for 16 hr. following addition of $NaPO_4$ (pH 7.5) and NP-40 to final concentrations of 50 mM and 1%, respectively. The reaction was stopped by addition of an equal volume of 2 × SDS-PAGE buffer (4% SDS, 50 mM Tris pH 6.8, 17.5% glycerol, 0.5% BME, and 0.025% bromophenol blue).

Solubilized microsomal proteins were fractionated by SDS-PAGE and transferred to PVDF or nitrocellulose sheets (Towbin et al., 1979). The nitrocellulose was quenched in 5% milk, 10% goat serum, and 0.1% Tween 20 in TBS (20 mM Tris, 137 mM NaCl, pH 7.6), followed by incubation with affinity purified human D_1 (1 mg/ml) or D_5 (1 mg/ml) antibodies. Blots were rinsed with TBS, 0.1% Tween 20, and then incubated with goat anti-rabbit IgG (1.6 mg/ml) (BRL). Bound antibody was detected by enhanced chemiluminescence (ECL) using an ECL kit (Amersham).

Tissue fixation and immunohistochemistry. Perfusion and preparation of brain tissue from five adult macaque monkeys (*Macaca mulatta*) was carried out as described previously (Mrzljak et al., 1993). Briefly, monkeys were anesthetized with sodium pentobarbital (Nembutal) (100 mg/ml). After initial saline perfusion, monkeys were perfused with 4% paraformaldehyde/0.1–0.15% glutaraldehyde/15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4. Tissue blocks were postfixed for 2 hr in the same fixative. The same immunohistochemical protocols were used for both light and electron microscopy. Sections were incubated for 48 hr (4°C) with either D_1 or D_5 antibodies (1:100 and 1:200), and processed by the avidin–biotin method using horseradish peroxidase (HRP) conjugated goat anti-rabbit biotinylated antibodies (Vector Labs) and ABC Elite kit (Vector labs). To visualize bound antibodies, sections were incubated with 0.05% diaminobenzidine (DAB) in the presence of 0.01% hydrogen peroxide in PB or with the DAB-glucose oxidase reaction in the presence of nickel-ammonium sulfate (Zaborsky and Heimer, 1989). Sections were dehydrated in ethanol, cleared with xylene, and photographed with a Zeiss Aristoplan microscope. Sections for electron microscopy were subsequently postfixed in 1% osmium tetroxide. Blocks containing area 46 and 9, the head of caudate nucleus and the CA1 field of the hippocampus were cut serially into ultrathin sections on an Ultramicrotome (Reichert), stained with lead citrate and uranyl acetate, and examined with a Jeol transmission electron microscope. To rule out the possibility of nonspecific immunoreactivity, we performed a series of control experiments including: (1) incubation of primary antibodies in the presence of cognate fusion proteins, (2) incubation of preimmune sera in place of primary antibodies, and (3) omission of primary antibodies. Under these conditions, we observed no detectable immunoreaction product when sections were analyzed by light or electron microscopy.

When double-labeled, sections were incubated in a cocktail of the rat D_1 monoclonal antibody (Mab) (RBI) (Levey et al., 1993; Smiley et al., 1994) and rabbit D_5 polyclonal antibodies (Bergson et al., 1995), washed, and incubated with CY3-conjugated, anti-rabbit antibodies (Jackson), and biotinylated anti-rat antibodies plus FITC-linked avidin-D (Vector) for detection of the D_5 and D_1 receptors, respectively. To control for possible cross-reactivity of the secondary antibodies, parallel sections were incubated with the D_1 or D_5 primary antibody, followed by incubation with the heterologous secondary antibody. No signal was produced by these antibody combinations or by incubating tissue with either secondary antibody alone.

Quantitative analysis of electron microscopic data. Quantitative analysis of D_1 and D_5 positive spines was performed in layer III of area 46 in three animals. Spines were defined as structures containing a spine apparatus and no mitochondria (Peters et al., 1991). A total of 257 electron micrographs at a final magnification 50,000× were sampled.

Results

Antibodies specific for the D_1 and D_5 receptors

Polyclonal antibodies raised against the divergent C-terminal sequences of the D_1 and D_5 DA receptors were affinity-purified and tested for subtype specificity. D_1 and D_5 antibodies were reacted with immunoblots of membrane preparations of recombinant baculovirus-infected insect *Sf*9 cells expressing a spectrum of G-protein coupled receptors including the D_1 D_2, D_3, D_4, and D_5 DA receptors. D_1 antibodies bound to a broad band (~46–55 kDa) present in cells expressing only the D_1 subtype and not any of the 14 other dopaminergic, muscarinic, adrenergic or serotonergic receptors on the immunoblot (Fig. 1*A*). Likewise, D_5 antibodies bound selectively to a broad band (~50–66 kDa) present only in the membrane fractions of D_5-expressing *Sf*9 cells (Fig. 1*B*). These results suggest that the antibodies are specific for the D_1 and D_5 receptors, respectively.

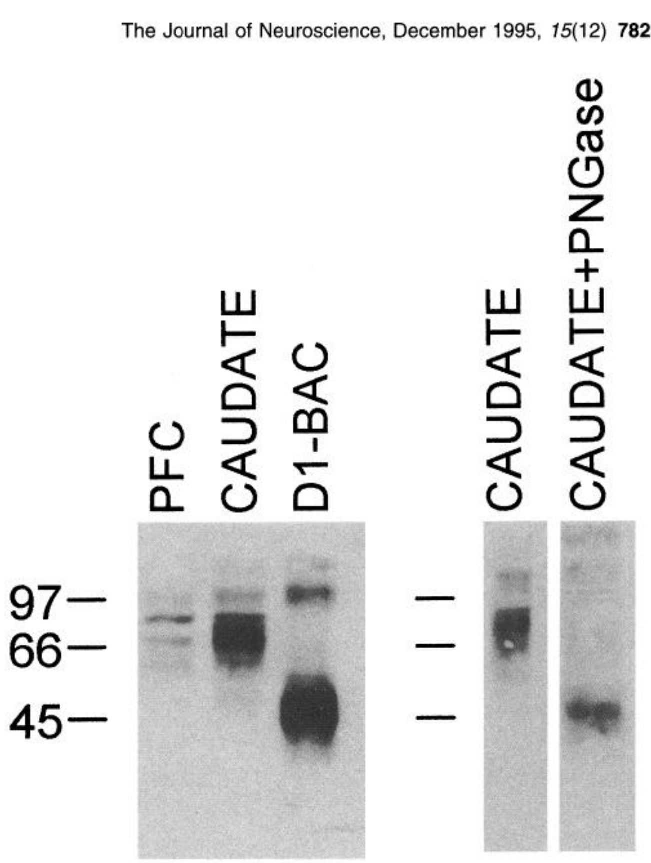

Figure 2. Expression of the D_1 dopamine receptor in macaque caudate and prefrontal cortex. *A*, Reaction of D_1 antibodies with solubilized membranes prepared from prefrontal cortex (*PFC*), caudate (*CAUDATE*), baculovirus-infected *Sf*9 cells expressing the human D_1 dopamine receptors (*D1-BAC*), caudate membranes incubated with *N*-glycosidase-F digestion buffer alone (*CAUDATE*) and caudate membranes incubated with *N*-glycosidase-F (CAUDATE+PNGase). Proteins were fractionated in an SDS containing 10% acrylamide gel. The positions of the molecular mass markers in kDa are indicated to the left.

The specificity of the D_1 receptor antibodies was further tested on immunoblots containing solubilized microsomes prepared from monkey caudate and prefrontal cortex. As shown in Figure 2, D_1 antibodies bound to bands exhibiting a broad molecular mass distribution (~58–85 kDa) in the caudate and to less prominent bands of similar M_r in the prefrontal cortex. Comparison of the mobility of the D_1 antibody reactive polypeptides in brain membranes with those expressed by the recombinant baculovirus indicated that in brain, the D_1 receptor is much more extensively glycosylated than the D_1 receptor expressed in *Sf*9 insect cells. Following treatment of caudate membranes with *N*-glycosidase F (Tarentino et al., 1985), D_1 receptor antibodies bound to a single band with an M_r of 47 kDa (Fig. 2). The mobility of this band is in good agreement with the predicted size of 49,060 for the core human D_1 receptor (Sunahara et al., 1990; Zhou et al., 1991). The specificity of the affinity-purified D_5 antibodies for the D_5 receptor has been described in detail previously by Bergson et al. (1995).

