Microdomains for Dopamine Volume Neurotransmission in Primate Prefrontal Cortex

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The explicit yet enigmatic involvement of dopamine in cortical physiology is in part volumetric (beyond the synapse), as is apparently the action of neuroleptics targeting dopamine receptors. The notion that nonsynaptic neuronal membranes would translate extracellular dopamine into receptor-specific spatiotemporal downstream signaling, similar to the chemical synapse, is intriguing. Here, we report that dopamine D₅ (but not D₁ or D₂) receptors in the perisomatic plasma membrane of prefrontal cortical neurons form discrete and exclusively extrasynaptic microdomains with inositol 1,4,5-trisphosphate-gated calcium stores of subsurface cisterns and mitochondria. These findings introduce a novel dopaminoceptive substratum in the brain and a unique D₅ receptor-specific signaling paradigm.

Key words: dopamine receptor; InsP₃ receptor; subsurface cistern; electron microscopy; neuronal calcium; microdomain; prefrontal cortex; working memory

Introduction

In the primate prefrontal cortex (PFC), dopamine (DA) signaling is implicated in complex cognitive operations, the actions of drugs of abuse, and devastating neuropsychiatric disorders, including schizophrenia (Brozoski et al., 1979; Goldman-Rakic, 1987, 2002; Lidow et al., 1998; Carlsson et al., 2001; Weinberger et al., 2001). Yet many of these explicitly specific actions result from DA signals reaching via gradient diffusion receptors on proximal and remote nonsynaptic membranes (Goldman-Rakic et al., 1997; Pickel, 2000) [see reviews edited by Agnati et al. (2000) for a comprehensive account on volume neurotransmission]. Efficacy and cellular compartmentalization of extrasynaptic DA receptors (DARs) is therefore presumed to account for distinctive neuromodulatory and neuropharmacological patterns via specific signal transduction mechanisms assigned to individual receptor subtypes (Goldman-Rakic et al., 1997; Missale et al., 1998).

Three decades after volumetric signaling was first introduced as a potent mechanism of interneuronal communication (for review, see Dismukes, 1977; Beaudet and Descarries, 1978; Agnati et al., 1995b), our understanding of receptors and effector systems mediating volume neurotransmission remains mostly incomplete. This is primarily because these receptors, unlike their synaptic counterparts, do not seem to operate within discrete signaling modules in the brain. These views have recently been

challenged by the evolving concept of the signaling microdomain. Elegant physiological and biochemical studies suggest the presence of microdomains that couple exogenous activation with a specific set of transduction molecules on intracellular membranes, thereby facilitating downstream signaling and encoding the stimulus spatiotemporal pattern and the signal transduction mechanisms of individual receptors (for review, see Berridge, 1998; Fagni et al., 2000; Delmas and Brown, 2002; Johnsen and Ehrlich, 2002; Augustine et al., 2003). In effect, a signaling microdomain would operate as a signal amplification module to ensure fidelity of transmission and potentially to translate local events into global responses by engaging universal second messengers. Of key significance is the compartmentalization of the local cytosol that sequesters ionic and biochemical pools, exemplified by the dendritic spine, and the phosphoinositide system mobilizing calcium (Ca²⁺) from intracellular stores via the 1,4,5-trisphosphate receptor (InsP₃R) (Berridge, 1993).

Given the importance of volume transmission mechanisms for DA physiology and neuropsychopharmacology (Zoli et al., 1999) and the potential of the signaling microdomain for maintaining input specificity and selectivity of regulation, we evaluated the plausibility of an evolving hypothesis that such modules may in fact exist in neurons to facilitate “diffuse” dopaminoception. We used high-resolution immunoelectron microscopy to study expression patterns of the D₁-like DARs (D₁-LRs; i.e., D₁ and D₅ subtypes) and the D₂ subtype, the prototypic receptor of the D₂-like subfamily (for review, see Missale et al., 1998), in the pyramidal perisomatic region. This compartment, unlike dendritic spines, includes a massive single cytosolic volume and, in the PFC of primates, bears rare association with DAergic afferents (Smiley and Goldman-Rakic, 1993; Sesack et al. 1995). More importantly, the perisomatic plasma membrane is uniquely furnished with subsurface cisterns (SSCs) (see Fig. 1 for an overview), which would ultimately ensure cytosol compartmentalization and serve as scaffold for molecules mediating downstream effects.

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Table 1. Receptor antibodies utilized in single (s) and dual (d) immunoprocures

Antibody	Immunogen	Host	Dilution	Source
Anti-D ₁ R	C terminus (human) aa 349–446	Clone 11- F11 S.E6	1:800 (s)	Sigma-RBI, St Louis, MO
Anti-D ₂ R	i3 loop (human) aa 284–311	Rabbit	1:500 (s)	Chemicon, Temecula, CA
Anti-D ₃ R	C terminus (human) aa 428–438	Rabbit	1:1000 (s) 1:1200 (d)	Dr. Z. U. Khan, University of Malaga, Spain
Anti-D ₅ R	C terminus (mouse) aa 377–477	Rabbit	1:1500 (s) 1:2000 (d)	Dr. R. Moratalla, Institute Cajal, Spain
Anti-InsP ₃ R	C terminus (human) aa 2492–2502	Clone IPR0.1	1:200 (d)	Calbiochem, San Diego, CA
Anti-InsP ₃ R	C terminus (human) aa 2492–2502	Clone IPR0.1	1:200 (d)	Chemicon
Anti-InsP ₃ R	C terminus (human) aa 2596–2608	Rabbit	1:250 (s)	Chemicon

