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Quantification of D₁ and D₅ Dopamine Receptor Localization in Layers I, III, and V of *Macaca mulatta* Prefrontal Cortical Area 9: Coexpression in Dendritic Spines and Axon Terminals

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Abstract

D1 family receptors (D1R) in prefrontal cortex (PFC) are critical for normal cognition and are implicated in pathological states such as schizophrenia. The two D1R subtypes, D₁ and D₅, cannot be pharmacologically distinguished but have important functional differences. To understand their contributions to cortical function, we quantified their localization in the neuropil of primate PFC. We identified different patterns of distribution for the two receptors that showed variation across cortical laminae. Although D₁ was enriched in spines and D₅ in dendrites, there was considerable overlap in their distribution within neuronal compartments. To determine whether the D₁ and D₅ receptors are localized to separate populations of synapses, we employed double-labeling methods. We found the two receptors colocalized and quantified the overlap of their distribution in spines and axon terminals of prefrontal cortical area 9 in the *Macaca mulatta* monkey. The two receptors are found in partially overlapping populations, such that the D₅ receptor is found in a subpopulation of those spines and terminals that contain D₁. These results indicate that dopamine activation of the two D1R subtypes does not modulate disparate populations of synapses onto dendritic spines in prefrontal cortical area 9; rather, dopamine can activate D₁ and D₅ receptors on the same spines, plus an additional group of spines that contains only D₁. The implications of these results for the dose-dependent relationship between D1R activation and PFC function are discussed.

Indexing terms

electron microscopy; circuitry; working memory; primate; ultrastructure; pyramidal cells; prefrontal cortex

Dopamine neurotransmission in prefrontal cortex (PFC) is crucial for normal cognition in humans and animals (Brozoski et al., 1979; Luciana et al., 1998; Castner et al., 2000; Harmer et al., 2001). In particular, activation of the D1 family of dopamine receptors (D1R) in PFC is critical for working memory (WM) performance (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995; Muller et al., 1998). Schizophrenia is associated with altered dopamine neurotransmission in the PFC (Weinberger et al., 1988; Akil et al., 1999;

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Abi-Dargham et al., 2002), and schizophrenic patients show pronounced impairments in WM performance (Park and Holzman, 1992; Goldman-Rakic, 1994; Callicott et al., 2003), which is strongly correlated with increased D1R availability in the PFC (Abi-Dargham et al., 2002). Furthermore, treatments that reduce D1R expression in the PFC impair WM performance in normal monkeys, and this is reversed by D1R agonist treatment (Castner et al., 2000). Given the importance of D1R activity for proper PFC functioning, it is critical to understand fully their roles within PFC cortical circuitry.

There are two subtypes of D1R, the D₁ and the D₅ receptors (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991). D₁ and D₅ share 80% homology in their transmembrane domain, and they both couple to G_{αs} (Kebabian and Calne, 1979; Tiberi et al., 1991). Currently available pharmacological tools do not differentiate D₁ and D₅. However, evidence for important functional differences between them has emerged (Tiberi and Caron, 1994; Dziewczapolski et al., 1998; Liu et al., 2000; Lee et al., 2002; Centonze et al., 2003; Laplante et al., 2004), including a tenfold higher affinity for dopamine exhibited by the D₅ receptor (Sunahara et al., 1991; Weinshank et al., 1991). Intriguingly, there is a complex relationship between D1R stimulation and WM performance, such that too little or too much D1R activation results in impaired WM abilities (for review see Goldman-Rakic et al., 2000). Although the importance of PFC circuitry in shaping WM abilities has been demonstrated (Rao et al., 1999, 2000; Kober and Dash, 2006), the specific roles of D₁ and D₅ within this circuitry cannot be addressed without a better understanding of how D1R subtypes are localized. A previous qualitative immunoelectron microscopic study in macaque PFC indicated that D₁ is localized predominantly to pyramidal cell spines, whereas the D₅ receptor is located on dendritic shafts (Bergson et al., 1995b), suggesting that the D1R subtypes play distinct roles in prefrontal circuitry. However, the pattern of D1R localization is more complex than suggested by this observation. For example, D₁ receptors have been localized in the axon terminals and dendrites of prefrontal interneurons of the macaque monkey (Muly et al., 1998) as well as axon terminals that make asymmetric synapses (Bergson et al., 1995b; Paspalas and Goldman-Rakic, 2005).

The work presented here was undertaken to determine whether the D₁ and D₅ dopamine receptors are located at separate or overlapping sites within area 9 prefrontal circuitry. This information is critical to determining how dopamine might affect specific cortical components via the D1R. To confirm the qualitative impressions of Bergson and colleagues (1995b), we have quantitatively determined the localization of D₁ and D₅ in layers I, III, and V of area 9 in the *Macaca mulatta* PFC. Moreover, to determine whether dopaminergic neurotransmission via the D1R can occur at distinct or overlapping axospinous synapses, we determined the extent of their colocalization to spines and axon terminals. For better correspondence to the available physiological data, we performed these experiments in layer III, area 9, of macaque PFC. Our data demonstrate that the D₁ and D₅ receptors are not restricted to spines and dendrites, respectively. Moreover, we show that they colocalize within dendritic spines and axon terminals such that the D₅ receptor is always present with the D₁ receptor in these elements, but not vice versa.