D_1 and D_5 antibodies were further examined by incubating affinity purified antibodies with coronal sections of rhesus monkey brain in the presence and absence of the complementary MBP-D_1 or MBP-D_5 fusion protein. In the absence of complementary fusion protein, incubation of tissue section with the D_1 or D_5 antibodies gave distinct staining patterns. In contrast, incubation with the complementary MBP fusion protein abolished

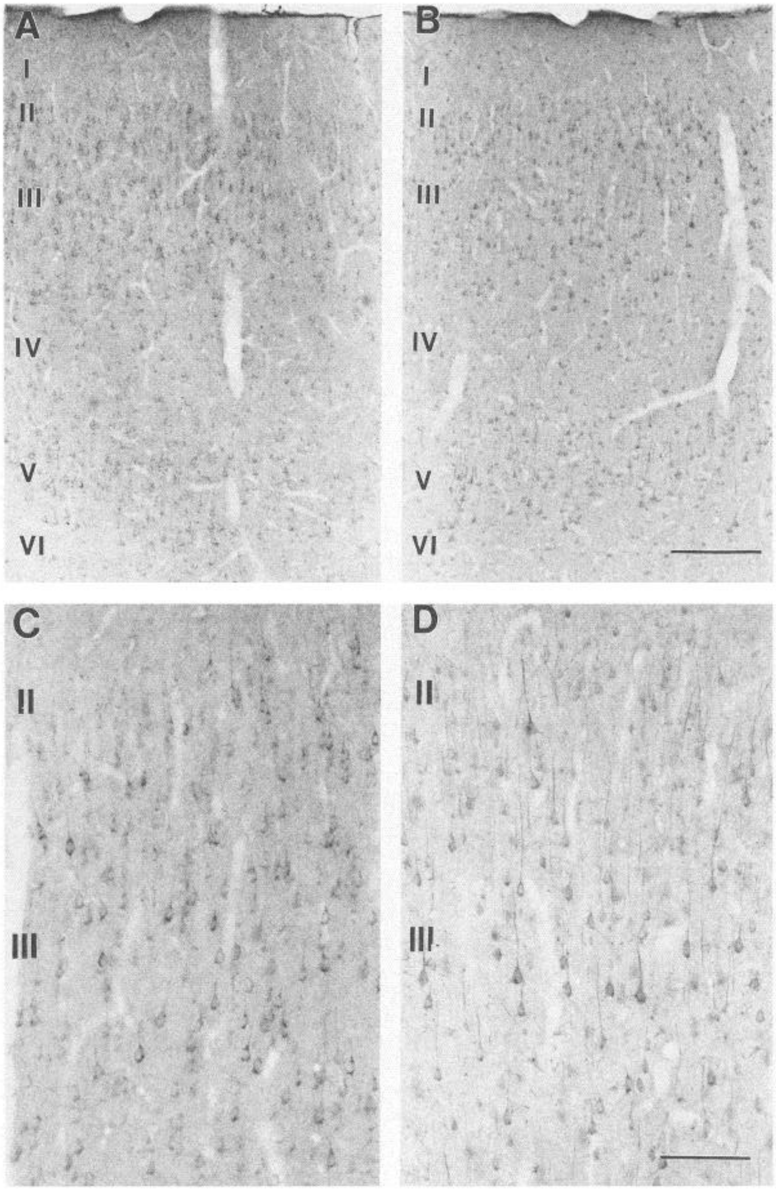

Figure 3. Immunohistochemical staining of DA D_1 and D_5 receptors in macaque neocortex. *A–B,* Micrographs of medial prefrontal cortex (area 9) stained with D_1 (*A*) or D_5 (*B*) antibodies. Neurons in layers II, III, and V are the most prominently stained with both antibodies. *C–D,* Higher magnification of D_1 (*C*) and D_5 (*D*) labeled neurons in layers II and III. D_5 immunostaining is more intense and exhibits Golgi-like staining of pyramidal cell apical and basal dendrites. Scale bars: *A–B,* 250 μm; *C–D,* 50 μm.

immunostaining, as did omission of the D_1 or D_5 antibodies from the immunohistochemical procedure. Together, these results establish that the D_1 and D_5 antibodies we developed are specific for their respective DA receptor subtypes.

D_1 and D_5 receptor immunoreactivity in cerebral cortex and hippocampus

We surveyed the distribution of the D1 and D5 receptors in specific cortical areas including prefrontal and premotor areas of the frontal lobe and the hippocampus; medial and lateral area of the temporal lobe; the motor, somatosensory and insular cortex; and the primary and secondary visual areas. Light microscopic analysis of D_1 and D_5 antibody staining revealed expression of D_1 and D_5 receptors in pyramidal neuronal populations in all of the neo- (prefrontal and premotor), meso- (cingulate and entorhinal) and archicortical (hippocampus and dentate gyrus) areas of monkey brain examined (Figs. 3, 4). The only apparent difference observed in the staining pattern was a more prominent staining of dendrites with D_5 receptor antibodies than with D_1 receptor antibodies. In the prefrontal (Walker areas 9, 10, 11, 12, and 46) and premotor (8, 6, and supplementary motor area) cortex, D_1 and D_5 immunoreactive neurons were visible in all cortical layers. However, labeled cells were most prominent in layers II, III and V (Fig. 3*A–D*) and in the pyramidal layer of CA1-CA3 subfields of the hippocampus and granular layer of the dentate gyrus (Fig. 4*A–D*). In the cortex and hippocampal subfields, labeling with D_1 and D_5 antibodies was predominantly associated with the soma and apical dendrites of pyramidal neurons (Figs. 3*C,D,* 4*C,D*), although some nonpyramidal neurons were labeled in the cortex and many were labeled in the polymorphic layer and hilar area of the hippocampus.

The similarity of the staining patterns in cortex obtained with the D_1 and D_5 receptor antibodies raised the possibility that the two receptors may be coexpressed within the same pyramidal neurons. To address this issue, we used double-label immunofluorescence microscopy. The results are shown in Figure 5. The soma and apical dendrites of pyramidal neurons in all cortical layers as well as in the hippocampal subfields and dentate gyrus

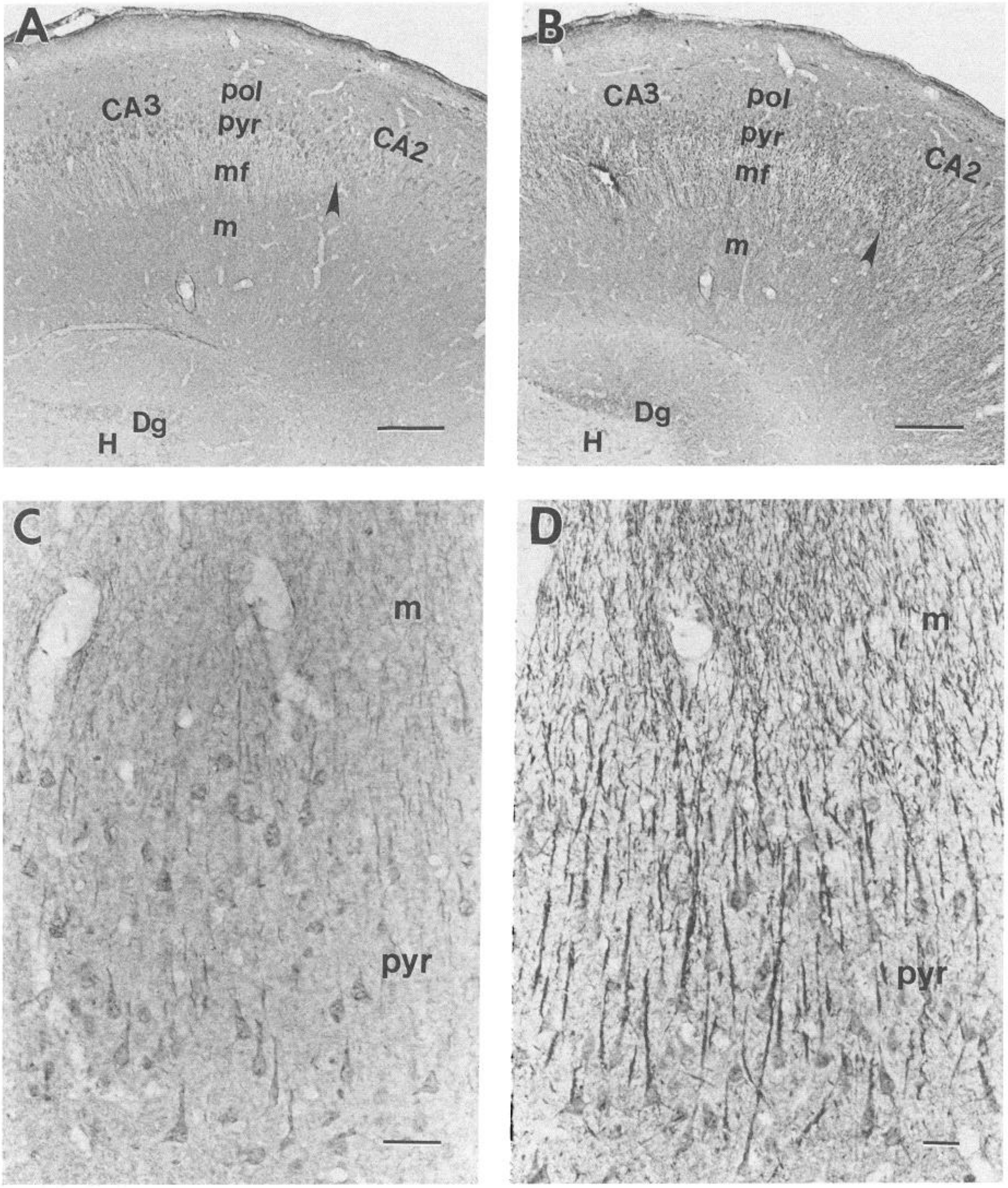

Figure 4. Immunohistochemical localization of D_1 and D_5 receptors in macaque hippocampus showing CA2 and CA3 subfields of hippocampus and dentate gyrus. Sections were reacted with D_1 (*A,C*) or D_5 (*B,D*) antibodies. *pol,* Polymorphic layer; *pyr,* pyramidal layer; *m,* molecular layer; *mf,* mossy fiber zone; *H,* hillus; *Dg,* dentate gyrus. Note rich D5 staining in apical dendritic trees of pyramidal neurons in the molecular layer (*D*). Scale bars: *A–B,* 250 μm; *C–D,* 50 μm.

were fluorescently labeled by both the D_1 and D_5 antibodies. Numerous pyramidal neurons in layers III and V and in the pyramidal layer of the hippocampus appear to express both the D_1 and D_5 receptor subtypes. Although more detailed quantitative analysis is necessary to determine what proportion of cells were single- or double-labeled, our preliminary analysis suggests that the vast majority of D_5-labeled neurons also contained D_1 receptors, but not all D_1-labeled cells appeared to contain D_5 receptors.