The amino acid sequence of immunogen peptides can be viewed on National Center for Biotechnology Information Sequence Viewer (free access) at: [http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=search for 118228 \(D₁R-human\), 71937 \(D₂R-human\), 32049 \(D₃R-human\), 29789042 \(D₅R-mouse\), and 10835023 \(InsP₃R-human\).](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=search for 118228 (D₁R-human), 71937 (D₂R-human), 32049 (D₃R-human), 29789042 (D₅R-mouse), and 10835023 (InsP₃R-human).)

Materials and Methods

Histology and immunoreagents. Two adult rhesus monkeys (*Macaca mulatta*) from the Yale Department of Neurobiology primate colony were anesthetized with sodium pentobarbital (100 mg/kg, i.v.) and perfused transcardially with oxygenated artificial CSF, followed by 4% paraformaldehyde/0.08% glutaraldehyde in phosphate buffer (PB; 100 mM) and aldehyde-free PB. Experimental procedures were conducted in accordance with federal and institutional guidelines. The brains were blocked coronally, vibrasliced at 60 μ m, cryoprotected, and stored frozen at -80°C . Prefrontal cortical sections were thawed in PB and preincubated for 45 min in 10% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and 2% bovine serum albumin (BSA; Vector Laboratories) in Tris-buffered saline (TBS; 50 mM) before being transferred to primary antibodies (Table 1). Nanogold- and biotin-SP-conjugated IgG fragments were purchased from Nanoprobe (Yaphank, NY) and Jackson ImmunoResearch (West Grove, PA), respectively. Unlabeled bridging antibodies and peroxidase–antiperoxidase complexes were from Sternberger Monoclonals (Lutherville, MD).

Single gold-based D₁R, D₂R, D₅R immunocytochemistry. Primary antibodies diluted in TBS plus 2% NGS (i.e., N-TBS) were applied for 36 hr at 4°C . Sections were then incubated for 2 hr in 1:500 species-specific biotinylated F(ab')₂ and for another 2 hr in 1:200 goat anti-biotin Fab' conjugated to 1.4 nm gold cluster. Immunocomplexes were fixed for 5 min in 1% buffered glutaraldehyde and, after a thorough wash in ultrapure water, nanogold was enhanced for 8 min with a silver autometallographic developer (HQ Silver; Nanoprobe).

Dual gold-based and peroxidase–gold-based D₅R/InsP₃R immunocytochemistry. Primary antibodies in N-TBS were simultaneously applied for 48 hr at 4°C . To visualize the D₅R, sections were preincubated for 30 min in N-TBS supplemented with 0.1% acetylated BSA (Aurion, Wageningen, The Netherlands), 0.1% fish skin gelatin and 0.07% Tween 20 (i.e., gold buffer), and transferred for 3 hr in nanogold-conjugated goat anti-rabbit Fab' (1:200 in gold buffer). After wash in ultrapure water and 20 mM sodium citrate, nanogold was enhanced for 12 min on ice with a gold developer (GoldEnhance; Nanoprobe). Subsequently, nanogold-conjugated goat anti-mouse Fab' (1:200) was applied for 4 hr to probe the InsP₃R primary antibody. Sections were finally postfixed in glutaraldehyde and transferred for 4 min to the gold developer to enhance the InsP₃R-bound nanogold but also to enhance further (i.e., 12 + 4 min in total) the D₅R gold signal of the first series (two-step autometallography). This sequential enhancement produced distinct, nonoverlapping particle-size groups (see Methodological Considerations). Alternatively, D₅Rs were labeled in the second series using species-specific bridging antibodies and peroxidase–antiperoxidase tertiary complexes (1:200 in N-TBS for 2 hr each). Peroxidase activity was visualized in 0.025% diaminobenzidine (DAB) in TBS with the addition of 0.007% hydrogen peroxide. In this case, InsP₃Rs were labeled as detailed above, with gold enhancement ranging from 3 to 8 min at 22°C .

Electron microscopy. Sections were postfixed in 1% buffered osmium tetroxide (15 and 30 min for silver- and gold-enhanced material, respectively), treated with ethanolic uranyl acetate *en block*, and finally embedded in Durcupan epoxy resin (Fluka, Steinheim, Switzerland) and polymerized at 58°C for 48 hr under vacuum. Layers II–IV and V–VI of the

Figure 1. *A, B*, Schematic representation of the relative topography of the SSC (*A*; arrowheads in frame and enlarged schema) and electron micrograph depicting oblique plane of section through the plasmalemma–SSC interface (*B*). As seen in *B*, SSCs typically consist of single or multiple flattened cisterns (electron opaque zone subjacent to the membrane; arrowheads), confluent through anastomoses with smooth reticular endomembranes (SER) and continuous with the rough endoplasmic reticulum (arrows). Therefore, an SSC derives from the reticular network of which is considered spatial and potentially functional modification. In the CNS and peripheral nervous system, SSCs are neuron specific and localized exclusively in perikarya and the most proximal portions of their processes (Rosenbluth, 1962; Henkart et al., 1976; Peters et al., 1991). Likewise, SSCs occur in perikarya and large-caliber proximal dendrites of cortical neurons (Rosenbluth, 1962; Buschmann, 1979; for review, see Peters et al., 1991) and are unique to the axon initial segment of principal cells (Palay et al., 1968; Benedekzy et al., 1994). Double arrowheads in *B* point to an axo-spinous synapse (ax-sp) next to the SSC. mit, Mitochondrion. Scale bar, 100 nm.

dorsolateral PFC (Walker's area 46) were sampled for thin sectioning and ultrastructural analysis under a JEM-1010 (Jeol, Tokyo, Japan) transmission electron microscope operated at 80 KV, with or without lead counterstaining.