MATERIALS AND METHODS

Antisera

Two antibodies were used in this study. The rat anti-D₁ antiserum (Sigma-Aldrich, St. Louis, MO; No. D187) was prepared against a 97-amino-acid synthetic peptide corresponding to the C-terminus of the human D₁ receptor. The antiserum stains two major bands at 40–45 and 65–75 kD (Hersch et al., 1995), and all staining at the light and electron microscopic levels was abolished when the antiserum was preincubated with 0.5 mg/ml of D₁-GST fusion protein (Smiley et al., 1994). The D₅ antiserum was a rabbit polyclonal antiserum raised against residues 428–438 of the D₅ receptor. This sequence is common to both rat and human D₅

receptors. This antiserum reacts to D₅-expressing recombinant Sf9 cells but not Sf9 cells expressing D₁, D₂, D₃, or D₄ (Khan et al., 2000). Western blot in macaque PFC, striatum, and hippocampus labeled a single band with a molecular weight of approximately 53–54 kD (Fig. 1A), in line with the predicted molecular weight of the D₅ receptor of approximately 53 kD (Sunahara et al., 1991; Tiberi et al., 1991). Finally, immunohistochemical staining of macaque PFC was abolished when the antiserum was preincubated with the cognate peptide (Fig. 1B,C).

Western blotting

Tissue from one male *Macaca mulatta* monkey, who was 1.13 years old at the time of death, was used for immunoblotting. The Western blotting was performed as described previously (Muly et al., 2004). Briefly, the animal was skilled by pentobarbital overdose (100 mg/kg), and blocks of various brain regions were frozen. Samples of PFC, striatum, and hippocampus were dounce homogenized in buffer containing 140 mM KCl, 10 mM glucose, 1.2 mM MgCl₂, 10 mM HEPES, pH 7.4, with a cocktail of protease inhibitors added. The homogenate was centrifuged, the pellet was discarded, and the supernatant was assayed for protein concentrations with a colorimetric assay (Bio-Rad, Hercules, CA). The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Each lane was loaded with 20 µg protein sample, and the gel was run for 50 minutes at 200 V; the gel was then transferred to PVDF membrane. The membrane was rinsed, blocked, and probed with rabbit anti-D₅ (used at 1:300). After rinsing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-goat anti-rabbit IgG; 1:10,000; Bio-Rad). Labeling was revealed by chemiluminescence. A ladder of markers was used to estimate the molecular weight of the labeled bands (SeeBlue plus 2; Invitrogen, Carlsbad, CA). Images of the Western blots in TIFF format were imported into an image processing program (Canvas 8; Deneba Software, Miami, FL), where the image was cropped and labels were added.

Animals and preparation of tissue for immunohistochemistry

Tissue from eight *Macaca mulatta* monkeys was used for this study. The care of the animals and all anesthesia and sacrifice procedures in this study were performed according to the National Institutes for Health *Guide for the care and use of laboratory animals* and were approved by the Institutional Animal Care and Use Committee of Emory University. The animals were killed with an overdose of pentobarbital (100 mg/kg) and then perfused with a flush of Tyrode's solution. The flush was followed by 3–4 liters of fixative solution of 4% paraformaldehyde/0.1–0.2% glutaraldehyde/0–0.2% picric acid in phosphate buffer (0.1 M, pH 7.4; PB). The brain was then blocked and postfixed in 4% paraformaldehyde for 2–24 hours. Coronal 50-µm-thick vibratome sections of prefrontal cortical area 9 (Walker, 1940) were cut and stored frozen at –80°C in 15% sucrose until immunohistochemical experiments were performed.

Single-label immunohistochemistry

Single-label immunoperoxidase labeling was performed using rat anti-D₁ at a 1:500 dilution or rabbit anti-D₅ antisera at 1:500. The single-label immunoperoxidase labeling for D₁ and D₅ was performed as described previously (Muly et al., 1998). Briefly, sections were thawed, incubated in blocking serum (3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% lysine in 0.01 M phosphate-buffered saline, pH 7.4) for 1 hour, and then placed in primary antiserum diluted in blocking serum. After 36 hours at 4°C, the sections were rinsed and placed in a 1:200 dilution of biotinylated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) for D₁ or goat anti-rabbit IgG (Vector, Burlingame, CA) for D₅ for 1 hour at room temperature. The sections were then rinsed, placed in avidin-biotinylated peroxidase complex (ABC; ABC Elite; Vector) for 1 hour at room temperature, and then processed to reveal peroxidase using 3,3' diaminobenzidine (DAB) as the chromagen. Sections were then postfixed

in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded in Durcupan resin (Electron Microscopy Sciences, Fort Washington, PA). Selected regions were mounted on blocks, and ultrathin sections were collected onto pioloform-coated slot grids and counterstained with lead citrate. Control sections processed as described above except for the omission of the primary immunoreagent did not contain DAB label upon electron microscopic examination.

Six *Macaca mulatta* monkeys in total were processed for D₁ layers I, III, and V, with four for each condition. Four of the six were female, and the age range was 2.14–14.75 years. No differences in receptor localization related to monkey age were observed. Four monkeys were processed for D₅ layers I and III, and two were male. The monkeys ranged from 2.14 to 9 years of age; again, no differences in receptor localization related to monkey age were observed. Three monkeys were processed for D₅ layer V, two of which were males. The monkeys ranged from 2.14 to 4.5 years of age.