Electron microscopic analysis revealed both presynaptic and postsynaptic localization of D_1 and D_5 receptors in the prefrontal cortex (area 46) and hippocampus, although postsynaptic localization was more frequently observed. As predicted from our light microscopic studies, both receptors were localized in cell bodies, dendrites and spines. However, the dominant ultrastructural feature of D_1 antibody staining was labeling of spines (Figs. 6*B*, 7). Approximately 20% of the total number of spines counted (147 out of 735) in the micrographs of layer III, area 46,

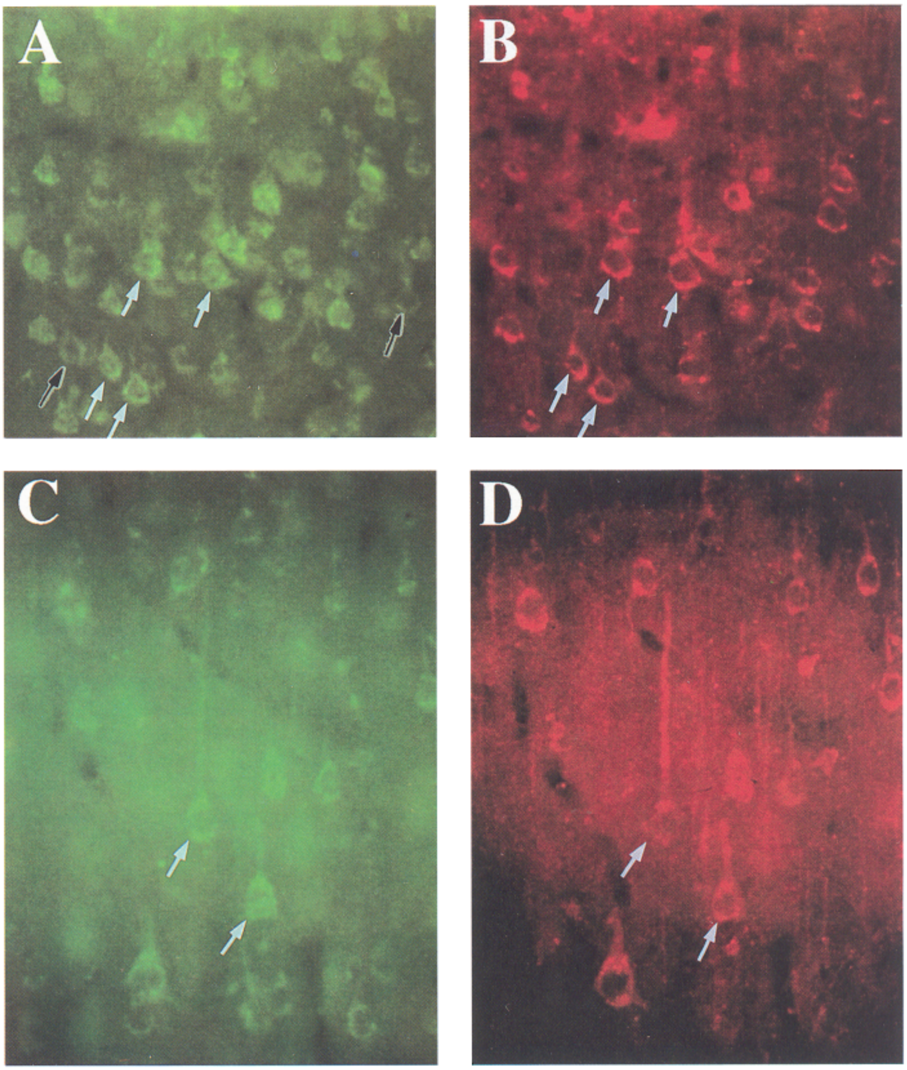

Figure 5. Double labeling immunofluorescence of D_1 (*A,C*) and D_5 (*B,D*). *A–B,* D_1 and D_5 receptor in the layer III of area 46. The two receptors are colocalized in numerous pyramidal cells (*white arrows*); D_1 receptor positive and D_5 negative neurons are also visible (*black arrows in A*); (*C–D*) D_1 and D_5 receptor in deep third of layer III and in layer V in area 6. Two of the double labeled pyramidal neurons are labeled with *white arrows.*

were labeled by D_1 antibodies. Within the labeled spines, D_1 receptor immunoreactivity was concentrated in the neck and head of dendritic spines (Fig. 7) and typically was slightly displaced from the asymmetric synapse or completely filled the spine head (Figs. 6*B,* 7).

In the prefrontal cortex, D_5 immunoreactivity was predominantly localized in dendritic shafts and to a lesser extent in dendritic spines (Figs. 6*A,* 8). By comparison with D_1 receptor antibodies, D_5 receptor antibodies labeled fewer spines (37 out of 796, 4.6%) in layer III area 46 of the prefrontal cortex. The different proportions of D_1 (20%) vs. D_5 (4.6%) labeled spines in the cortex was highly significant ($\chi^2 = 85.17$, $p = 0.0001$). Like the D_1 labeled spines, D_5 receptor-containing spines were nearly always targets of asymmetric synaptic input (Fig. 8*B,C*). The immunoreaction product in D_5-positive spines was either distant from the synaptic specialization (Fig. 8*B,C*) or diffusely distributed in the spine. Labeled spines arose from dendrites which either exhibited or lacked D_5 immunoreaction product. (Fig. 8*C*). D_5 antibody staining of dendritic shafts was either distributed throughout the cytoplasm (Fig. 6*A*), or found in patches confined to the cytoplasmic face of dendritic membranes (Fig. 9*A,B*). A similar postsynaptic localization of the D_5 receptor was observed in the CA1 field of the hippocampus (Fig. 9*A,B*).

When localized presynaptically, D_1 receptor immunoreactivity appeared as small patches of reaction product in axon terminals. These terminals often formed asymmetric synaptic specializations and the immunoreaction product was most often found at a distance from the synaptic specialization (Fig. 10). In contrast, presynaptic D_5 antibody labeling was observed not only in terminals, but also in the initial axonal segments of pyramidal neurons (Fig. 8*A*). D_5 receptors were present in axon terminals forming both symmetric (Figs. 8*B,* 9*C–E*) and asymmetric synapses (Fig. 9*A*). D_5-positive axons formed asymmetric synapses on spines (Fig. 9*A*); those making symmetric contacts were found both on spines and dendrites (Figs. 8*B,* 9*C–E*).

D_1 and D_5 receptor immunoreactivity in subcortical structures

We next examined D1 and D5 receptor expression in subcortical structures. In the caudate nucleus and putamen, both D_1 and D_5 receptor antibodies labeled medium-sized neurons (Fig. 11*D,E*). However, D_1 antibodies labeled many more neurons of this class. In contrast to the staining pattern obtained in cortex, D_1 labeling of dendrites was much more intense than the staining produced