Distinction between pyramidal and nonpyramidal neurons at the level of the perisomatic region was made on the basis of the established ultrastructural criteria for neuron-class identification in the neocortex, including the unique synaptology of each class (i.e., symmetric axosomatic and proximal axo-dendritic synapses for pyramids versus both symmetric and asymmetric synapses for interneurons) (Colonnier, 1981; Peters and Kara, 1985a,b).

Antibodies. The full characterization of the affinity-purified DAR antibodies has been detailed previously (Boundy et al., 1993; Levey et al., 1993; Khan et al., 2000; Centonze et al., 2003). The immunogen peptide for each subtype-specific antibody (Table 1) shows no sequence homology with other identified DARs. The D₅R antibody (aa 377–477) has been characterized also in D₁R null (–/–) mice and shown not to cross-react with the homologous D₁R subtype (Centonze et al., 2003). Various groups have provided additional characterization and applied these antibodies for the study of DARs in rodents, cats, humans, and nonhuman primates with various fixation protocols (Smiley et al., 1994; Bergson et al., 1995b; Yung et al., 1995; Rivera et al., 2002; Wang and Pickel, 2002). Both D₅R antibodies labeled selectively plasmalemma/SSC complexes and revealed in the neuropil (data not shown) an expression pattern complementary to that of the D₁R, as described with antibodies raised against the 375–477 residue of the human D₅R protein (Bergson et al., 1995b). The affinity-purified InsP₃R monoclonal antibodies (Bourguignon et al., 1993, 1994) yielded indistinguishable labeling patterns in both the neuropil and the perisomatic region, targeting selectively the cytoplasmic face of reticular endomembranes as predicted from the transmembrane topology of the immunogen peptide (i.e., cytoplasmic C terminus sequence of the InsP₃R) (see Fig. 5A–D). To confirm the specificity of immunolabeling for the InsP₃R, we used an affinity-purified polyclonal InsP₃R antibody raised against a different amino acid sequence (aa 2596–2608 vs 2492–2502) (Table 1) of the human InsP₃R C-terminal domain (Bourguignon et al., 1994; Wang et al., 2004). Nanogold immunocytochemistry with this antibody revealed strong but sporadic InsP₃R expression in dendritic spine apparatus (supplemental Fig. 1A, available at www.jneurosci.org; compare with Fig. 5A) and prominent immunolabeling of reticular endomembranes (supplemental Fig. 1B–D, available at www.jneurosci.org), which is consistent with the labeling patterns of the monoclonal InsP₃R antibodies (Fig. 5B–D) (see Results) and previous descriptions (Sharp et al., 1993). For all immunoprocures, we monitored specificity and selectivity with a series of omission–substitution controls, including reversal of the dual labeling sequence (see Fig. 6C) and prolonged gold enhancement (10 min at 22°C or 16 min on ice) after omitting the nanogold conjugates.

Methodological considerations. The single and dual gold-based immunoprocures yielded distinctive particulate signals. The DAR single immunolabeling produced particle aggregates (Figs. 2D–G, 3A–E) by introducing to the immunocomplex the long form SP-biotinylated antibody and multiple nanogold-tertiary antibodies. In dual immunolabeling, in contrast, a two-layer procedure with a stoichiometry close to 1

Figure 2. Differential distribution of DAR subtypes in the perisomatic region. Dashed lines and arrowheads outline uncomplexed plasma membrane and plasmalemma–SSC complexes, respectively. *A*, D₁Rs are exclusively associated with the Golgi apparatus with a marked predilection for certain Golgi complexes. *B*, *C*, Similarly, the D₂Rs comprise an intracellular pool, often in association with endosomes (*B*, arrows) but also a plasmalemmal component on uncomplexed membranes (*C*, arrows). *D*–*G*, In contrast, D₅Rs form distinct cytoplasmic clusters, including clathrin-coated vesicles (*D*, arrows) subjacent to plasmalemma–SSC complexes; note in *D* a nearby immunonegative complex. D₅Rs are additionally expressed on membranes flanking SSCs (*F*, *G*), as contrasted with uncomplexed and synaptic (*D*, double arrowheads) membranes. *E* and *F* depict consecutive sections. Arrows in *D*, inset, point to the plasmalemma and to individual distal profiles; a particle aggregate (arrowhead) is likely displaced from the somatic membrane, for apposed cellular profiles were rarely immunoreactive. ax, Axon; den, dendrite; lys, lysosome; mvb, multivesicular body. Scale bars: *A*, *B*, *D*–*G*, 400 nm; *C*, *D*, inset, 200 nm.