Double-label immunogold/DAB immunohistochemistry

To examine the possibility of colocalization of the two D1R subtypes, we performed double-label immunogold/DAB experiments. In one condition, D₁ was labeled with immunogold, and D₅ was labeled with DAB. In a second condition, the chromagens were reversed. PFC tissue sections from area 9 were thawed and rinsed in PBS. They were incubated in blocking serum (3% normal goat serum, 1% bovine serum albumin, 0.1% glycine, 0.1% lysine, and 0.5% fish gelatin in 0.01 M phosphate-buffered saline, pH 7.4) for 1 hour and then placed in the primary antiserum diluted at the same concentrations as in the single-label experiments overnight at 4°C. The sections were removed from the primary antiserum, rinsed in PBS, and placed in secondary antiserum [1-nm gold-conjugated goat anti-rat, used at 1:100 (Nanoprobes, Yaphank, NY), and biotinylated goat anti-rabbit, used at 1:200 (Jackson Immunoresearch); 1-nm gold-conjugated goat anti-rabbit, used at 1:100 (Nanoprobes), and biotinylated donkey anti-rat, used at 1:200 (Jackson Immunoresearch)] overnight at 4°C. The tissue was then rinsed in PBS, placed in 2% glutaraldehyde for 20 minutes, rinsed in PBS, rinsed in 2% acetate buffer, silver-intensified for 4 minutes (HQ silver; Nanoprobes), then rinsed in acetate buffer and in PBS. The sections were incubated in ABC overnight at 4°C and reacted in the same manner as for the single-label material.

Double-label cocktail immunohistochemistry

To quantify the extent of colocalization of D₁ and D₅, tissue sections were incubated in a cocktail of the primary immunoreagents rat anti-D₁ and rabbit anti-D₅ at a dilution of 1:500 and compared with tissue sections that were incubated with rat anti-D₁ alone or rabbit anti-D₅ alone at a dilution of 1:500. Four *Macaca mulatta* monkeys in total were used for these experiments, with three monkeys processed for each condition. Three of the four monkeys were male, and the monkeys ranged from 2.83 to 9 years of age. This cocktail procedure has been described in detail elsewhere (Muly et al., 2001) and has been used in subsequent studies (Lei et al., 2004; Mitrano and Smith, 2007). Briefly, the tissue sections in the cocktail condition were incubated with both primary antisera, then in a cocktail of biotinylated secondary IgGs and in ABC to reveal D₁ and D₅. DAB was used as the chromagen for both D₁ and D₅. The D₁-alone and D₅-alone conditions were processed as described above.

Analysis of material

The single-label DAB material was analyzed as previously described (Muly et al., 2003). Blocks of tissue from layers I, III, and V of cortical area 9 were made and cut in ultrathin sections that were examined using a Zeiss EM10C electron microscope. Regions of the grids containing neuropil were selected based on the presence of label and adequate ultrastructural preservation. Fields of immunoreactive elements in the neuropil were randomly selected, and

images were collected at a magnification of $\times 31,500$ with a Dualvision cooled CCD camera ($1,300 \times 1,030$ pixels) and Digital Micrograph software (version 3.7.4; Gatan, Inc., Pleasanton, CA). Images selected for publication were saved in TIFF format and imported into an image processing program (Canvas 8; Deneba Software). The contrast was adjusted, and the images were cropped to meet size requirements. For D₁, 295 micrographs in total representing $1,800 \mu\text{m}^2$ of layer I were analyzed across four monkeys. Five hundred twenty-eight labeled profiles were counted, and each monkey contributed 123–147 profiles. Three hundred sixty micrographs representing $2,196 \mu\text{m}^2$ of layer III were analyzed across four monkeys. Five hundred forty-four labeled profiles were counted. Three of the monkeys contributed 100–141 labeled profiles each, and one monkey contributed 263. Two hundred ninety-five micrographs representing $1,800 \mu\text{m}^2$ of layer V were analyzed across four monkeys. Five hundred sixteen labeled profiles were counted, and each monkey contributed 108–177 labeled profiles.

For D₅ 290 micrographs in total representing $1,769 \mu\text{m}^2$ of layer I were analyzed across four monkeys. Four hundred seventy labeled profiles were counted. Two of the monkeys contributed 116 labeled profiles each, one contributed 70 labeled profiles, and one contributed 168 labeled profiles. Three hundred forty micrographs representing $2,074 \mu\text{m}^2$ of layer III were analyzed across four monkeys. Four hundred ninety-eight labeled profiles were counted, and each monkey contributed 104–149 labeled profiles. Finally, 316 micrographs representing $1,928 \mu\text{m}^2$ of layer V were analyzed across three monkeys. Four hundred eighty-nine labeled profiles were counted, and the monkeys contributed 73, 205, and 211 labeled profiles each.

On each micrograph, DAB-labeled profiles were identified and classified as spines, dendrites, terminals, axons, glia, or unknown based on ultrastructural criteria (Peters et al., 1991) as previously described (Muly et al., 2003). Profiles that could not be clearly characterized based on these criteria were considered unknown profiles. The number of immunoreactive profiles was tabulated, and the distributions (excluding the unknown profiles) were compared with a χ^2 analysis.