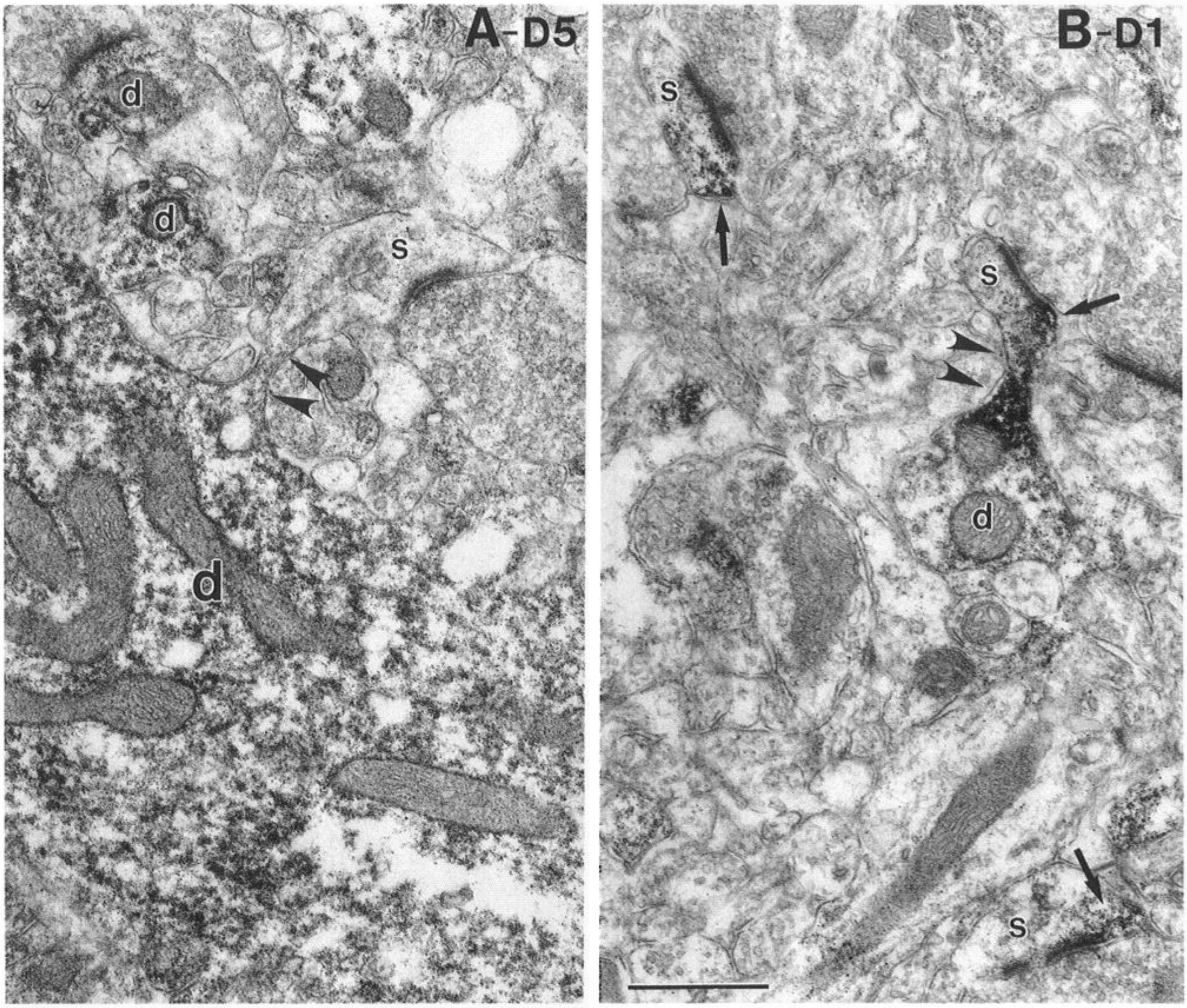

Figure 6. Electron microscopy of D_1 and D_5 receptor immunostaining in macaque cerebral cortex. *A,* D_5-immunoreactivity in dendritic shafts (*d*), but not in a dendritic spine (*s*) arising from a labeled shaft. The spine neck (*arrowheads*) is similarly devoid of label. *B,* D_1-immunoreactivity in dendritic spines, either in the spine heads (*arrows*), or spine neck (*arrowheads*) arising from a dendritic shaft (*d*). Immunoreactivity was visualized with the glucose oxidase-DAB method. Scale bar, 0.5 μm.

by D_5 antibodies (Fig. 11). Also, in caudate, large neurons with the morphological characteristics of cholinergic interneurons were labeled, but only by the D5 antibody (Fig. 11*B,E*).

The differential cellular distribution of the D_1 and D_5 receptors suggested by light microscopy was supported by ultrastructural analysis. D_1 receptor labeling was prominent in spines that were postsynaptic to asymmetric synapses. The labeling was intense and often present throughout the spine head and neck as well as at the postsynaptic density (Fig. 12). As was the case in cortex, some spines were labeled with D_5 antibodies, but much less frequently than spines labeled with D_1 antibodies (Fig. 13). D_1 and D_5 receptor immunoreaction product was also observed at the postsynaptic densities of small synapses characteristic of DA terminals (Bouyer et al., 1984; Freund et al., 1984; Smith et al., 1994). However, the dense immunoreactivity at these synapses precludes their precise morphological identification. Presynaptic labeling of axons with both D_1- and D_5-specific antibodies was also observed in the caudate (Fig. 13). Both antibodies labeled axons that formed asymmetric synapses, and the D_5 receptor antibodies also labeled some axon terminals making symmetric contacts (Fig. 13).

Distinct regional and cell-specific patterns of D_1 and D_5 receptor expression were also observed in other subcortical nuclei including neurons of the globus pallidus, thalamus (anterior, ventrolateral, ventroposterolateral and mediodorsal subdivisions, and lateral geniculate body), amygdala (cortical, central, and basolateral parts), and the basal forebrain (medial septal, nuclei of diagonal band of Broca, and nucleus basalis). For example, D_5 receptor antibodies labeled cholinergic neurons of the basal forebrain more intensely than did D_1 antibodies. In contrast, in the mesencephalon, D_1 antibodies strongly labeled neuropil in the pars reticulata of the substantia nigra, whereas D_5 receptors were undetectable in these fibers (Fig. 14*A,B*).

Discussion

The present light and electron microscopic study of D_1 and D_5 DA receptor antibody staining of monkey brain has demonstrat-

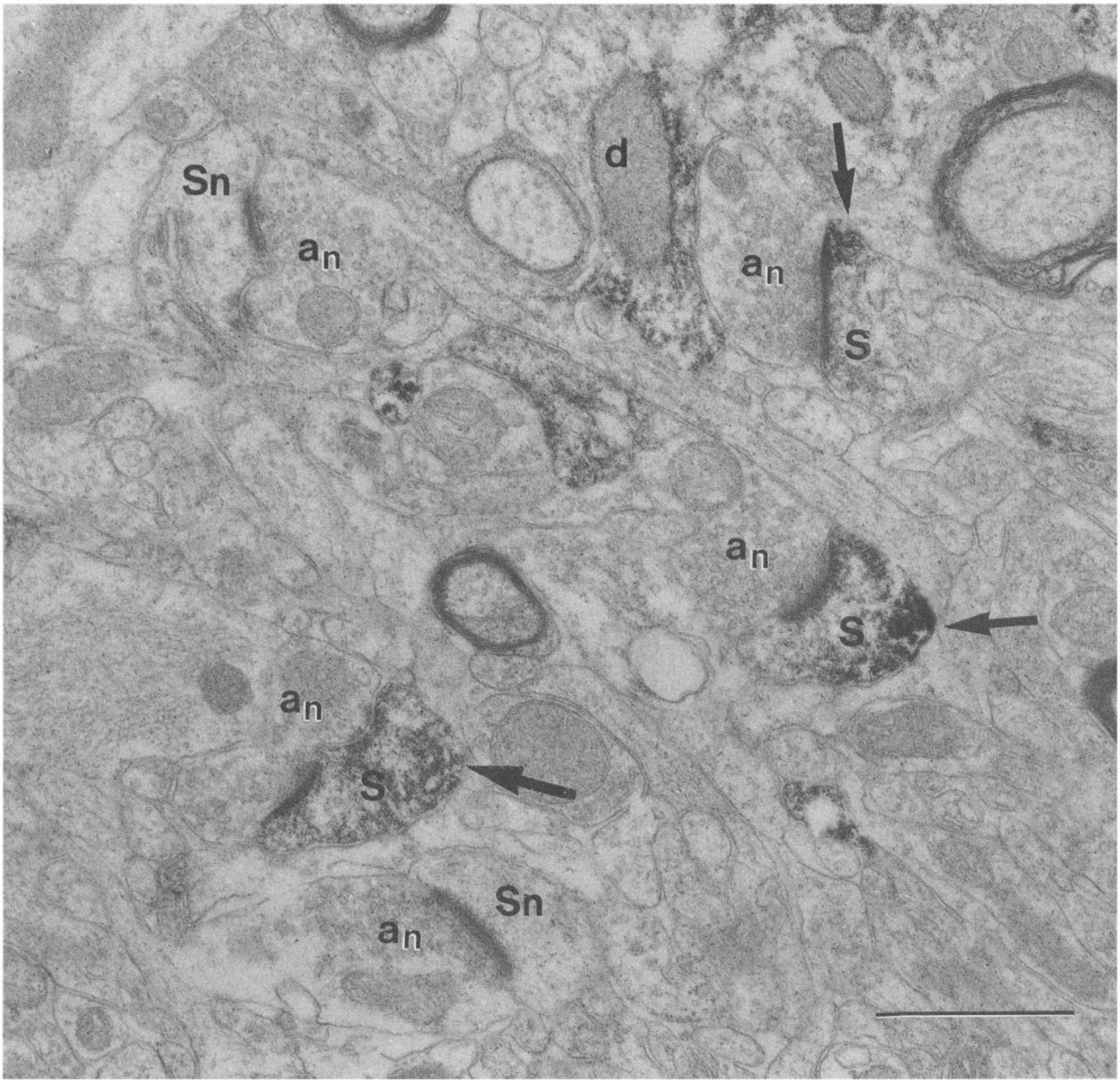

Figure 7. Postsynaptic localization of the D_1 receptor in the macaque prefrontal cortex. Note labeling of multiple dendritic spines (*s*) receiving asymmetric input from unlabeled axons (a_n). *Arrows* point to immunoreaction product near or slightly displaced from the synapse. *d,* Labeled dendrite; s_n, unlabeled spine. Scale bar, 0.5 μm.

ed distinct differences with respect to the cellular and subcellular distribution of these two members of the D_1 family of DA receptors. We find that these two receptors are expressed within different neuronal populations in subcortical brain regions, and they exhibit significant differences in subcellular localization in pyramidal neurons of cerebral cortex. Differences in the cellular and subcellular localization strongly suggests that although D_1 and D_5 receptors exhibit similar pharmacological properties, they are not functionally redundant. On the contrary, our data provide the first evidence that these receptors are likely to subserve distinct pre- and postsynaptic functions and involve distinct pathways of neuronal signalling.

