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Ultrastructural Localization of D_2 Receptor-like Immunoreactivity in Midbrain Dopamine Neurons and Their Striatal Targets

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Potential cellular substrates for functions ascribed to the dopamine D_2 receptor were examined in rat brain using immunoperoxidase for localization of a D_2 receptor peptide and immunogold staining for the catecholamine biosynthetic enzyme tyrosine hydroxylase (TH). Specificity of the rat polyclonal antiserum, raised against a 15 amino acid fragment from the third intracellular loop of the D_2 receptor, was shown by immunoblot analysis and by selective labeling of cultured Chinese hamster ovary cells permanently transfected with the cDNA for the D_2 receptor. Although the light microscopic distribution of immunolabeling for the D_2 peptide was diffuse, it was selectively localized to regions containing dopamine cells (substantia nigra and ventral tegmental area) or their forebrain projections (dorsal and ventral striatum, nucleus accumbens, and olfactory tubercles). Electron microscopic examination of the medial substantia nigra and ventral tegmental area revealed readily detectable peroxidase immunoreactivity for the D_2 peptide, primarily associated with the smooth endoplasmic reticulum and plasmalemmal surfaces of dendrites. Many D_2 peptide–immunoreactive dendrites also contained immunogold labeling for TH, although some dendrites were singly labeled for either marker. In the medial and dorsolateral striatum, immunoperoxidase product for the D_2 peptide was localized most extensively in dendrites, with the greatest intensity of immunolabeling seen in spines. A number of striatal dendrites exhibiting D_2 peptide labeling were contacted by axon terminals immunoreactive for TH. Additionally, D_2 peptide immunoreactivity was distributed to some synaptic vesicles and portions of the plasmalemmal surface in unmyelinated axons and in axon terminals. Most D_2 peptide–immunoreactive terminals either lacked detectable membrane specializations, or formed thin, symmetric synapses in single sections. A few D_2 peptide–labeled terminals formed asymmetric junctions on dendritic spines. In dually labeled sections, most D_2 peptide–immunoreactive terminals lacked detectable immunolabeling for TH. However, in fortunate planes of section, peroxidase product for D_2 peptide immunoreactivity was occasionally seen in preterminal portions of axons whose terminal varicosities contained immunogold labeling for TH. These ultrastructural results are consistent with the localization of a dopamine D_2 receptor-like protein that is strategically positioned to subserve (1) autoreceptor functions at the level of dendrites in the midbrain and presynaptic axon terminals in the striatum, as well as (2) postsynaptic actions on striatal spiny dendrites and other nondopamine terminals.

[Key words: dopamine, receptor, substantia nigra, ventral tegmental area, striatum, caudate nucleus, tyrosine hydroxylase, ultrastructure, endoplasmic reticulum]

The dopamine D_2 receptor has been the subject of considerable investigation since its links with mental illness were first suggested (Creese et al., 1976; Seeman, 1981). Although additional dopamine receptor subtypes have been discovered and redefined on a molecular basis (Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989; Sokoloff et al., 1990; Sunahara et al., 1990, 1991; Zhou et al., 1990; Dearry et al., 1991; Grandy et al., 1991; Van Tol et al., 1991, 1992), those with greatest homology to the D_2 class continue to be implicated in the therapeutic effects of antipsychotic drugs (Sokoloff et al., 1990; Civelli et al., 1991; Van Tol et al., 1991; Sibley and Monsma, 1992). The mechanisms through which dopamine systems contribute to normal cognitive and emotive processes, or the pathological disturbances of psychosis, are not yet known. However, a number of important cellular functions have been associated with central D_2 receptors, including autoreceptor regulation of dopamine transmission, heteroreceptor regulation of nondopamine transmitter release, and postsynaptic modulation of neuronal excitability and/or protein synthesis (Chesselet, 1984; Mercuri et al., 1985; Young et al., 1986; Lacey et al., 1987; Seeman and Grigoriadis, 1987; Wolf and Roth, 1987; Freedman and Weight, 1988; Drukarch et al., 1989; Williams et al., 1989; Gerfen et al., 1991).