Analysis of the immunogold/DAB material was performed on blocks from layer III of area 9. We examined ultrathin sections from the surface of each block where both immunoperoxidase label and immunogold label were visible. Because immunogold label can be noisy, we sought to avoid false-positive labeling by avoiding the very surface of each block, where nonspecific gold particles tend to accumulate. We compared the immunogold label in a given structure to the surrounding background level of immunogold labeling as well as the size of the silver-intensified gold particles. If the profile qualitatively contained more immunogold label than the background level, we deemed that acceptable immunogold labeling.

Blocks of tissue from layer III cortical area 9 were made for the D₁/D₅-cocktail, D₁-alone, and D₅-alone conditions. Fields of the neuropil were randomly selected, and images were collected at a magnification of $\times 20,000$. An ANOVA sample-size analysis (SigmaStat, version 2.03; SPSS Inc.) indicated that the minimum sample size required to have a statistical power of 80% and a minimum detectable difference in group means of seven was 239 images; therefore, we analyzed 239 images in the D₁-alone condition, 297 images in the D₅-alone condition, and 279 images in the D₁/D₅-cocktail condition. In each experimental condition, the numbers of micrographs analyzed from each monkey were similar. On each micrograph, spines and axon terminals were identified by using the previously described ultrastructural criteria (Peters et al., 1991), then classified as immunopositive or immunonegative, and the percentage of identified spines or terminals that were immunopositive was calculated. The mean percentages of immunopositive spines and axon terminals were tabulated for each condition and compared across antigen conditions with an ANOVA. The results are reported as mean $\pm$ SE.

RESULTS

Laminar- and subtype-specific variation in subcellular distributions of D1R

The localization of D₁ and D₅ in the PFC has been previously described in detail at the light microscopic level (Bergson et al., 1995a,b; Muly et al., 1998; Khan et al., 2000). Briefly, D₁ immunolabeling is found in the Golgi apparatus of labeled perikarya and extends into the proximal dendrites. D₅ immunolabeling is also present in the cell soma and strongly labels dendrites. At the electron microscopic level, we identified label for both D₁ and D₅ in the soma. D₁ immunoreactivity (-IR) was associated with internal membranes, namely, the Golgi apparatus (Fig. 2A). D₅-IR was also associated with internal membranes of the soma (Fig. 2B) as well as the plasma membrane (Fig. 2C).

We examined the localization of D₁ and D₅ in *Macaca mulatta* PFC neuropil in layers I, III, and V of prefrontal cortical area 9 to determine whether their localization patterns differ across cortical layers. Each receptor was seen in spines, dendrites, axon terminals, preterminal axons, and glia in each cortical layer (Figs. 3, 4); however, the degree to which each receptor was localized in these compartments appeared to differ. Accordingly, we quantified the distribution of each receptor in three different layers of PFC. The pattern of D₁ localization in various cellular compartments in layers I, III, and V differed significantly (Fig. 5A; $\chi^2 = 41.728$, $p < 0.0001$). Post hoc testing revealed that D₁ was more frequently identified in spines of layer III than in spines of layer I or V, in preterminal axons of layer V than of layer I or III, and in glia of layer I than of layer III or V. In addition, the pattern of D₅ localization in cellular compartments in layers I, III, and V differed significantly (Fig. 5B; $\chi^2 = 45.986$, $p < 0.0001$). Post hoc testing revealed that D₅ was more frequently identified in dendrites of layer III than in dendrites of layer I, in axon terminals of layer I than of layer III or V, in preterminal axons of layer V than of layer I or III, and in glia of layer I than of layer III or V. Thus, in layer III, both D1R subtypes are enriched in dendritic structures where they can modulate the response of neurons.

The distributions of D₁ and D₅ that we observed in our laminar analyses appeared to be markedly different, and we tested this by comparing the distributions of D₁ and D₅ in layer III of area 9. Layer III was chosen for the remaining experiments because it is a major site of cortical integration (Rockland and Pandya, 1979; Maunsell and van Essen, 1983; Kritzer and Goldman-Rakic, 1995) and is altered in patients with schizophrenia (Glantz and Lewis, 2000; Lewis et al., 2003). Within layer III of PFC area 9, the patterns of D₁ and D₅ labeling differed significantly (Fig. 6; $\chi^2 = 74.592$, $p < 0.0001$). Post hoc testing revealed that D₁ immunoreactivity was more commonly found in spines and preterminal axons, whereas D₅ immunoreactivity was more commonly found in dendrites and glia.

Our quantitative analyses indicate that, although the D₁ receptor is found in spines to a greater extent than D₅, both D₁ and D₅ are found to a large degree in both dendritic spines and shafts. Thus, once activated by dopamine, both are well positioned to modulate inputs to dendritic spines as well as the propagation of these signals through the dendritic shafts to the cell soma. Furthermore, though less prominent, both D1R subtypes are positioned to play a role in modulating presynaptic actions.