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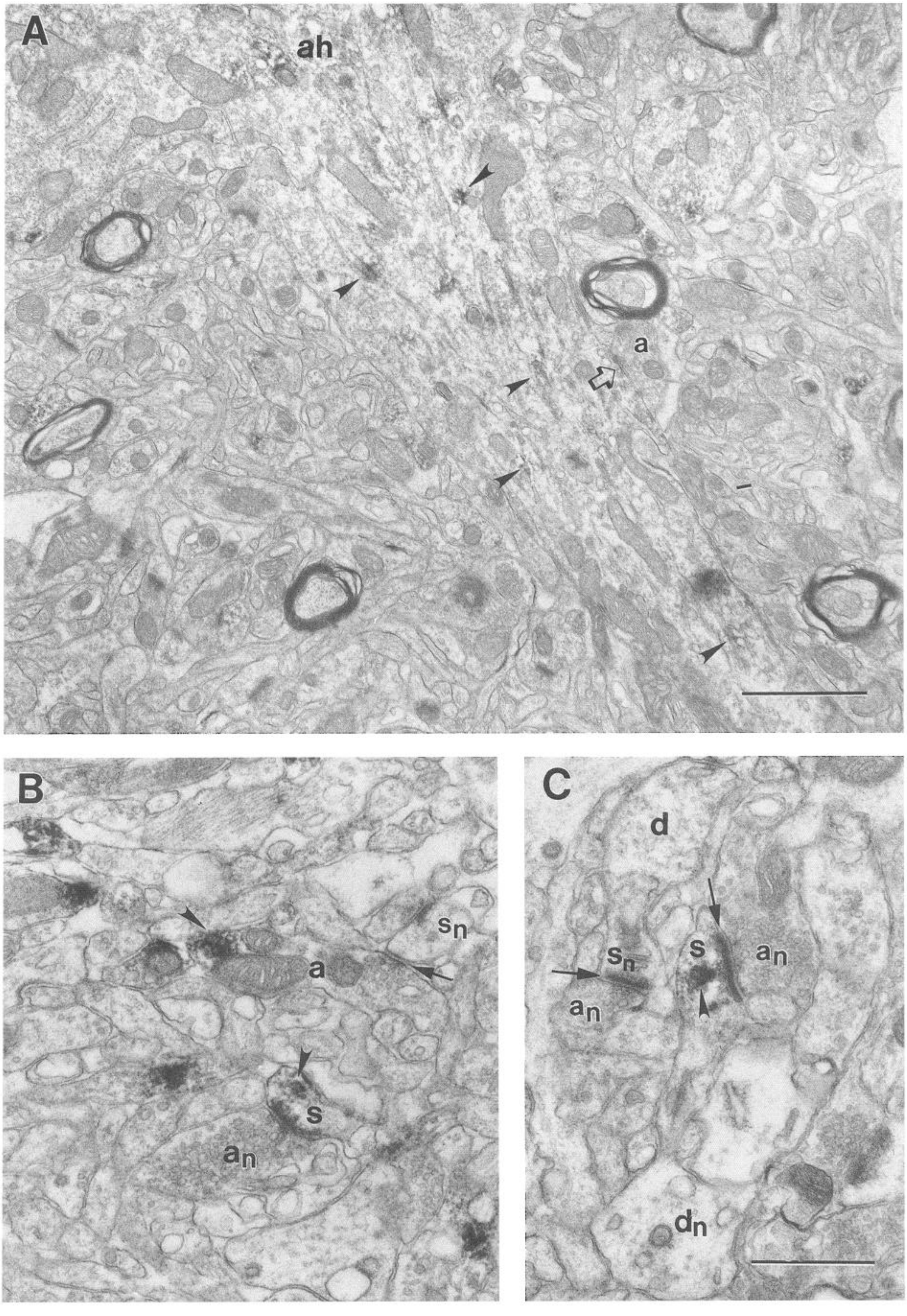
Figure 8. Pre- and postsynaptic localization of the D_5 receptor in layer III of area 46 of macaque neocortex. *A,* Initial axonal segment of a D_5 labeled pyramidal neuron. Immunolabeling is not limited to the axon hillock (*ah*) but is present throughout initial segment towards more distal part of the axon. *Open arrow* points to the symmetric axo-axonic synapse. *Arrowheads* point to electron dense material associated with membranes. *B,* Labeled axon (*a, arrowhead*) forms symmetric synapse on unlabeled spine (s_n, *arrow*). Labeled spine (*s*) receives asymmetric input from an unlabeled axon (a_n). *C,* Labeled dendrite (*d*) giving rise to unlabeled spine (s_n) and unlabeled dendrite (d_n) giving rise to labeled spine (*s*). Both spines are postsynaptic elements of asymmetric synapses (*arrows*) formed by unlabeled axons (a_n). *Arrowhead* points to immunoreaction product. Scale bars: *A,* 1 μm; *B–C,* 0.5 μm.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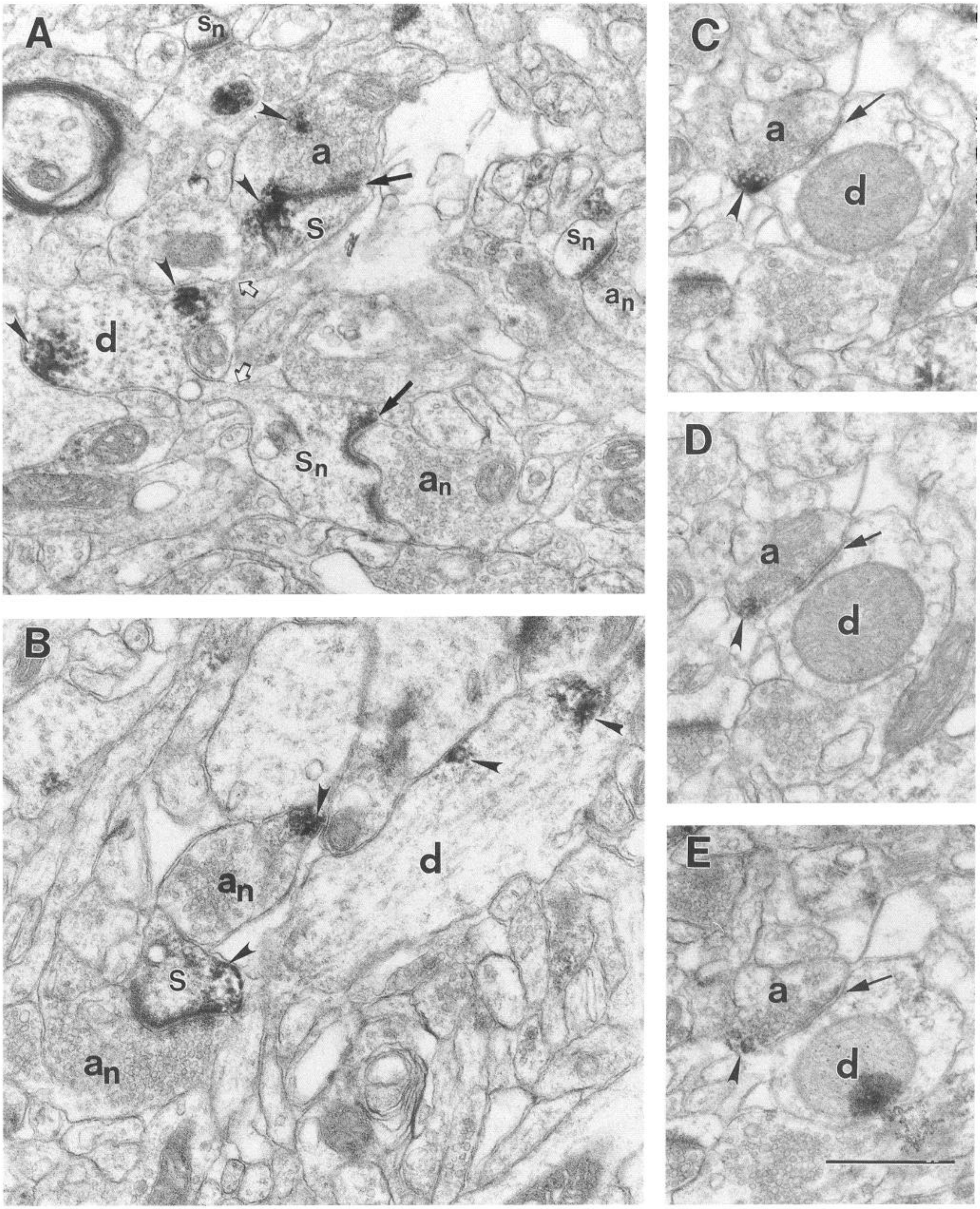

Figure 9. Pre- and postsynaptic localization of D_5 receptor protein in the pyramidal layer of the CA1 field of the macaque hippocampus. *A,* Immunopositive dendrite (*d*) gives rise (*open arrows*) to a labeled (*s*) and an unlabeled spine (s_n). *Arrowheads* point to the reaction product. Both spines are postsynaptic elements of asymmetric synapses (*arrows*) formed by a labeled axon (*a*) and unlabeled axon (a_n). *B,* Unlabeled axons (a_n) form asymmetric synapses on a labeled spine (*s*). Note the association of immunoreaction product with the cytoplasmic surface of the membrane in axon and dendrite (*d*). *C–E,* Serial sections through a labeled axon (*a*) forming a symmetric synapse (*arrow*) on a dendrite (*d*). Immunoreaction product is present as a small patch distant from the synaptic specialization (*arrowheads*). Scale bar, 0.5 μm in *A–E.*

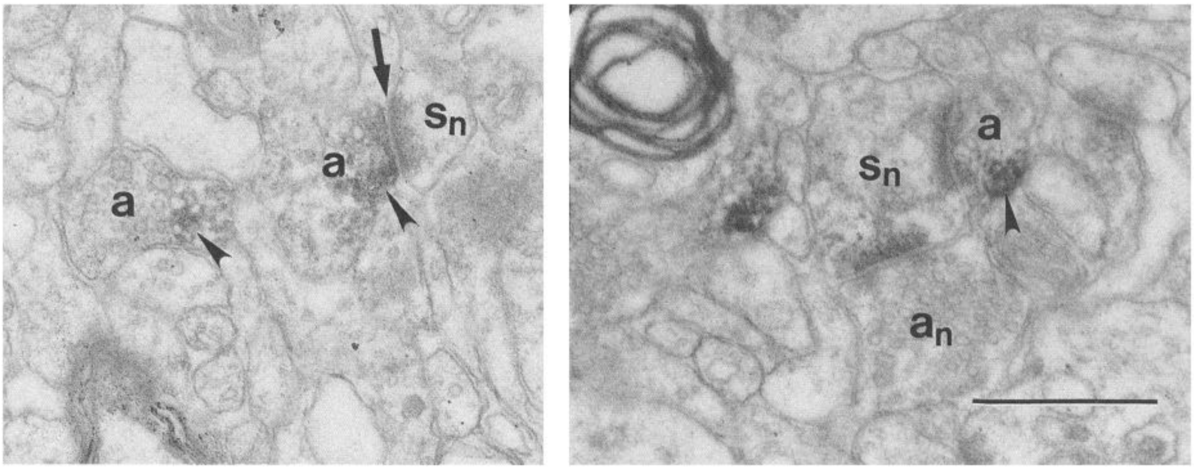

Figure 10. Presynaptic localization of the D_1 receptor in layer III of area 9 of the macaque neocortex. *A* and *B*, D_1 labeled axons (*a*) which form asymmetric synapses (*arrow*) with unlabeled spines (s_n). *Arrowheads* point to immunoreaction product which is distant from the synaptic specialization. a_n, unlabeled axon. Scale bar, 0.5 µm.