A more thorough understanding of the sites and mechanisms through which dopamine mediates its crucial modulatory effects can be gained from a comprehensive examination of the neuronal elements and their subcellular components that express dopamine receptors. Studies using *in situ* hybridization for receptor mRNA have provided a sensitive means for localizing cells that express dopamine receptor subtypes (Mengod et al., 1989; Gerfen et al., 1990; Mansour et al., 1990; Bouthenet et al., 1991; Fremeau et al., 1991; Meador-Woodruff et al., 1991; Weiner et al., 1991). However, this method is inadequate for

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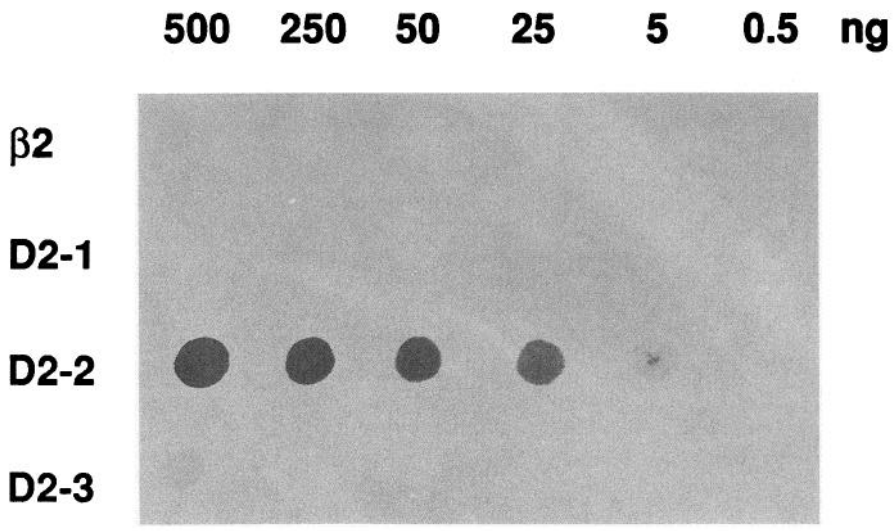

Figure 1. Immunodot blots showing specificity of the D_2 receptor peptide antiserum. Serial dilutions of unconjugated peptides corresponding to amino acids from the third cytoplasmic loop of the D_2 or β_2 adrenergic receptors were blotted onto filter paper, incubated in a 1:1000 dilution of rat anti–D2-2 antiserum, and processed with the ABC method. The corresponding amino acid sequences are, for *β2*, 226–239; *D2-1*, 220–233; *D2-2*, 273–287; and *D2-3*, 258–269 (numbers based on the long form of the rat D_2 receptor). A dense peroxidase product is seen only against the immunizing D2-2 peptide at concentrations of 25–500 ng. Cross-reaction with the highest concentration (500 ng) of the D2-3 peptide is even less than that seen at 5 ng of the D2-2 peptide.

determining the sites of receptor insertion into membrane, which are often at considerable distance from the cell soma (Mansour et al., 1990). Ligand-based autoradiography is the method traditionally used to localize available receptors at the light microscopic level (Boyson et al., 1986; Bouthenet et al., 1987; Mansour et al., 1990). However, autoradiography is sometimes limited in resolution and sensitivity (Lidow et al., 1988) and, furthermore, has proved difficult to adapt to ultrastructural investigations of receptor localization. Although this technique has been carefully applied to electron microscopic localization of peptide receptors (Hamel and Beaudet, 1984; Dana et al., 1989), interpretation of the results in these studies has depended on the strength of statistical analyses defining the most probable source of autoradiographic signal (Beaudet et al., 1988). The ligand-based autoradiographic method may also be less useful for revealing the subcellular distribution of receptors prior to insertion in, or following internalization from, the plasma membrane. Moreover, even these limited methods have not yet been applied to the ultrastructural investigation of dopamine receptor expression and localization.

Recently, antibodies directed against receptor peptide fragments, or purified receptor proteins, have been successfully employed for immunocytochemical localization of putative catecholamine receptors in the brain (Aoki et al., 1987, 1989; McVittie et al., 1991; Aoki, 1992; Ariano et al., 1993). The cloning of genes coding for dopamine receptors (Bunzow et al., 1988; Sokoloff et al., 1990; Sunahara et al., 1990, 1991; Zhou et al., 1990; Dearry et al., 1990; Van Tol et al., 1991) has permitted the production of antisera directed against peptide sequences that have limited homology to other G-protein–linked receptors (cf. McVittie et al., 1991). We have produced a polyclonal antiserum in rat directed against a synthetic peptide fragment corresponding to a portion of the third intracellular loop of the dopamine D_2 receptor (common to both the long and short forms) (Bunzow et al., 1988; Giros et al., 1989; Monsma et al., 1989). We have utilized this antiserum for a detailed ultrastructural investigation of the cellular and subcellular localization of D_2 receptor-like protein in both the midbrain regions that contain dopamine neurons and their postsynaptic targets in the forebrain. The distribution of the receptor peptide has been further examined in relation to presumed dopamine neurons and processes, using a dual, preembedding immunolabeling method (Chan et al., 1990) to localize D_2 peptide and tyrosine hydroxylase (TH), the catecholamine synthetic enzyme, within single sections.