Distribution of D₁ and D₅ in cortical spines and axon terminals

Our quantitative analyses show that both receptors are present in spines and axon terminals and, as such, are positioned to mediate dopaminergic modulation of axospinous inputs to pyramidal cells both pre- and postsynaptically. An important question is whether the D₁ and D₅ receptors are located in the same or different populations of spines and axon terminals. Because of their different affinities for dopamine and the dose-response relationship of D1R

stimulation and working memory function, we hypothesized that D₁ and D₅ receptors would be found in different populations of dendritic spines and axon terminals. To test this hypothesis, we used a double-label approach in which one receptor was revealed with preembedding immunogold and the other with DAB. Contrary to our hypothesis, we found spines containing both immunogold and DAB, and double-labeled spines and axon terminals could be identified regardless of which chromagen was utilized (Fig. 7). These experiments suggest that D₁ and D₅ are colocalized in dendritic spines of prefrontal cortical area 9. However, there are reasons to be cautious in interpreting these experiments. Although the different labels are distinguishable when examined with the electron microscope, preembedding immunogold labeling is less sensitive than immunoperoxidase labeling because of limited penetration of 1-nm gold conjugates as well as the instability of silver intensifier in osmium-treated material. This is especially problematic when lower-abundance antigens are examined, such as dopamine receptors in neocortex, compared with calcium-binding proteins (Galvan et al., 2006). In addition, it is our experience that immunoperoxidase labeling is less robust in tissue that has been previously silver intensified. However, if silver intensification is performed after the immunoperoxidase reaction, it can nonspecifically deposit onto DAB, as demonstrated by the use of silver solutions to intensify DAB labeling (Smiley and Goldman-Rakic, 1993; Teclemariam-Mesbah et al., 1997). Thus it is very difficult to interpret the significance of single-labeled profiles in this double-labeled material. For these reasons, we cannot determine the extent of colocalization of the two receptors with these methods.

To quantify the degree to which the two receptors colocalize in spines and axon terminals, we used a cocktail labeling approach that has been successfully used to identify overlapping distributions of proteins (Muly et al., 2001; Mitrano and Smith, 2007) as well as distinct distributions (Lei et al., 2004). The advantage of this procedure is that the labeling method used for both receptors (immunoperoxidase labeling) has the best and equal sensitivity and penetration (Wouterlood et al., 1993; Galvan et al., 2006). We randomly imaged material labeled with antiserum to D₁, D₅, or a cocktail of antisera to both receptors and calculated the percentage of spines and terminals labeled for each receptor individually as well as for the two receptors combined. These values were then compared with an ANOVA. The percentage of spines in layer III of area 9 labeled individually for the two receptors or the cocktail differed significantly (Fig. 8A; $F_{2,812} = 8.418$, $p = 0.0002$), and post hoc Scheffe tests confirmed that the percentage of spines labeled for D₅ was significantly less than for D₁ ($p = 0.0030$) and the cocktail ($p = 0.0016$). However, there was no significant difference between the percentage of spines labeled for D₁ or the cocktail of D₁ and D₅ ($p = 0.9996$). The finding that the percentage of spines labeled by D₁ and a cocktail of D₁ and D₅ is not significantly different demonstrates that the D₅ receptor is found in a subpopulation of the D₁-positive spines. If D₁ and D₅ labeled separate populations of spines, the cocktail condition would label a higher percentage of spines than D₁ alone. These data indicate that both D₁ and D₅ are found together in approximately 14% of dendritic spines and that D₁ is found in an additional 7% of prefrontal cortical area 9 layer III spines.

We also performed a double-label cocktail analysis for axon terminals in prefrontal cortical area 9 within layer III, as we had for dendritic spines. As seen in dendritic spine labeling, the percentage of axon terminals labeled for D₁, D₅, or the cocktail differed significantly (Fig. 8B; $F_{2,811} = 25.598$, $p < 0.0001$), and post hoc Scheffe tests confirmed that the percentage of D₅-labeled terminals was significantly less than the percentages of D₁ ($p < 0.0001$) and the cocktail ($p < 0.0001$); however, there was no significant difference between D₁ and the cocktail ($P = 0.3396$). These data indicate that both D₁ and D₅ are found together in approximately 4% of axon terminals, and that D₁ is found in an additional 6% of prefrontal cortical area 9 layer III terminals.

We examined the synaptic type and postsynaptic structures of D1R-labeled axon terminals. Among the 29 D₁-positive axon terminals that made identifiable synapses, 26 were asymmetric and three were symmetric. Twenty-three of the asymmetric synapses were onto unlabeled spines, and the remaining three were onto unlabeled dendrites. Two of the symmetric synapses were onto unlabeled dendrites, and the remaining D₁-positive axon terminal formed a symmetric synapse onto an unlabeled spine. Among the 24 D₅-positive axon terminals that made identifiable synapses, 23 were asymmetric and one was symmetric. Twenty-one of the asymmetric synapses were onto unlabeled spines, and the remaining two were onto unlabeled dendrites. The symmetric synapse was formed onto a spine. Taken together, our data demonstrate that there are three populations of spines and axon terminals in area 9 of the macaque PFC defined by their presence or absence of D1R: those that contain both D₁ and D₅, those that contain only D₁, and those that contain neither D1R subtype.