Antibody specificity

A major goal of our laboratory is the localization of the molecules which mediate DA neurotransmission in primate brain. Here we report the development of subtype specific antibodies directed at divergent sequences in the C-terminal regions of the D_1 and D_5 DA receptor polypeptides. These molecules have previously been localized by the D_1 class of dopaminergic ligands which do not discriminate between individual receptor subtypes. The subtype specificity of the antibodies was demonstrated by a variety of biochemical and immunological criteria: (1) the D_1 and D_5 antibodies specifically react with the corresponding glycosylated and unglycosylated forms of the receptors expressed in recombinant baculovirus infected insect cells and do not cross react with the other member of the dopamine family of receptors, or with other catecholaminergic and muscarinic receptors; (2) the antibodies recognize unique proteins expressed in monkey prefrontal cortex and caudate of M_r corresponding to the core and glycosylated forms of the receptors (see also Bergson et al., 1995) as predicted from molecular cloning (Sunahara et al., 1990; Grandy et al., 1991; Sunahara et al., 1991; Zhou et al., 1991) and biochemical studies (Amlaiky et al., 1986; Niznik et al., 1988; Sidhu, 1990); and (3) immunoreactivity with proteins on immunoblots and brain tissue is specifically inhibited when the antibodies are incubated in the presence of the cognate fusion proteins, but not in the presence of unrelated fusion proteins. While these results do not eliminate the possibility that our antibodies detect unrelated proteins of similar molecular weight, the correspondence between the immunohistochemical localization of the D_1 and D_5 receptors described here with the *in situ* localization of D_1 and D_5 mRNA in the monkey cortex and caudate reported previously (Huntley et al., 1992; Rappaport et al., 1993) makes this possibility highly unlikely.

Differential expression of D_1 and D_5 receptors in subcortical structures

The clearest indication of differences in the distribution of D_1 and D_5 receptors was observed in subcortical brain regions. In the caudate, for example, we found that only D_5 antibodies stain neurons with large cell bodies. Preliminary double label experiments indicate that these large neurons are also positive for the enzyme choline acetyltransferase indicating that D_5 receptors are expressed in cholinergic interneurons (L.M. and C.B., unpublished observations). The localization of D_5 receptors in cholinergic interneurons in caudate suggests a selective role for D_5 in modulating axonal input to, or release of ACh from these neurons. Although both D_1 and D_5 receptor antibodies label spiny medium-sized GABAergic neurons in caudate, the D_1 receptor is localized in a large number of these neurons, whereas the D_5 receptor appears to be expressed in relatively few of them. Differences in expression of D_1 and D_5 receptors were also evident in the substantia nigra where only D_1 antibodies label the neuropil of the pars reticulata. D_1/dynorphin/substance P containing striatal neurons (reviewed in Gerfen, 1992) project to the substantia nigra where they inhibit tonic activity in GABAergic neurons of the substantia nigra pars reticulata (Chevalier et al., 1985). The localization of D_1, and not D_5 receptors in the neuropil of the pars reticulata indicates that if D_5 receptors are expressed in D_1-containing medium-sized spiny neurons of the caudate, then only D_1 receptors are transported to striato-nigral terminals. Alternatively, the lack of D_5 receptor in terminals in the pars reticulata may indicate that the D_5 receptor is expressed in a population of medium-sized spiny neurons which do not express the D_1 receptor. Each of these possibilities is consistent with the differential distribution of the D_1 and D_5 receptors in striatal medium sized spiny neurons either due to differences in D_1 versus D_5 gene expression or to differences in vesicular transport. Taken together, the localization of D_1 and D_5 in different neuronal cell types and elements in the caudate and mesencephalon strongly suggests the D_1 and D_5 receptors may perform distinct pre- and postsynaptic functions.

Differential distribution of the D_1 and D_5 receptor subtypes in the cerebral cortex and hippocampus

Both D_1 and D_5 receptor antibodies prominently labeled pyramidal cells in layers II, III, and V in association (prefrontal, premotor) and mesolimbic (cingulate, entorhinal) cortical areas and pyramidal and granule cells in archicortex (hippocampus and dentate gyrus) of rhesus monkey brain. Furthermore, double-label localization experiments indicated that the D_1 and D_5 receptor subtypes are frequently coexpressed in the same pyramidal neurons both in the neocortex and hippocampus. This localization of both D_1-like receptor proteins in the cerebral cortex

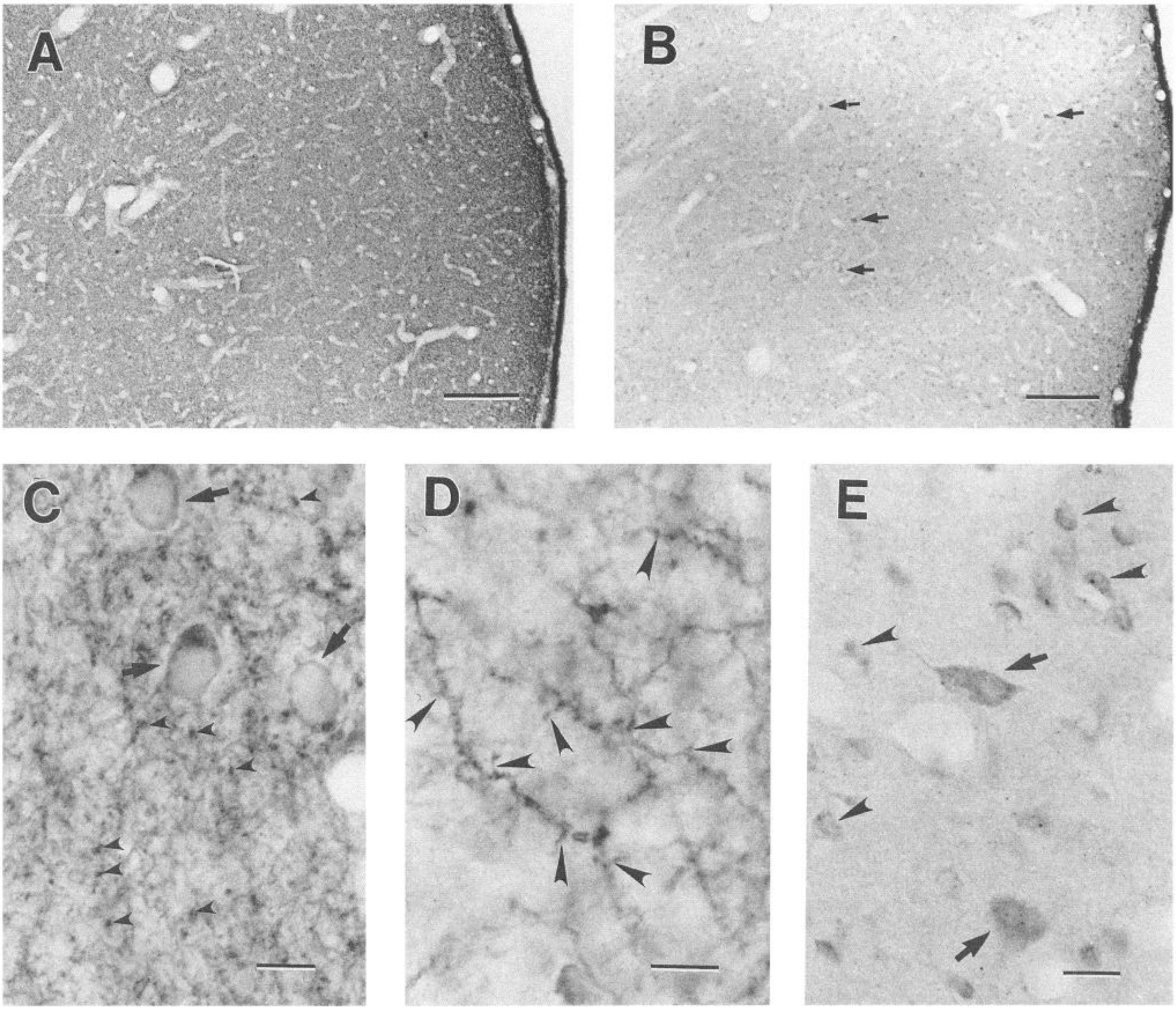

Figure 11. Immunohistochemical staining of D_1 and D_5 receptors in macaque caudate nucleus. Tissue reacted with (*A*) D_1 and (*B*) D_5 antibodies; *arrows* indicate large cells, presumably cholinergic interneurons. *C,* D_1 antibody staining of medium-sized neurons (*arrows*). Dendritic spines are indicated by *arrowheads. D,* Higher magnification of *A. E,* D_5 staining of medium-sized (*arrowheads*) and large (*arrows*) neurons. Scale bars: *A* and *B,* 600 μm; *C,* 10 μm; *D,* 5 μm; *E,* 20 μm.