(i.e., 1 nanogold/immunocomplex) resulted in single immunoparticles (Fig. 4A–D, compare with Fig. 2E–G). In effect, the multiparticle approach combined with the more sensitive silver autometallography generally increased detection sensitivity, whereas the single-particle procedure provided higher spatial resolution because of the smaller immunocomplex and particle size. Finally, note the use of Tween 20 permeabilization in single-particle immunoprocures for increasing the penetration of immunoprobosc and facilitating labeling between tightly apposed membranes (Fig. 4A–C).

The peroxidase–gold methodology produced readily distinguishable immunocytochemical signals (amorphous precipitates vs particulate gold) (Fig. 6A, inset). However, immunoperoxidase, which can be a valuable auxiliary marker for initially exploring and confirming patterns of receptor expression, performs poorly when spatial resolution is required at the subcellular level (Novikoff, 1980). Therefore, only immunolabeling with gold probes was used for quantification throughout the study.

The sequential enhancement of nanogold with autometallography is based on the descriptions of Bienz and Egger (1998) for colloidal gold and was developed in our laboratory for receptor colocalization and independently reported by another group for cytoskeletal and vesicular

Figure 3. D₅R expression in the perisomatic region. All panels depict nonpermeabilized material, hence the lack of immunolabeling in the plasmalemma–SSC interface (compare with Fig. 4). *A–E*, In perikarya (*A, B, E*) and primary dendrites (den; *C, D*), D₅Rs are selectively clustered subjacent to plasmalemma–SSC complexes (*A, B*, arrowheads) or disposed on flanking membranes. Uncomplexed and synaptic (*A, B*, double arrowheads) membranes are devoid of immunoreactivity. Sections shown in *C* and *D* belong to a series and are 280 nm apart. Notice how the mitochondrion (mit) in *C* appears in *D* directly apposed to the SSC. In both micrographs, glial leaflets (asterisks) separate the plasmalemma–SSC complex from an apposed varicosity (ax) forming an asymmetric axo–spinous synapse (double arrowheads). In *C–E*, arrowheads point to periodic cross-bridges in the plasmalemma–SSC interface of similar size and periodicity to the tetrameric protrusions described in Purkinje SER membranes with freeze-fracture/replication (Kanaseki et al., 1998). It has been suggested that these electron-dense protrusions, which also appear in plasmalemma–SSC complexes of hippocampal pyramids (Benedek et al., 1994), may correspond to the fourfold symmetry of the tetrameric InsP₃R (Kanaseki et al., 1998; Jiang et al., 2002). Scale bars: *A*, 300 nm; *B–E*, 200 nm.

Figure 4. D₅R immunoreactivity visualized with single particles after membrane permeabilization. *A–D*, Arrows point to the receptor cytoplasmic pool, and arrowheads point to the plasmalemmal component on membranes overlying (*A–C*) or flanking (*D*) SSCs. Notice how this architecture confines a 10–15 nm wide cytosolic microcompartment (double arrowheads) between the plasma membrane and the cisternal outer limiting membrane. White arrowheads point to an oblique section of the plasmalemma (and the SSC); the cytosolic microcompartment is not visible. Scale bars, 200 nm.

proteins (Yi et al., 2001). To determine the optimal conditions for this experimental system, gold conjugates were sequentially introduced and gold enhanced, either being linked to antigens (supplemental Fig. 2C–G, available at www.jneurosci.org) or fixed directly on tissues with glutaraldehyde (supplemental Fig. 2A, B, available at [## Results](http://www.jneurosci.org).</p>
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This study pertains to the neuronal perisomatic region (i.e., perikaryon, axon initial segment, and proximal primary dendrites) known to include reticular specializations in the form of SSCs (see Fig. 1 for an overview). The smallest dendrite containing an SSC in our material had a diameter (minor axis) of 1.2 μ m and displayed rough endoplasmic reticular profiles (data not

Table 2. Extracellular milieu of the D₅R microdomain

Glut-like varicosities ^a	Non-glut-like varicosities ^a	Dendrites	Intervaricose axon segments	Glia ^b	ND	Total
53–17	22–8	34–11	17–4	8–3	11–3	145–46

Paired values represent the number of D₅R microdomains found in juxtaposition with individual cellular profiles in single and serial sections, respectively. Classification is based on form, size, organelle content, synaptology, and continuity with an identifiable structure [see Peters et al. (1991) for criteria]. Glut, Glutamatergic; ND, nonidentified cellular profiles.

^aGlia not including intervening processes separating axons.

^bDirect apposition or apposition with intervening glial processes.

shown), which would accordingly correspond to a primary shaft, and generally SSCs were present in more proximal primary branches (>1.6 μ m) continuous with the perikaryon. The SSCs were almost exclusively localized in the perisomatic region of pyramidal cells, whereas they occurred rarely in perikarya and were virtually absent from the proximal processes of interneurons (see Materials and Methods for identification criteria). These rarely occurring SSCs in nonpyramidal neurons were never seen in association with DARs [see Khan et al. (2001) for D₂R, Muly et al. (1998) for D₁R]. In addition, both the D₅R protein and D₅R mRNA are localized predominantly in pyramidal neurons (Bergson et al., 1995a; Ariano et al., 1997; Ciliax et al., 2000). Therefore, in the following sections, we describe the subcellular expression patterns of DAR subtypes in the pyramidal perisomatic region.