DISCUSSION

We have quantified the distributions of the D₁ and D₅ receptors within *Macaca mulatta* prefrontal cortical area 9 neuropil and determined their colocalization in dendritic spines and axon terminals. Our data confirm the previously reported relative enrichment of D₁ in spines and D₅ in dendrites (Bergson et al., 1995b). However, our quantitative data indicate each receptor has a complex localization throughout the neuropil, including the D₁ receptor labeling spines and dendrites at equivalent frequencies. Furthermore, we demonstrate laminar specificity in the distributions of the two D1R subtypes in macaque prefrontal cortical area 9, a region where they are critical for working memory function. These laminar differences suggest a potential circuit specificity in their actions. Finally, a key finding of the current study is that the D1Rs are extensively colocalized to area 9 prefrontal cortical pyramidal cell spines and axon terminals, such that D₅ is always localized with D₁. This final result demonstrates that dopaminergic activation of the two D1R can modulate overlapping populations of synapses both presynaptically on terminals and postsynaptically on dendritic spines.

One finding in these studies is that the distribution of each D1R subtype differs across the area 9 cortical layers. The six layers of neocortex are heterogeneous in their cellular makeup and extrinsic and intrinsic connections (Gilbert, 1983; Swadlow, 1983), and these laminar differences are likely related to the observed differences in D1R localization. For example, the increased D1R labeling of glia in layer I compared with layers III and V may reflect the increased presence of glia in layer I versus other layers (Dombrowski et al., 2001). The extent of glial labeling observed in our study (10–25%) was unexpected; however, the presence of dopamine receptors, including D₁ and D₅, in cortical and striatal glial cells is well documented (Zanassi et al., 1999; Reuss and Unsicker, 2001; Brito et al., 2004; Miyazaki et al., 2004; Kumar and Patel, 2007), although one study did not find D₅ glial labeling in rat tissue (Ciliax et al., 2000). Dopamine has been shown to increase intracellular calcium levels in cortical glial cells (Reuss et al., 2001; Reuss and Unsicker, 2001), an effect that is blocked by pretreatment with atypical neuroleptics (Reuss and Unsicker, 2001). The D1R agonist SKF 38393 stimulates G-protein coupling in rat spinal white matter (Venugopalan et al., 2006); induces cAMP production in rat, monkey, and human striatal glia (Vermeulen et al., 1994) and rat cortical glia (Zanassi et al., 1999); and increases PKA activity in striatal glia (Li et al., 2006). Additionally, brain-derived neurotrophic factor specifically stimulates D₅ expression in mouse striatal astrocyte cultures (Bruto et al., 2004). Moreover, recent research suggests that methamphetamine has significant effects on gliogenesis in the medial PFC of rats (Mandyam et al., 2007). Thus, although research is ongoing regarding glial D1R, a picture is emerging in which dopaminergic agents and D1R activity in glia play a role in proper CNS functioning and can be dysregulated in many CNS diseases (for review see Maragakis and Rothstein, 2006).

The D₅ receptor is present in layer I axon terminals to a greater extent than in layers III and V. Although our data do not address the source of these axon terminals, layer I in particular receives input from the intralaminar thalamic nuclei (Jones, 1975; Rausell and Avendano, 1985), which express the D₅ receptor in rat, monkey, and human brain (Ciliax et al., 2000; Clinton et al., 2005). The layer I D₅-positive terminals identified in the current study form asymmetric synapses predominantly onto unlabeled dendritic spines, as would be expected from thalamic nuclei, which also form predominantly asymmetric axospinous synapses. Interestingly, lesions of the intralaminar nuclei in rats cause specific deficits in memory tasks (Mair et al., 1998), and D₅ receptors on the axon terminals from these nuclei may contribute to D1R modulation of cognition. D₁ and D₅ were also present in preterminal axons of layer V more so than in layers I and III. The enrichment of these receptors in preterminal axons may represent a reservoir of D1R (Shakiryanova et al., 2006) destined for the axon terminals of corticostriatal or corticocortical projections.

Perhaps the most striking finding of the quantitative laminar analyses undertaken in this study is that D₁-labeled spines and D₅-labeled dendritic shafts are particularly common in prefrontal cortical area 9 layer III compared with layers I and V. Layer III is a major site of cortical integration (Rockland and Pandya, 1979; Maunsell and van Essen, 1983; Kritzer and Goldman-Rakic, 1995), and prefrontal cortical layer III pyramidal cells are modulated by dopamine and D1R antagonists (Henze et al., 2000; Urban et al., 2002). Furthermore, patients with schizophrenia show specific alterations in layer III (Glantz and Lewis, 2000; Lewis et al., 2003). The enrichment of D1R subtypes at sites of postsynaptic integration suggests that D1R activity plays a particularly important role in modulating responses in layer III of the PFC.

In comparing the localization of D₁ and D₅, we show that the D₁ receptor antiserum labels spines more frequently than D₅ and that the D₅ receptor antiserum labels dendrites more frequently than D₁. However, it is important to note that the D₁ receptor labels dendritic shafts at equivalent and greater frequencies than it labels dendritic spines across layers I, III, and V. Electrophysiological studies have emphasized the importance of D1R dendritic localization in modulating PFC input in a spatially dependent manner (Seamans and Yang, 2004). For example, activation of D1R on apical dendrites attenuates high-threshold Ca²⁺ spikes, thus attenuating the effects of inputs to these apical dendrites (Yang and Seamans, 1996). The present study indicates that both the D₅ and the D₁ receptors are present in dendritic shafts, so both may be modulating ionic conductances on pyramidal cell dendrites. Although we have not directly addressed whether both the D1Rs are expressed on the dendrite plasma membrane, studies in the rodent basal ganglia and basolateral amygdala and monkey PFC demonstrate the presence of the D₁ receptor on the plasma membrane of dendritic shafts (Dumartin et al., 2000; Paspalas and Goldman-Rakic, 2005; Pickel et al., 2006; Hara and Pickel, 2007).