is similar to the laminar distribution of D_1 ligand binding sites in primate cortex obtained by receptor autoradiography (reviewed in Lidow, 1993). In addition to the perikaryal localization of both receptors, we observed an abundance of D_5 receptor, and noticeably less D_1 receptor in apical dendritic shafts. Examination by electron microscopy revealed marked differences with respect to the postsynaptic localization of the D_1 and D_5 receptors. When we quantified the number of D_1 labeled to unlabeled spines and D_5 labeled to unlabeled spines in layer III of area 46, we found a statistically significant difference in the frequency of D_1 [~20% (147/735)] and D_5 [~5% (37/796)] receptors localized in spines. Assuming equivalent penetration of dendrites and spines by both antibodies and relatively similar antibody sensitivities, our light and electron microscopic data are consistent with the idea that the postsynaptic pool of D_1 receptors is preferentially transported from the perikarya to spines, whereas the majority of postsynaptic D_5 receptors are sorted to dendritic shafts. The observed differences in D_1 versus D_5 immunostaining of pyramidal cell dendritic shafts may further indicate differences in the rates of D_5 versus D_1 biosynthesis, transport, or stability. Differences in immunostaining may also reflect selective transport of the D_1 and D_5 receptors to a specific subcellular compartment as has been noted for the N- and L-type calcium channels (Westenbroek et al., 1992). Regardless of mechanism, the pattern of D_1 versus D_5 receptor antibody staining visible at the light and electron microscopic level suggests that the two receptors are differentially localized within individual pyramidal neurons.

The differential subcellular localization of the D_1 and D_5 receptor subtypes in cortical pyramidal neurons is a relative finding, but one which may have considerable functional implications. Pyramidal cell dendritic spines are the major postsynaptic targets of glutamatergic inputs to the cortex (Sloper et al., 1978; Winfield et al., 1982; Conti et al., 1988, 1989; McGuire et al., 1991; reviewed in Harris and Kater, 1994). In contrast, cortical dendritic shafts are more likely to receive inhibitory inputs from

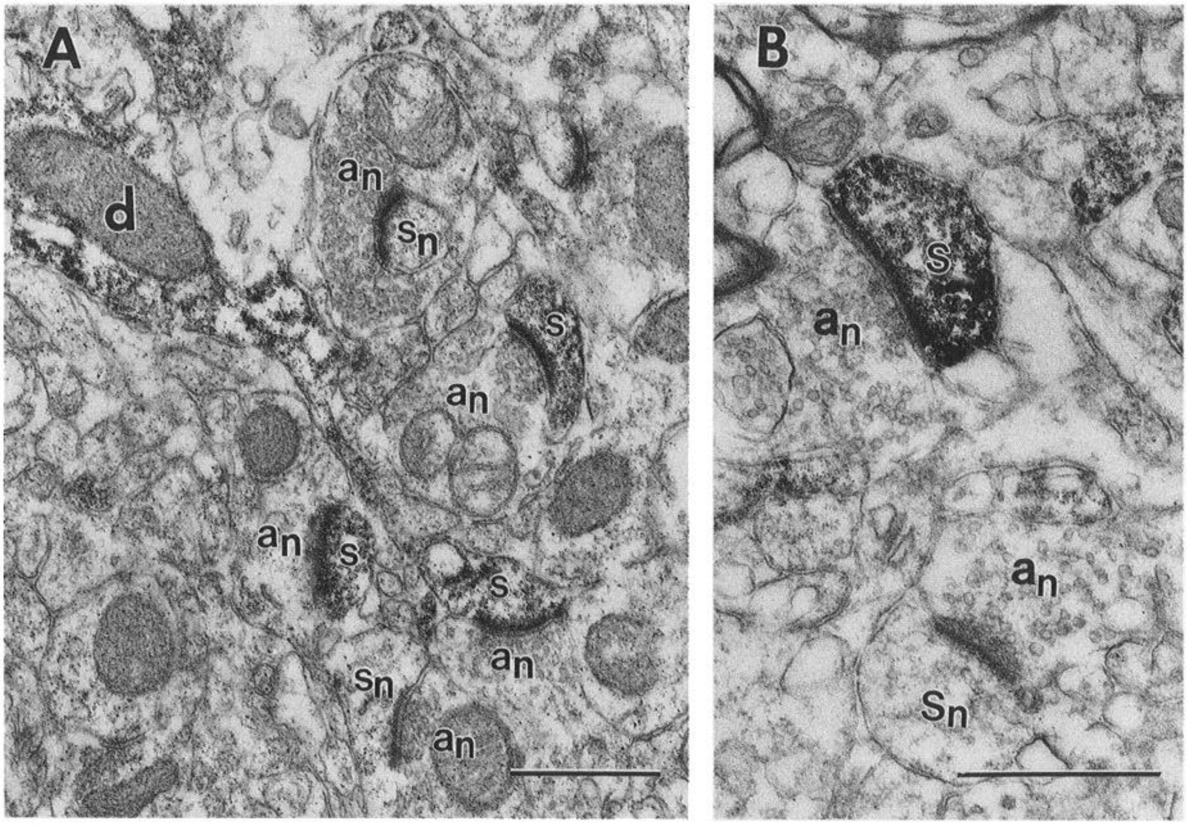

Figure 12. Electron micrographs of D_1 receptor immunoreactivity in macaque caudate. *A*, D_1 antibodies prominently stain dendrites (*d*) as well as numerous spines (*s*) which make asymmetric synapses with unlabeled axons (a_n). Unlabeled spines are also visible (s_n). *B*, High power electron micrograph of D_1 labeled (*s*) and unlabeled (s_n) spines. Immunoreaction product is visible throughout the cytoplasm and postsynaptic density. Scale bars: *A* and *B*, 0.5 μm.

GABAergic interneurons (Jones, 1993), although axon terminals of some interneurons (double bouquet cells) also target dendritic spines (Somogyi and Cowey, 1984). In addition to receiving different synaptic input, studies of pyramidal neurons in slice preparations of hippocampus suggest that spines and shafts are autonomous compartments (Guthrie et al., 1991; Muller and Connor, 1991), providing additional evidence that spines and shafts are involved in independent pyramidal cell functions. It is therefore tempting to speculate that the major function of the D_1 receptor may be to modulate excitatory neurotransmission (Smiley et al., 1994). On the other hand, the frequent localization of D_5 receptors to shafts of pyramidal neurons suggests that a main function of this receptor may be modulation of terminals that target shafts rather than spines and possibly even a selective subset of inhibitory inputs. A test of this hypothesis awaits a more exhaustive analysis of synaptic arrangements with D_1 and D_5 receptor antibodies as well as a better understanding of the sequence elements within the D_1 and D_5 receptors that specify their different functional properties.

Target-terminal interactions

DA terminals in the macaque cortex establish predominantly symmetric synapses on pyramidal cell spines and shafts and rarely on GABAergic nonpyramidal cells. (Goldman-Rakic et al., 1989; Smiley and Goldman-Rakic, 1993). However, we observed very few D_1 or D_5 labeled profiles postsynaptic to symmetric, potentially dopaminergic, synapses. This result is consistent with our previous double labeling immuno-EM analysis of the D_1 receptor and tyrosine hydroxylase (TH) expression. These data showed D_1 labeled spines and TH labeled axons in close proximity to each other, but rarely in direct contact (Smiley and Goldman-Rakic, 1993). It is possible that the diffuse DAB-peroxidase immunoreaction product may have obscured detection of D_1 or D_5 labeled dopaminergic synapses which are small and clearly visible only in 1–2 serial sections (Smiley and Goldman-Rakic, 1993). The observed labeling profile could also represent a case for diffusion or "volume transmission" of DA (Descarries et al., 1991) from nearby axons as the primary mode of dopaminergic innervation of D_1 and D_5 receptors in dendritic spines and shafts in cortex. It must be borne in mind, however, that DAB reaction product shows a high degree of diffusion with the immunocytochemical technique employed in the present study, and it would not be prudent to draw strong conclusions about the precise localization of the antigen with respect to postsynaptic densities at the present time. Whether the receptors studied are extrasynaptic or directly apposed to presynaptic terminals awaits the application of a more suitable method such as an immunogold technique (see Yung et al., 1995).

Presynaptic localization in cortex

Electron microscopic analysis revealed localization of the D_1 and D_5 receptors in axon terminals in cortex. A considerable proportion of D_1- and D_5-positive axons form asymmetric synapses which are characteristic of excitatory glutamatergic synapses, possibly representing thalamocortical or cortico-cortical axons (Sloper et al., 1978; Winfield et al., 1982; Conti et al., 1988).