The D₁Rs comprised a highly immunoreactive cytoplasmic pool in specific association with the Golgi apparatus in perikarya and dendrites (Fig. 2A). Plasmalemmal expression, predominant in dendritic spines of the neuropil (Smiley et al., 1994), was not identified in the perisomatic region. In contrast, D₂Rs were not generally localized in the perisomatic region, with the exception of certain pyramids expressing weak immunoreactivity in rough endoplasmic reticular (RER) membranes, the Golgi, and in association with endosomes (Fig. 2B), consistent with the rare presence of D₂Rs in PFC efferents (Wang and Pickel, 2002). In these cells, nonsynaptic D₂Rs were additionally distributed on “uncomplexed” portions of the plasmalemma (i.e., lacking juxtaposed endomembranes; see below) (Fig. 2C). Overall, the perisomatic region expressed high levels of D₅R immunoreactivity, with the exception of the axon initial segment. Indeed, labeling was rarely seen beyond the axon hillock (data not shown). The D₅R immunoparticles labeled RER profiles deeper in the cytoplasm and formed distinct clusters below the cell surface (Fig. 2D–G) often in association with clathrin-coated vesicles (Fig. 2D). More importantly, the specific clustering of D₅Rs subjacent to the plasma membrane invariably predicted the presence of a juxtaposed SSC (Figs. 2D–G, 3, 4). In addition to the cytoplasmic pool, we observed and examined in detail a plasmalemmal D₅R component.

For the three-layer immunoprocures, membrane-bound immunoparticles were defined as within 25 nm from the inner aspect of the plasmalemma. Proximity criteria were based on the size of the immunocomplex used for probing the intracellularly protruding C terminus of the receptor protein; equivalent to one IgG and two Fab' fragments, including the long-form biotin conjugate (see Materials and Methods). In two-layer immunoprocures, D₅R particles that appeared on the cytoplasmic face of the plasma membrane were considered plasmalemmal. We identified 618 membrane-bound immunoparticles in 26 perisomatic profiles of individual pyramids midsectioned through the apical dendrite (final magnification, 60,000 $\times$). Ninety-three percent (577 particles) were

distributed on membranes that overlay or flanked a juxtaposed SSC, at a distance no more than 80 nm of its margins (Figs. 2*F*, *G*, 3*A*, *B*, *D*, 4*A–D*). Spatial specificity of D₅R expression was also manifest among the plasmalemma–SSC complexes of a single neuron, and occasionally highly immunopositive and immunonegative complexes were merely 0.5 μ m apart (Fig. 2*D*). The latter appeared characteristically devoid of the subjacent D₅R cytoplasmic cluster, which also suggests that this cluster could represent an exchanging D₅R pool locally associated with an overlying plasmalemma–SSC complex.

To determine whether this spatially restricted expression of D₅Rs along the perisomatic membrane is in response to synaptic input or other specific cellular apposition, we examined D₅R plasmalemma–SSC complexes (called D₅R microdomains hereafter) in single and successive sections (Table 2). Although the more frequently encountered appositions involved axons often forming asymmetric synapses with profiles other than the perisomatic membrane (Fig. 3*C*, *D*) (Alonso-Nanclares et al., 2004), the D₅R microdomain was never directly associated with the symmetric synapses that typically converge on the pyramidal perisomatic region (Beaulieu and Colonnier, 1985) (Figs. 2*D*, 3*A*, *B*). This is of particular interest because cisternal structures have indeed been shown to accompany symmetric synapses (GABAergic) on the axon initial segment of hippocampal pyramids (Benedeczy et al., 1994) (Fig. 5*D*). In two single cases, we identified microdomains in somato-somatic plasma membrane juxtapositions (Fig. 6*H*).

Given the central role of reticular endomembranes (and the SSC) in Ca²⁺ homeostasis (Park et al., 2000; Meldolesi, 2001) and the capacity of D₁-LRs to mobilize Ca²⁺ stores (for review, see Missale et al., 1998), we next examined whether the D₅R microdomain could be a potential physical substrate for the phosphoinositide system by also expressing InsP₃Rs. In the neuropil (Fig. 5*A*, *B*), InsP₃Rs were sporadically expressed in dendritic spine apparatus and in slender vesicular structures within dendrites and rarely axonal varicosities. In the perisomatic region, we detected InsP₃Rs on reticular endomembranes and rarely on Golgi complexes (Fig. 5*C*, *D*) (for Golgi as a Ca²⁺-signaling apparatus, see Pinton et al., 1998). Coexpression of D₅Rs and InsP₃Rs in individual microdomains was confirmed with different dual immunoprocures (i.e., gold–gold- and peroxidase–gold-based; see Materials and Methods). Despite the inherent limitations of immunoperoxidase (discussed in Materials and Methods), DAB immunocytochemistry for the D₅R labeled intensely plasmalemma–SSC complexes and often the subjacent cytoplasm, whereas precipitation over uncomplexed membranes and the juxtaposed cellular profiles was minimal or absent (Fig. 6*A*, compare with the gold labeling pattern in *B–H*). The InsP₃R immunoparticles labeled the lateral vacuolar expansions as well as central portions of the SSC limiting membranes (Fig. 6*A–H*).