The results of this study have been reported in a preliminary form (Sesack et al., 1991).

Materials and Methods

Production of polyclonal antiserum

A synthetic peptide fragment corresponding to amino acids 273–287 of the rat dopamine D_2 receptor sequence (third intracellular loop of the long and short forms) was obtained from Multiple Peptide Systems (San Diego, CA) and conjugated through glutaraldehyde to keyhole limpet hemocyanin (Sigma). The conjugate was dialyzed, homogenized with Freund's adjuvant (Calbiochem), and injected subcutaneously into rats. A total volume of 0.5 ml containing 0.5 mg of peptide was injected. Serum samples were drawn prior to immunization and 10–14 d after each boost injection. Following three or four boosts, animals were deeply anesthetized with sodium pentobarbital and killed by exsanguination. The final serum was stored at −70°C.

Immunoblot and Western blot analysis

For determination of antibody titer and specificity, immunoblot analysis modified from the method of Larsson (1981) and standard Western blot techniques were used. For the immunoblot method, immunizing and nonimmunizing peptides from the dopamine D_2 receptor and the structurally related β_2 adrenergic receptor (Fig. 1) were serially diluted in 0.01 M phosphate-buffered saline (PBS), pH 7.4, blotted onto Whatman #1 filter paper, and fixed with paraformaldehyde vapors at 60°C for 1 hr. Papers were then rinsed in 0.05 M Tris-buffered saline (TBS), pH 7.6, blocked in 1% bovine serum albumin (BSA) in TBS for 30 min, and then incubated in a 1:1000 dilution of rat antiserum in 1% BSA-TBS for 1 hr at 37°C. Papers were rinsed in TBS and processed using the avidin-biotin peroxidase (ABC) staining method (Hsu et al., 1981). Briefly, papers were incubated in 1:100 biotinylated donkey anti-rat IgG (Jackson Labs) or 1:400 biotinylated goat anti-rat IgG (Amersham) for 30 min, rinsed, and then incubated in 1:100 avidin-biotin peroxidase complex (Vector Elite). The bound peroxidase was visualized by reaction with 22 μg of 3,3′-diaminobenzidine (DAB) (Aldrich) and 10 μl of 30% H_2O_2 in 100 ml of TBS for 6 min.

For Western blot analysis in three experiments, homogenates of proteins from various brain regions were prepared, run on 7–15% gradient polyacrylamide gels, and transferred to nitrocellulose papers (Towbin et al., 1979). The nitrocellulose papers were incubated for 1 hr at 37°C

in dilutions of the rat anti-D_2 peptide antiserum ranging from 1:500 to 1:10,000. The bound antibody was visualized using the ABC method as described above. In a fourth experiment, a Western transfer of proteins in enriched membrane fractions (Wu et al., 1986) from striatal and cerebellar homogenates was prepared. The concentration of the anti-D_2 peptide antiserum was increased to 1:250, the incubation was run overnight at room temperature, a secondary goat anti-rat IgG directly conjugated to peroxidase (Cappel; dilution 1:500) was used, and the bound antibody was visualized using 4-chloro-1-naphthol (Hawkes et al., 1982) as the chromogen for the peroxidase reaction.

Immunocytochemistry

Cultured cells. Chinese hamster ovary (CHO) cells stably transfected with the cDNA for the rat dopamine D_2 receptor (McVittie et al., 1991) were generously donated by Drs. Marjorie Ariano (Chicago Medical School) and David Sibley (NIH-NINDS). The ability of rat polyclonal anti-peptide antiserum to recognize dopamine D_2 receptor was tested in this cell line. CHO cells grown on coverslips were dehydrated, and then fixed with 4% paraformaldehyde for 10 min, rinsed in PBS, treated with 0.01% saponin in PBS three times for 5 min each, and rinsed again. Cells were blocked with 1% BSA in 0.1 M TBS and incubated for 18 hr at room temperature, or 42 hr at 4°C, in a 1:500 dilution of rat anti-D_2 peptide antiserum. Untransfected cells were included as controls for nonspecificity of antibody reaction. In addition, transfected cells were incubated in immune serum preadsorbed for 1 hr at room temperature with 100 μg/ml of immunizing peptide. The ABC method described above was used to visualize the bound antibody. Coverslips were then rinsed, dehydrated through graded ethanols, and mounted onto glass slides.