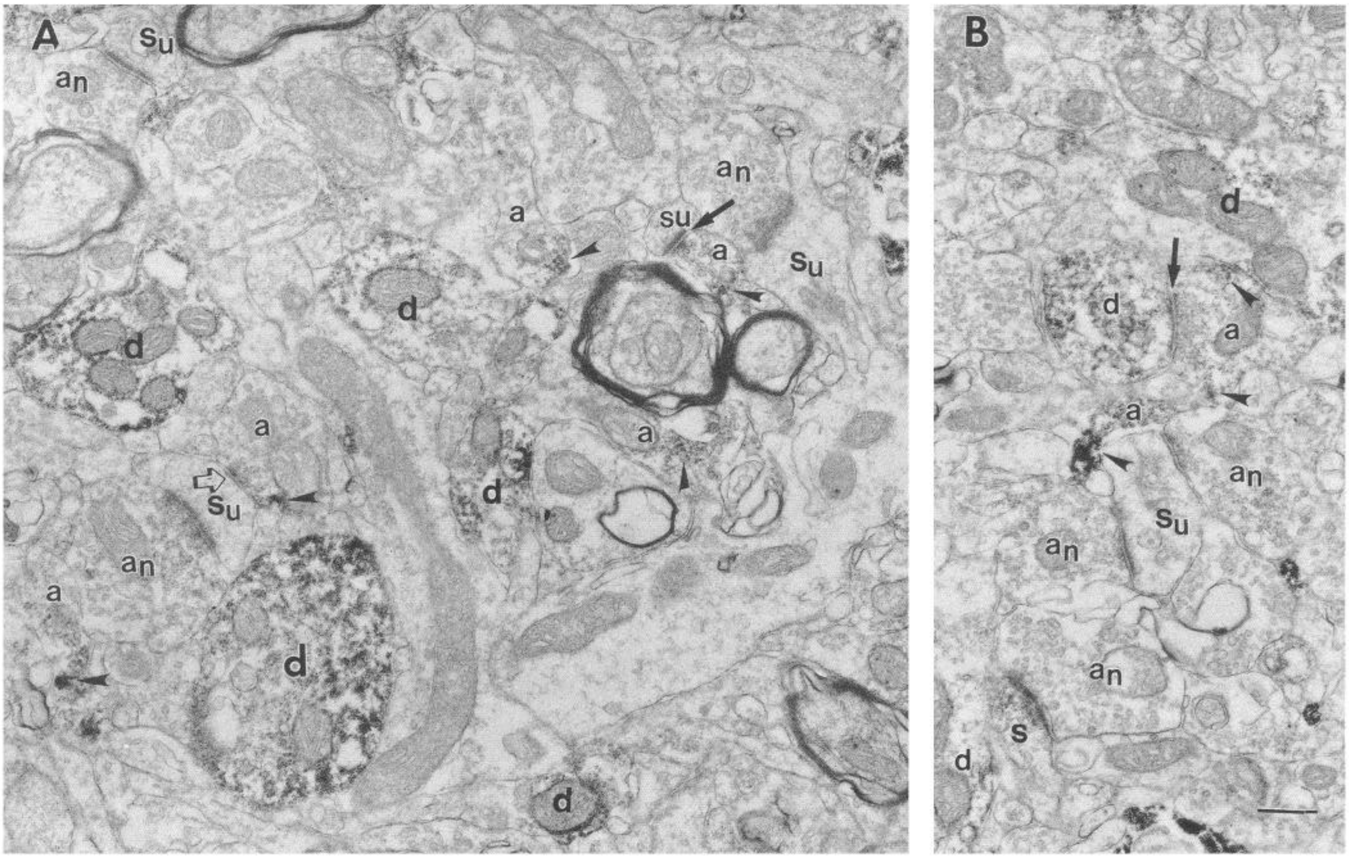

Figure 13. Electron micrographs of D_5 receptor immunoreactivity in macaque caudate. *A* and *B*, Dendrites (*d*) labeled by D_5 antibodies. Unlabeled spines (s_u) contacted by unlabeled axons (a_n) forming asymmetric synapses. *B*, D_5 labeled spine (*s*) and axon (*a*). *Arrowheads* point to labeled axoplasm. Labeled axons form either asymmetric synapse (*solid arrows in A* and *B*) or symmetric synapse (*open arrows* in *A*). Scale bars in *A* and *B*, 0.5 μm.

Consistent with this anatomical architecture, D_5 immunoreactivity was localized to the initial segments of some layer III pyramidal neurons which are known to be the origin of corticocortical pathways (Conti et al., 1988). Therefore, in addition to the postsynaptic mechanism described above, the localization of the D_1 and D_5 receptors in these axons suggest a presynaptic mechanism for the dopaminergic modulation of excitatory neurotransmission in cortex.

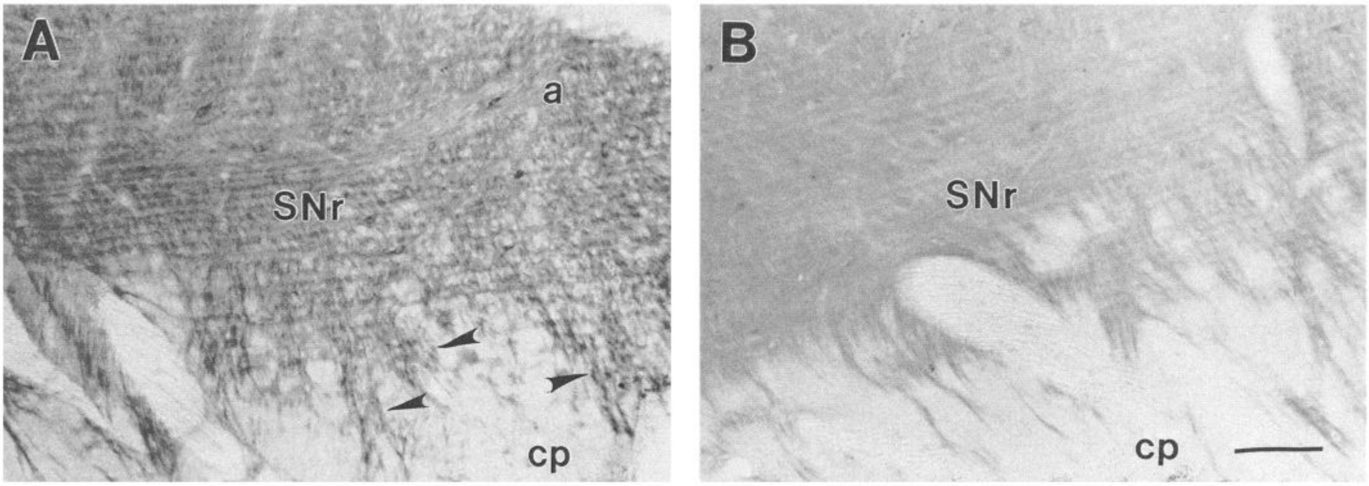

Figure 14. D_1 and D_5 receptor immunostaining in macaque mesencephalon. Vibratome section stained with D_1 (*A*) or D_5 (*B*) antibodies. D_1 receptor antibodies selectively label neuropil in the pars reticulata of the substantia nigra (SNr) (*A*), which is not stained with D_5 antibodies (*B*). *B* was overexposed relative to *A* to give enough contrast to visualize tissue. Occasional labeled neurons are present in both D_1 and D_5 immunostaining of sections. *Arrowheads* indicate D_1-stained fibers. *pc,* Cerebral peduncle. Scale bar, 150 μm.

Differences in the presynaptic localization of the D_1 and D_5 DA receptors were also observed as the D_5 receptor was localized in symmetric, as well as asymmetric, axon terminals in the prefrontal cortex. It seems unlikely that these symmetric profiles are DA terminals since the dopaminergic cell bodies of the ventral tegmental area and substantia nigra are unlabeled by D_5 receptor antibodies, but labeled by D_2 antibodies (Levey et al., 1993; Sesack et al., 1994). As we were unable to localize D_5 receptors in symmetric terminals on pyramidal cell bodies or at axo-axonic synapses which are typically formed by local circuit interneurons (Hendry et al., 1983), it is possible that these are either GABAergic or cholinergic terminals of basal forebrain afferents (Freund and Meskenaite, 1993; Mrzljak et al., 1995). Consistent with this idea, we observed strong D_5 antibody labeling of basal forebrain neurons. Taken together these results would suggest that the D_5 receptors expressed in basal forebrain neurons are transported to terminals formed in cortex where the receptor presumably would function in modulating cholinergic input.

Functional specificity of D_1-like receptors in prefrontal cortex

The role of the prefrontal cortex in cognition has begun to be understood in terms of the cellular processes underlying working memory (Goldman-Rakic, 1987). Recordings from prefrontal neurons in monkeys performing memory tasks have revealed a class of cell that possesses "memory fields," that is, increases their rate of discharge as monkeys recall a specific location (Funahashi et al., 1989), feature or face (Wilson et al., 1993), when these stimuli are no longer present in the animals' environment. Partial blockade of D_1 receptors by the D_1-specific antagonist, SCH39166, has been found to enhance the memory-related activity of these prefrontal neurons (Williams and Goldman-Rakic, in press). Remarkably, the rate of discharge for a neuron's preferred or "best" direction was enhanced, the normal pattern of inhibition of activity to the opponent direction was also enhanced, and neuronal activity during recall of all other nonpreferred directions was unchanged (Williams and Goldman-Rakic, in press). The specificity of the drug's action in this study was explained in terms of dopaminergic modulation of excitatory transmission at closely associated spine synapses ("triads" as described in Goldman-Rakic et al., 1989). The present ultrastructural localization of D_1 receptors to spines, together with findings from a previous study using a different D_1 antibody (Smiley et al., 1994) place D_1 receptors directly at the spines which receive excitatory terminals from cortico-cortical and/or thalamo-cortical fiber systems. As SCH39166 has a high affinity for both the D_1 and D_5 receptor subtype, it has not yet been possible to ascribe the effects of this drug on a neuron's "memory field" to the D_1 and/or D_5 receptor proteins. The preferential spine localization of the D_1 receptor favors its role in modulating the excitatory synapses on these spines in order to enhance the neuron's activation for remembered "best" directions. On the other hand, the preferential localization of the D_5 subtype in dendritic shafts may indicate its role in modulating the inhibition associated with the neurons' response to the remembered opponent direction. These and other hypotheses concerning the molecular basis of higher cortical function in primates are currently under active investigation.

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