Next, we sought to quantify the extent of colocalization of D₅Rs and InsP₃Rs in the pyramidal perisomatic region. However, note that this is a semiquantitative approach, for several factors discussed in the following section may influence differentially the immunolocalization of individual antigens. Because the number

Figure 5. Subcellular distribution of InsP₃Rs (arrows) in the neuropil and the perisomatic region (see supplemental Fig. 1, available at www.jneurosci.org) and coexpression with D₅Rs (arrowheads). *A*, The spine apparatus (asterisk) of a dendritic spine (sp) displays InsP₃R immunoreactivity with a predilection for saccules facing the postsynaptic density of an apposed synaptic varicosity; the obliquely sectioned synaptic cleft is not visible. *B*, Inset, InsP₃R immunoparticles decorate slender SER tubules in dendrites (den). In the perisomatic region, InsP₃Rs were rarely visualized on the *cis*-face of Golgi complexes (*C*), whereas the main body of immunoreactivity appears on the cytoplasmic face of reticular endomembranes (*D*; compare with the large D₅R immunoparticles of the cytoplasmic pool). Note the loose association of a cisternal profile (*D*, double arrowheads) with an axo-somatic symmetric synapse. Elaborate cisternal appositions have been described to accompany symmetric GABAergic synapses on the axon initial segment of hippocampal pyramids and to sequester free Ca²⁺ (Benedeczy et al., 1994). ax, Axon. Scale bars, 200 nm (applies also to *B*, inset).

of SSCs in individual perisomatic profiles varies depending on the length of visible plasma membrane, we examined perisomatic regions midsectioned through the nucleus and the apical primary dendrite. In D₅R–InsP₃R-immunolabeled material, we identified a total of 210 plasmalemma–SSC complexes (referred to as complexes) in 24 perisomatic profiles (8.8 ± 2.7 complexes/profile; mean $\pm$ SD; range, 4–14). The majority (158 complexes; 75%) expressed InsP₃R immunoreactivity on SSCs as described in the previous section. D₅R immunoreactivity was identified in 96 of the complexes (4 ± 1.7 complexes/profile; mean $\pm$ SD; range, 1–9), equal to 46% of total complexes. With the exception of two perisomatic profiles containing exclusively D₅R complexes, immunopositive and immunonegative complexes for the D₅R generally coexisted in individual profiles (Fig. 2*D*). Eighty-one (84%) of D₅R complexes expressed InsP₃Rs immunoreactivity on SSCs. The remaining were immunonegative (Fig. 6*H*) or more frequently displayed InsP₃Rs in association with other endomembranes in the vicinity of the SSC.

Having established the association of D₅Rs with the InsP₃R-expressing cisternal endomembranes, we finally investigated whether the InsP₃-sensitive stores of the microdomain could be additionally linked to the mitochondria, an important cytoplasmic component of the intracellular Ca²⁺ network (Rizzuto et al., 1994, 1998; Babcock et al., 1997). In 21% ($n = 100$) of single-sectioned and 32% ($n = 46$) of serially sectioned D₅R microdomains (Fig. 3, compare *C* and *D*), a single mitochondrion was directly apposed to the SSC, separated by a sparsely bridged 12–20 nm electron-lucent zone (Fig. 7*A–C*). In favorably sectioned appositions (Fig. 7*C*), we observed InsP₃R immunoparticles within the minute cleft separating the cisternal from the mitochondrial outer membrane, apparently labeling the cisternal face because mitochondria are not known nor were they observed here to express InsP₃Rs.

Discussion

Although the perisomatic occurrence of the SSC has been documented in central and peripheral neurons (see Fig. 1 for review) and corroborated here for the macaque PFC, there is no compelling evidence until now, with respect to SSCs and neuronal classification. The original descriptions either do not distinguish between pyramidal and nonpyramidal neurons (Rosenbluth,

Figure 6. Coexpression of D₅Rs and InsP₃Rs in microdomains. In *A*, D₅Rs were visualized with immunoperoxidase precipitates. Despite the diffuse nature of the immunomarker, two plasmalemma–SSC complexes (framed) and the subjacent cytoplasm demonstrate increased labeling density compared with uncomplexed membranes or neighboring cellular profiles. InsP₃Rs were simultaneously visualized with nanogold at high magnification (inset, arrows). To avoid artifactual contrasting of membranes, this material was processed with minimal lead counterstaining. For illustration purposes, image contrast in *A* and inset has been enhanced digitally (Photoshop 7.0; Adobe Systems, San Jose, CA). When detected with nanogold probes (arrowheads), D₅Rs were visualized between the plasmalemma and juxtaposed cisternal endomembranes (*C*, inset, *G*), and predominantly at the periphery of plasmalemma–SSC complexes (*D*, *E*). The InsP₃R-immunoreactive profiles (arrows) included the lateral vacuolar expansions of SSCs (*B*–*E*) and their middle portion (*A*, *F*, *G*, inset, *H*). Note in *C* the reversal of the immunolabeling sequence for D₅R (small particles; main panel and inset) and InsP₃R (large particles); double arrowheads point to the plasmalemma–SSC interface. *H* illustrates a rare case of somato-somatic membrane juxtaposition furnished with two SSCs (white arrowheads); one SSC is confluent with the reticular network (double arrowheads). Notice that the plasmalemma–SSC complex, which is immunoreactive for both D₅Rs and InsP₃Rs, also coincides spatially with the widening of the perineuronal space (asterisks), perhaps to allow access via extracellular diffusion. White arrow in *B* points to a dense-cored vesicle within a DAergic-like axon (ax) deployed *en passant*. Scale bars: *A*, 500 nm; *A* inset, *B*, *D*–*G*, 200 nm; *G* inset, 20 nm; *C*, 100 nm; *C* inset, 25 nm.