Fixed rat brain tissue. Fifteen male Sprague–Dawley rats (250–300 gm) were anesthetized with pentobarbital, and of these, 11 were perfused transcardially with 10 ml of heparin saline (1000 U/ml), followed first by 50 ml of 3.75% acrolein (Polysciences), then 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and then by an additional 200 ml of 2% paraformaldehyde. The remaining rats were perfused either with 4% paraformaldehyde (one animal), 4% paraformaldehyde with 0.2% glutaraldehyde (one animal), or 5% glutaraldehyde (two animals), all in 0.1 M phosphate buffer, pH 7.4. Brains were postfixed for 30 min in the final fixative. The composition of fixation solution appeared to exert little effect on the intensity of peroxidase immunoreactivity for the D_2 peptide. Thus, the results reported here are primarily from acrolein-fixed tissue.

The brains were sectioned at a thickness of 40 μm on a vibratome. Sections fixed either with acrolein or 5% glutaraldehyde were treated with 1% sodium borohydride in 0.1 M phosphate buffer (Leranth and Pickel, 1989). Some vibratome sections were cryoprotected with 25% sucrose and 10% glycerol in 0.05 M phosphate buffer and subjected to a rapid freeze-thaw using liquid Freon, followed by liquid nitrogen. Tissue was then rinsed in phosphate buffer, followed by 0.1 M TBS, and blocked for 1 hr in 1% BSA in TBS, to which 0.05% Triton X-100 was added in a few cases. Sections were incubated for 18 hr at room temperature, or 42 hr at 4°C, in a 1:100 to 1:2000 dilution of (1) rat D_2 peptide antiserum (with or without Triton), (2) preimmune serum, or (3) immune serum to which 100 μg/ml of immunizing peptide had been added. The sections were processed using the ABC method (described above) or double-bridged, peroxidase-antiperoxidase (PAP) staining (Ordronneau et al., 1981). For PAP labeling, tissue was incubated in 1:100 mouse anti-rat IgG (Accurate) and then 1:100 rat PAP (Sternberger Meyer), and the steps were repeated. Peroxidase was visualized using DAB as described above. Incubations were performed at room temperature with continuous agitation and sections were rinsed in TBS between incubations.

Dual immunoperoxidase-gold labeling. In some cases, alternate vibratome sections were dually labeled for (1) D_2 peptide, using peroxidase immunostaining, and (2) TH, using a preembedding immunogold-silver method (Chan et al., 1990). Tissue was co-incubated in a mixture of 1:1000 rat anti-D_2 peptide and 1:2000 rabbit anti-TH (Joh et al., 1973). Following DAB reaction, sections were rinsed in 0.01 M PBS, blocked in 0.8% BSA and 0.1% gelatin in PBS, and incubated for 3 hr in 1:50 goat anti-rabbit IgG conjugated to 1 nm gold particles (Amersham). After rinsing in PBS and 0.1 M sodium citrate buffer (pH 7.4), the bound gold was intensified by treatment with silver solution (Amersham) for an empirically determined time (usually 4–7 min).

To control for potential cross-species reaction of secondary antisera, sections incubated separately in either primary antiserum were subsequently treated with the nonmatching secondary IgG species and processed for that marker (i.e., rat anti-D_2 peptide followed by gold-conjugated anti-rabbit IgG and silver enhancement; or rabbit anti-TH followed by biotinylated anti-rat IgG and ABC processing). In initial studies, the goat anti-rat IgG from Amersham cross-reacted with rabbit anti-TH, producing detectable peroxidase immunoreactivity in substantia nigra and ventral tegmental area perikarya. In the case of biotinylated donkey anti-rat IgG from Jackson Labs, or gold-conjugated goat anti-rabbit antiserum from Amersham, no evidence of species cross-reaction was detectable by light or electron microscopy. Therefore, the results of dual labeling studies presented here derive only from experiments using the latter combination of secondary antisera.

Light and electron microscopy

Singly and dually labeled sections were either slide mounted for light microscopy or further processed for electron microscopic examination. In the latter case, tissue was postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 (Leranth and Pickel, 1989). Ultrathin sections cut from this material were stained with uranyl acetate and lead citrate and examined in a Phillips 201 electron microscope.