D1R activation in the PFC is critical for WM performance (for review see Goldman-Rakic, 1995), and D1R activation has a dose-dependent relationship with WM ability and neuronal activity, such that low levels of D1R activation results in low ability and activity, medium levels of D1R activation result in optimal WM ability and higher activity, and high levels of D1R activation result in diminished WM ability and lower activity (Vijayraghavan et al., 2007). In this context, it is particularly germane to note that D₅ has been reported to have a tenfold greater affinity for dopamine; however, the individual contributions of D₁ and D₅ to WM processes cannot be determined with currently available pharmacological tools. We have found that the D₁ receptor is located in approximately 20% of PFC spines (Bergson et al., 1995b; Muly et al., 2001) and that the D₅ receptor is located in approximately 14% of PFC spines. We expected the D1R subtypes to be found in different populations of spines, similar to the selective localization of the D₅ receptors, but not the D₁ or D₂ receptors, in the vicinity of subsurface cisterns of cell bodies in the macaque PFC (Paspalas and Goldman-Rakic, 2004). However, by using a cocktail of the D₁ and D₅ antibodies, we have determined that the

D₅ receptor is present in a subpopulation of those spines that contains the D₁ receptor. This observation has a number of implications regarding the dose-dependent relationship between D₁R activation and PFC functioning. First, the spines with both D₁R subtypes will have a larger dynamic response range to varying dopamine concentrations than spines expressing only D₁. However, it remains unknown whether the responses to D₁ and D₅ stimulation are simply additive within a spine or whether they result in distinct actions. Both receptors couple through G_s, so increasing levels of dopamine should result in a larger cAMP response. Additionally, there is growing evidence that G-protein-coupled receptors can signal via heterodimers or oligomers (Bouvier, 2001; Prinster et al., 2005), including a D₁-D₂ heterodimer (Dziedzicka-Wasylewska et al., 2006). Our quantitative cocktail and immunogold/DAB data demonstrate that D₁-D₅ heterodimers are possible (Fig. 6B). Biochemical studies will be required to determine whether such interactions occur in the primate PFC.

Alternately, there is evidence that the two D₁R subtypes can couple to different G-proteins (Kimura et al., 1995; Sidhu et al., 1998; Wang et al., 2001), can differentially signal via protein kinase C and phospholipase C (Yu et al., 1996; Paolillo et al., 1998; Jackson et al., 2005; Zhen et al., 2005), and are physically linked to different effector proteins (Liu et al., 2000; Lee et al., 2002). Moreover, precise targeting of signal transduction proteins via scaffolds has been shown to play an important role in neuronal signaling (Westphal et al., 1999; Yan et al., 1999; Chen et al., 2004). These factors raise the possibility that D₁ and D₅ could induce distinct intracellular signals within the same spine, contributing to the complex relationship between D₁R and WM. Indeed, the group I metabotropic glutamate receptors 1 and 5 are frequently colocalized, even though they both classically signal via the same second messenger cascade. From the use of subtype-specific pharmacological tools, it is now known that activation of each receptor leads to discrete neuronal responses when the two are colocalized (for review see Valenti et al., 2002; Poisik et al., 2003).

Electrophysiological studies indicate that activation of presynaptic D₁R generally decreases excitatory and inhibitory neurotransmission (Momiya and Sim, 1996; Behr et al., 2000; Gao et al., 2001; Young and Yang, 2005). Twenty-six of the twenty-nine D₁-only-containing axon terminals made asymmetric synapses, and 11.5% (3 of 26) of these D₁-positive axon terminals making asymmetric synapses targeted dendrites, whereas the remainder formed asymmetric synapses onto spines. These data are in strong agreement with a previously published study of D₁-positive axon terminals in the macaque PFC, which found that 11.7% of D₁-positive axon terminals formed asymmetric synapses onto dendrites (Paspalas and Goldman-Rakic, 2005). It is important to recognize that the data reported here on the distribution of D₁R in the neuropil primarily reflect the distribution of these receptors in the most common cellular element in the PFC, which is the pyramidal projection neuron. Because certain populations of interneurons also show tuned delay responses during WM tasks (Rao et al., 1999, 2000), have been shown to contain the D₁ receptor (Muly et al., 1998), and can strongly modulate pyramidal cell output (Lund and Lewis, 1993; DeFelipe, 1997; Zaitsev et al., 2004; Gonzalez-Burgos et al., 2005), determining the localization of D₁R to specific classes of interneurons could prove to be helpful in understanding the circuitry mechanisms of D₁R activation on neural activity. Understanding more fully how each of these dopamine receptors contributes to prefrontal functioning will require an examination of inhibitory interneurons.

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Fig. 1.