1962) or report SSCs primarily in pyramids (Buschmann, 1979). Here, we confirmed the predominant localization of SSCs in pyramidal cells as opposed to interneurons. Moreover, the rarely occurring SSCs in nonpyramidal perikarya were never associated with DARs.

Within pyramidal neurons (Fig. 2), the DAR subtypes formed cytoplasmic pools (D₁R, D₂R, and D₅R) and a membrane-bound component (D₂R and D₅R). The latter would represent the “functional” component of the perisomatic region, because active receptors signal from the plasma membrane and only these recep-

tors would be available to extracellular DA. Another possibility to consider is that DARs could be additionally localized within postsynaptic densities of DAergic synapses preventing immunodetection with the pre-embedding method, as repeatedly demonstrated by Somogyi’s group for ionotropic glutamate receptors [see Baude et al. (1995) for discussion on pre-embedding vs post-embedding approaches]. Even for these receptors, however, one would expect the pre-embedding method to occasionally visualize relatively “unmasked” antigens, especially at the edges of the synapse (Baude et al., 1995), which was never observed here. Although it seems unlikely, considering the rare occurrence of perisomatic DAergic input (Goldman-Rakic et al., 1997), we cannot exclude this possibility. Likewise, we cannot preclude a biased immunodetection because of physical barriers (i.e., the tightly apposed plasmalemma and cisternal membranes) or other factors that may interfere with the dual immunolabeling, such as loss of gold signal of the first labeling series, reduced antigenicity in the second series, nonuniform preservation of antigenicity for D₅Rs and InsP₃Rs after fixation or simply different potencies of the individual antibodies. Therefore, one should bear in mind these inherent limitations when interpreting immunocytochemical findings.

We have established that D₅Rs exhibit strict spatial specificity along the pyramidal perisomatic plasmalemma and form discrete and exclusively extrasynaptic microdomains. Moreover, this expression pattern appears to be unique to the D₅R, as manifested not only between subtypes of distinct DAR subfamilies (D₅Rs but not D₂Rs) but also between the structurally and pharmacologically homologous subtypes of the D₁-LR subfamily (D₅Rs but not D₁Rs). To the best of our knowledge, the D₅R microdomain is the first evidence for the existence of intricately structured neurotransmission modules in the brain exhibiting spatial-, receptor-, and cell-class/compartmentspecificity beyond the chemical synapse.

Evidently, such modules would never meet the exquisite “one-to-one” spatio-temporal requirement of the synapse.

Therefore, they should not be viewed as an alternative to “wired” communication to compensate for the rare occurrence of perisomatic DA synapses but rather as a synergistic mechanism for the modulation of cellular assemblies (for review, see Agnati et al., 2000). Consistent with these views, the dopaminergic microdomain is strategically positioned to influence the neuronal excitability centrally by influencing directly the signal relay compartment of the cortical principal cell, as opposed to DAergic modulation of individual synapses on distal dendrites mediated primarily via the D₁R (for review, see Goldman-Rakic et al.,

Figure 7. The D₅R microdomains establish intimate associations with mitochondria (mit). *A*, *B*, D₅Rs (black arrowheads) are localized at the edges of the microdomain and on a neuropil profile (*A*). A dendritic spine (sp) receiving an asymmetric synapse (between arrowheads in *B*) is apposed to the microdomain with intervening glial processes (asterisks); double arrowheads point to cross-bridges (Fig. 3 legend) in the plasmalemma–SSC and SSC–mitochondrion interface. *C* depicts InsP₃R expression (two attached immunoparticles; arrows) within the minute cleft separating the cisternal from the outer mitochondrial membrane. Scale bars, 200 nm.

2000). The selective expression of the high-affinity D₅R (its affinity for DA is an order of magnitude higher than that of the D₁R) (Sunahara et al., 1991) will then favor perisomatic dopaminoception at relatively lower transmitter levels and potentially long-range volumetric signaling. Inevitably, the dopaminoceptive microdomain should be regarded as a potent substratum for D₁-LR pharmacological manipulation. It is noteworthy that chronic treatment with typical and atypical antipsychotics downregulates D₅R mRNA in the PFC (Lidow et al., 1997), although it is not currently known whether this involves neuropilar or perisomatic D₅Rs.