Data analysis

Analysis of single labeling for the D_2 peptide, and its codistribution with TH immunoreactivity, was confined to the outer 1–3 μm of tissue, where maximal penetration of antibodies and immunological reagents was achieved. Furthermore, in dually stained material, only micrographs containing both peroxidase and gold markers were included for analysis. Quantitative analysis of immunogold particles was not necessary, as specific labeling was considerably greater than background particles in the surrounding neuropil (Chan et al., 1990; Sesack and Pickel, 1992).

Definition of neuronal and synaptic elements

The classification of neuronal and synaptic elements in this study followed the descriptions of Peters et al. (1991). Perikarya were identified as regions of the cell containing the nucleus. Dendrites were defined as processes that exhibited densities postsynaptic to axon terminals. Such processes having diameters greater than 0.7 μm and containing rough endoplasmic reticulum were classified as proximal; dendrites with smaller diameters and fewer ribosomes were considered distal. Dendritic spines, which typically arose from distal processes, were often smaller than dendrites and typically did not exhibit mitochondria, microtubules, or rough endoplasmic reticulum. In some planes of section, a spine apparatus was apparent in the head or neck of the spine. Axon terminals contained numerous synaptic vesicles and measured at least 0.2 μm in diameter, while the preterminal portions of axons were typically smaller, and contained few vesicles. Asymmetric synapses were identified by their thickened postsynaptic densities, while symmetric synapses had thin densities (Gray, 1959). Appositions were defined as parallel membrane associations that showed no conventional synaptic morphology, but nevertheless were not separated by astrocytic processes.

Results

Dopamine receptor recognition and antibody specificity

By immunoblot analysis, rat antiserum (at 1:1000 dilution) specifically recognized the immunizing D_2 peptide with a threshold of 5 ng, but did not cross-react appreciably with other nonimmunizing peptides from adjacent positions on the D_2 or β_2 adrenergic receptors (Fig. 1). In cultured CHO cells, the rat anti-D_2 antiserum produced a dense perinuclear immunolabeling of cells permanently transfected with the cDNA for the D_2 receptor (Fig. 2*A*), but not untransfected CHO cells (Fig. 2*C*). Preadsorption of the immune serum with 100 μg/ml of D_2 peptide greatly reduced the intensity of immunoreaction product (Fig. 2*B*).

For Western blot analysis, in the three experiments where bound antibody was visualized using the ABC method, no bands