A: Western blot of *Macaca mulatta* prefrontal cortex (PFC), striatum (STR), and hippocampus (HIPP) showing that the D₅ antibody reacts only with a single protein band at a molecular weight of approximately 53 kD. **B:** Light electron microscopic image of typical D₅ staining in macaque PFC. **C:** D₅ staining is abolished in macaque PFC when the antiserum is preincubated with the cognate peptide. Scale bar = 200 μ m.

Fig. 2. Electron micrographs illustrating typical somatic labeling. D₁ diaminobenzidine label (arrows) was limited to the Golgi apparatus in cell bodies (A), whereas D₅ immunoreactivity was associated with internal membranes such as the endoplasmic reticulum (B) as well as the plasma membrane (C). Scale bar = 500 nm.

Fig. 3. Electron micrographs illustrating examples of D₁ immunoreactivity in PFC area 9 neuropil. Diaminobenzidine label (arrows) was identified in spines (**A**), dendrites (**B**), axon terminals (**C**), preterminal axons (**D**), and glia (**E**) in each layer examined. Scale bar = 500 nm.

Fig. 4. Electron micrographs illustrating examples of D₅ immunoreactivity in PFC area 9 neuropil. Diaminobenzidine label (arrows) was identified in spines (A), dendrites (B), axon terminals (C), preterminal axons (D), and glia (E) in each layer examined. Scale bar = 500 nm.

Fig. 5.

A: Histogram showing the relative abundance of D₁ in layers I, III, and V in area 9 of the PFC. In layer I, 528 profiles in 295 micrographs were examined; in layer III, 544 profiles in 360 micrographs were examined; and, in layer V, 516 profiles in 295 micrographs were examined. The distribution of the D₁ receptor differed significantly across layers ($\chi^2 = 41.728, p < 0.0001$).

B: Histogram showing the relative abundance of D₅ in layers I, III, and V in area 9 of the PFC. In layer I, 470 profiles in 290 micrographs were examined; in layer III, 498 profiles in 340 micrographs were examined; and, in layer V, 489 profiles in 316 micrographs were examined. The distribution of the D₅ receptor differed significantly across layers ($\chi^2 = 45.986, p < 0.0001$). Comparisons that are significantly different by post hoc tests are indicated by an asterisk.

Fig. 6.

Histogram comparing the relative abundance of D₁ and D₅ in layer III of area 9 of the PFC. The distribution of D₁ and D₅ differed significantly in layer III ($\chi^2 = 74.592$, $p < 0.0001$). Post hoc testing revealed that spines and preterminal axons were more commonly labeled for D₁, whereas dendrites and glia were more commonly labeled for D₅. Comparisons that are significantly different by post hoc tests are indicated by an asterisk.

Fig. 7. Electron micrographs of double-label immunogold (arrow-heads) and DAB (arrows) images of D₁ and D₅ in PFC. **A,B:** Dendritic spines immunogold labeled for D₁ and DAB labeled for D₅. **C,D:** Dendritic spines immunogold labeled for D₅ and DAB labeled for D₁. Scale bar = 500 nm.

Fig. 8.

A: Histogram showing the percentage of PFC area 9 layer III spines labeled by D₁, by D₅, or by both D₁ and D₅. For the D₁-alone condition, 239 micrographs containing 1,053 spines were analyzed. For the D₅-alone condition, 297 micrographs containing 1,284 spines were analyzed. For the D₁/D₅-cocktail condition, 279 micrographs containing 1,258 spines were analyzed. ANOVA revealed that the percentage of spines labeled for D₁ (21.38% ± 1.438%), by D₅ (14.635% ± 1.211%), or by both D₁ and D₅ (21.438% ± 1.487%) differed significantly ($F_{2,812} = 8.418$, $p = 0.0002$), and post hoc Scheffe tests confirmed that the percentage of D₅ labeled spines was significantly less than that of D₁ ($p = 0.0030$) or the cocktail ($p = 0.0016$). However, there was no significant difference between D₁ and the cocktail ($p = 0.9996$). These data demonstrate that the D₅ dopamine receptor is found in a subset of the D₁ labeled spines.

B: Histogram showing the percentage of axon terminals labeled by D₁, by D₅, or by both D₁ and D₅ in layer III area 9 the PFC. For the D₁-alone condition, 239 micrographs containing 2,033 axon terminals were analyzed. For the D₅-alone condition, 297 micrographs containing 2,483 axon terminals were analyzed. For the D₁/D₅-cocktail condition, 279 micrographs

containing 1,842 axon terminals were analyzed. ANOVA revealed that the percentage of axon terminals labeled for D₁ ($10.066\% \pm 0.858\%$), D₅ ($3.595\% \pm 0.411\%$), and both D₁ and D₅ ($8.620\% \pm 0.756\%$) differed significantly ($F_{2,811} = 25.598$, $p < 0.0001$), and post hoc Scheffe tests confirmed that the percentage D₅ labeled terminals was significantly less than that of D₁ ($p < 0.0001$) and the cocktail ($p < 0.0001$). However, there was no significant difference between D₁ and the cocktail ($p = 0.3396$). These data indicate the D₅ dopamine receptor is found in a subset of the D₁ labeled axon terminals. Comparisons that are significantly different by post hoc Scheffe tests are indicated by an asterisk.