The question naturally arises as to whether the D₅Rs could be organized in discrete microdomains independently of extrinsic signals (i.e., in response to the intracellular milieu) or, conversely, whether extrinsic signals may trigger clustering of the D₅R and juxtaposition of cisternal membranes. Apparently, the clustered D₅R expression is not in response to synapses (either DAergic or heterosynapses), and local extrasynaptic release of DA across the microdomain might seem unlikely considering the frequent juxtaposition of non-DAergic profiles (i.e., glutamatergic-like axons, dendrites, glia) (Table 2) (however, see the DAergic-like *en passant* axon in Fig. 6*B*). In fact, analyses of the DAergic innervation of the frontal cortex have confirmed the rare association of DA axons with the perisomatic region (Smiley and Goldman-Rakic, 1993; Sesack et al. 1995; for review, see Goldman-Rakic et al., 1997). Nonetheless, this evidence does not necessarily imply that the perisomatic membrane is uniformly exposed to “circulating” DA. Glial barriers or heterogeneous distribution of DA transporters within individual axons (Lewis et al., 2001) could favor diffusion anisotropies (Barbour and Häusser, 1997; Gonon

et al., 2000; Syková, 2001), which may ultimately result in spatially restricted elevations of DA levels (“hot spots”) along the perisomatic membrane. These DAergic axons could be located in the relative vicinity of a microdomain or even more remotely within the parenchyma. Whether the nonuniformity of the plasmalemma with regard to extracellular DA may apply *in vivo* is only speculative. What is very interesting, however, is that both the SSC and mitochondria are most certainly dynamic structures, unlike their static images captured in electron microscopy. Early ultrastructural studies have demonstrated that SSCs are capable of plastic changes after axotomy and during development (Sumner, 1975; Buschmann, 1979), and recent evidence indicates that local Ca²⁺ dynamics may affect physical coupling between the smooth endoplasmic reticulum and mitochondria (Wang et al., 2000). Finally, we should consider the possibility of a dynamic trafficking between the plasmalemmal component and the cytoplasmic pool of the D₅R, as might indicate its selective clustering below the microdomain, often in association with clathrin-coated vesicles (Fig. 2*D–G*). Compared with the D₁R, which undergoes dynamin-dependent endocytosis involving clathrin-coated pits (Bloch et al., 1999; Vickery and Zastrow, 1999), little is known about the D₅R regarding its handling after agonist stimulation. Although it appears to undergo agonist-induced internalization, similar to the D₁R, recent evidence suggests that after endocytosis the D₅R is sorted for degradation in the lysosomes (Heydorn et al., 2004).

Although more studies, both anatomical and physiological, would be needed to elucidate these key issues, it is obvious that ligands (endogenous DA and pharmacological analogs) would reach the D₅R microdomain, as well as other dopaminoceptive membranes, via volumetric diffusion (Zoli et al., 1999; Pickel, 2000). Even unoccupied D₅Rs may retain substantial capacity for activating adenylyl cyclase by behaving as constitutively active receptors (Tiberi and Caron, 1994). Yet, unlike any other brain dopaminoceptive membranes, the D₅R microdomain presents a unique cellular feature: the distinctive alignment of the D₅R-expressing membranes with the InsP₃R-furnished SSC and the mitochondrion, implying their involvement in similarly restricted intracellular signaling mechanisms to maintain input specificity and selectivity of regulation. It has been suggested, for example, that InsP₃Rs expressed in the reticulum–mitochondrion interface, as shown here, would generate the essential Ca²⁺ hot spots for the low-affinity, high-capacity mitochondrial Ca²⁺ uniporter to operate, leading to sharp rise of matrix Ca²⁺ levels in response to InsP₃ (Rizzuto et al., 1994, 1998). In essence, the D₅R microdomain constitutes the equivalent of a nonprojecting “somatic spine” by sequestering the cytosol and by supplying the reticular scaffold, reminiscent of the spine apparatus, for specific macromolecular arrays, including the InsP₃R, to assemble and function (Berridge, 1998; Blackstone and Sheng, 1999; Delmas et al., 2002; Augustine et al., 2003).

Previous studies in behaving primates have established that the D₁-LRs are critically involved in working memory operations (Sawaguchi and Goldman-Rakic, 1994; Williams and Goldman-Rakic, 1995; Castner et al., 2000), even though the lack of specific ligands for D₁R and D₅R subtypes has hitherto precluded consideration of their potentially distinct roles within the cortical microcircuitry (Gao and Goldman-Rakic, 2003). Moreover, their signal transduction mechanisms (for review, see Missale et al., 1998) have most commonly been linked to activation of adenylyl cyclase. In addition to the cAMP pathway, however, D₁-LR activation increases the formation of inositol phosphates in the brain (Mahan et al., 1990; Undie and Friedman, 1990) and stimulates

Ca²⁺ release from InsP₃-sensitive stores in perikarya of dissociated cortical neurons (Lezcano and Bergson, 2002). It appears that the involvement of D₁-LRs in the phosphatidylinositol turnover is independent of the cAMP pathway and is likely attributed to a subtype distinct from the prototypic D₁R (Friedman et al., 1997). The discovery here of a specialized microdomain that facilitates selective physical coupling of the D₅R and the phosphoinositide system introduces a unique signaling paradigm for the volumetric regulation of cortical functions by DA and identifies new targets (Agnati et al., 1995a) that could be involved in the numerous neuropsychiatric disorders affecting the brain DAergic system. This link can be particularly tempting in light of the recently discovered Ca²⁺-binding, DAR-interacting proteins and the proposed involvement of Ca²⁺ in the pathophysiology of schizophrenia (Bergson et al., 2003; Lidow, 2003).

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