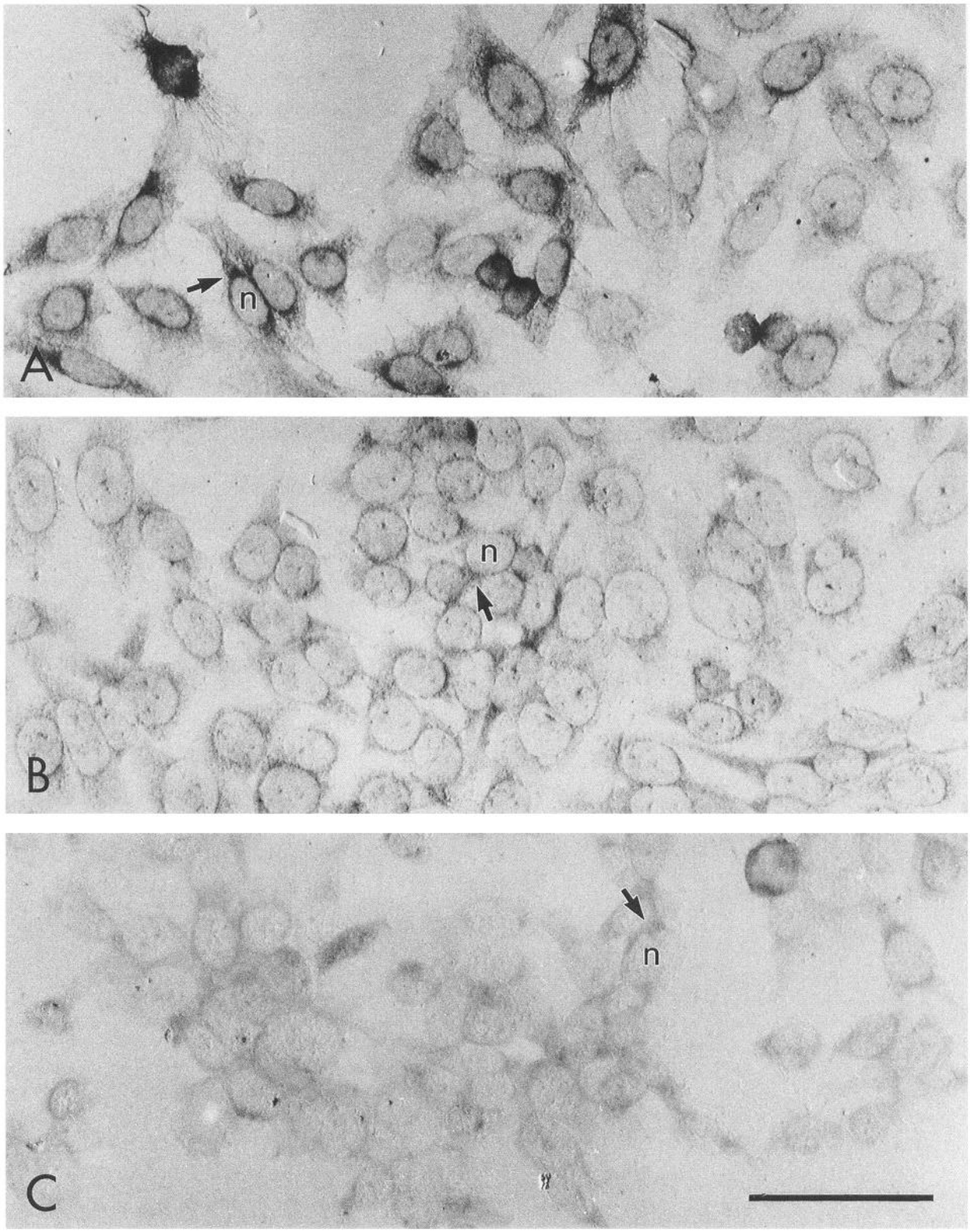

Figure 2. Light micrographs of cultured CHO cells processed immunocytochemically for the D_2 peptide. *A,* CHO cells permanently transfected with the cDNA for the dopamine D_2 receptor exhibit dense perinuclear immunoreaction product (*arrow*) when incubated in the polyclonal D_2 peptide antiserum and processed by the ABC method. *B,* Perinuclear immunostaining (*arrow*) is reduced by preadsorption of the primary antiserum with 100 μg/ml of D_2 peptide conjugated to hemocyanin. *C,* Untransfected CHO cells exhibit little perinuclear immunolabeling (*arrow*), comparable to unstained cells (compare to *A* and *B*). *n*, nucleus. Scale bar, 50 μm.

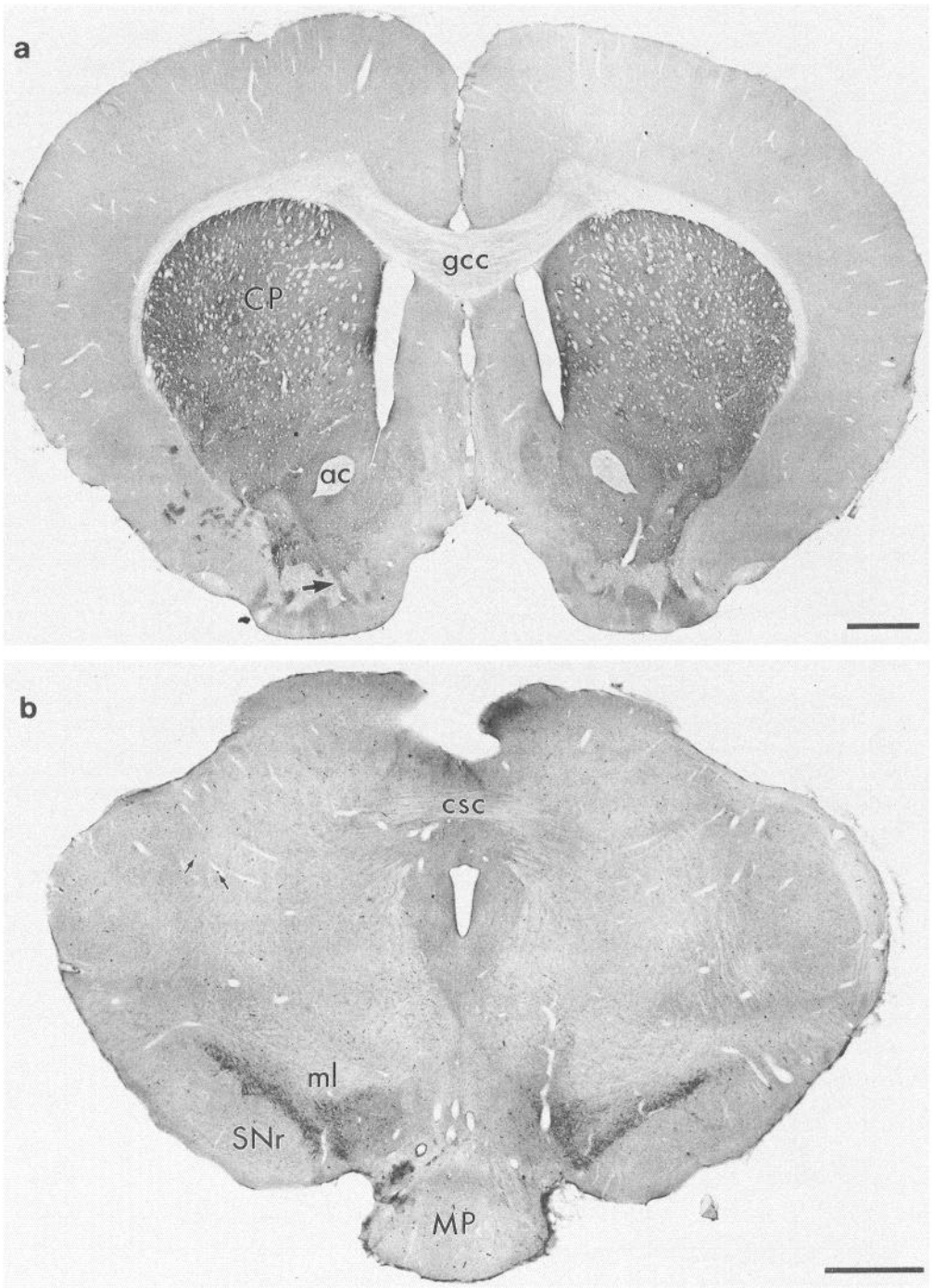

Figure 3. Low-power light micrographs of D_2-LI in the forebrain (*A*) and midbrain (*B*). *A*, In a coronal section through the genu of the corpus callosum (*gcc*), peroxidase D_2-LI is diffusely and heterogeneously localized throughout the dorsal and ventral extent of the caudate putamen nucleus (*CP*). D_2-LI is also distributed throughout the nucleus accumbens septi (surrounding the anterior commissure, *ac*) and in the olfactory tubercles. Peroxidase product can be seen in the cell bridges that connect the latter structures (*arrow*). *B*, In a midbrain section at the level of the commissure of the superior colliculus (*csc*) and the posterior mammillary nucleus (*MP*), D_2-LI is selectively distributed to the region bounded dorsally by the medial lemniscus (*ml*) and ventrally by the substantia nigra zona reticulata (*SNr*). Light immunoreaction product also extends medially into the ventral tegmental area, but does not appear in the lateral aspects of the substantia nigra. Other mesencephalic structures contain only background labeling. Small, darkly staining puncta (*small arrows*) are blood cells that appeared as well in control sections. Scale bars, 1 mm.

other than those seen in control lanes incubated without primary antibody were detected for 1:500–1:10,000 dilutions of anti-D_2 peptide antiserum. In the remaining experiment, for which membrane fractions and overnight incubation in 1:250 antiserum were used, chromogen reaction for 5 min also produced no labeled bands. However, between 6 and 20 min of incubation in the reaction solution, a weakly labeled band developed in both striatal and cerebellar preparations that had an approxi-

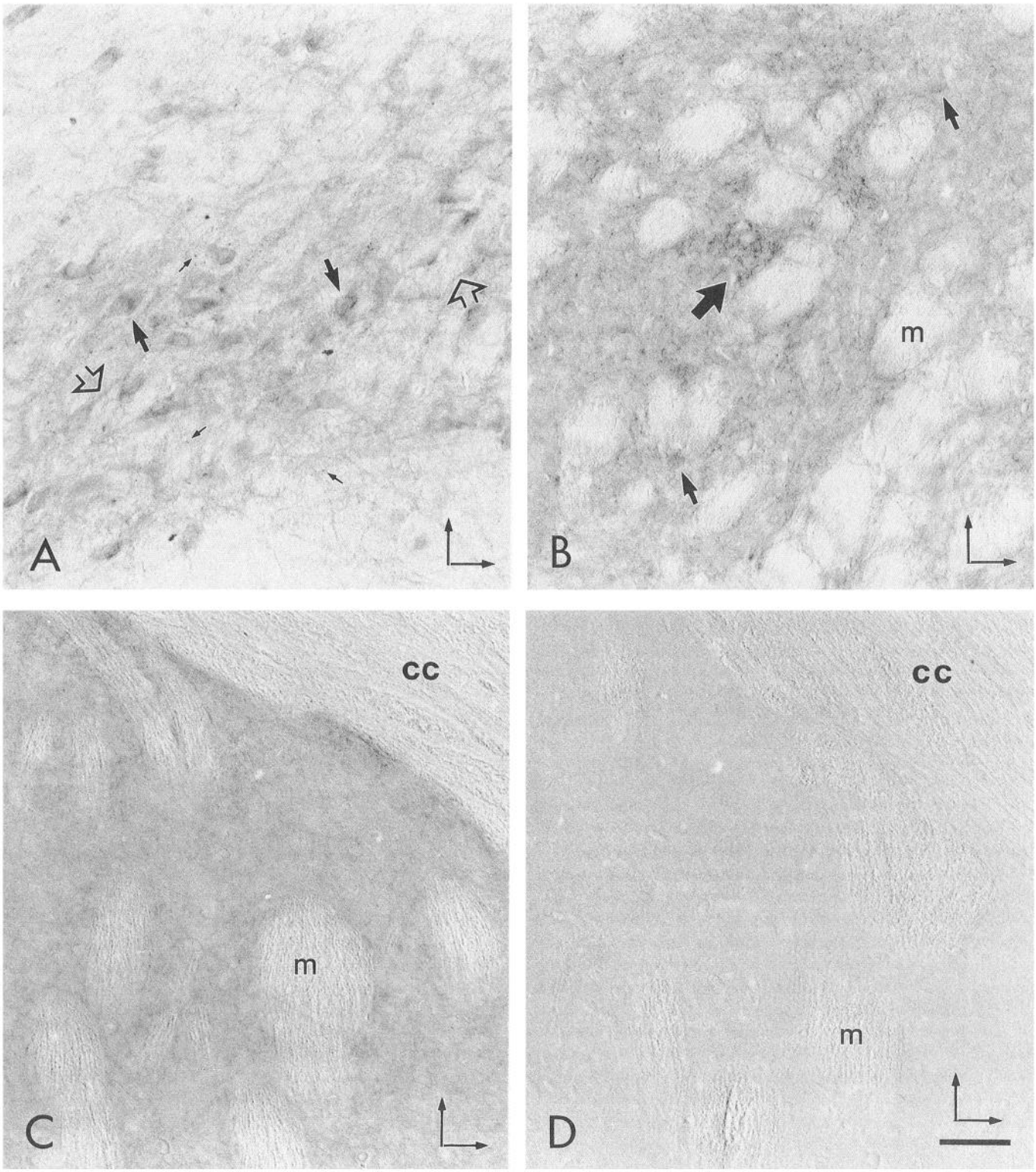

Figure 4. High-magnification light micrographs of D_2-LI in midbrain and forebrain regions. *A,* In sections through the substantia nigra/ventral tegmental area, peroxidase reaction product for D_2-LI is primarily localized to perikarya (*large solid arrows*) and thick processes (*open arrows*) having the morphological appearance of dendrites. Less numerous, punctate processes are also visible in the neuropil (*small solid arrows*). *B,* Within the medial striatum, D_2-LI is diffusely and heterogeneously localized within the neuropil, but is absent from myelinated bundles (*m*). Patches of more intense staining are apparent (*large arrow*), as are lightly labeled perikarya (*small arrows*). *C,* Diffuse D_2-LI is localized throughout the neuropil of the dorsolateral striatum, but is absent from the corpus callosum (*cc*) and myelinated bundles (*m*). *D,* Adjacent striatal sections incubated in D_2 peptide antiserum to which 100 μg/ml of immunizing peptide had been added prior to immunocytochemical processing fail to exhibit detectable immunoreactivity. *Arrows* indicate dorsal (upward) and lateral (rightward) orientation in the brain. Scale bar, 100 μm.

mate molecular weight of 69 kDa (data not shown). This band was not visible in nitrocellulose papers incubated in the absence of the primary antiserum. We consider this band to have resulted from nonspecific cross-reaction of the antiserum under the excessive labeling conditions used in this experiment. In contrast, adjacent lanes run in parallel showed bands strongly immunoreactive for a neural cell adhesion molecule (Chung et al., 1991) within 1 min of incubation in chromogen.

Light microscopic localization of D_2-LI

Diffuse peroxidase product for D_2 receptor-like immunoreactivity (D_2-LI) was specifically localized in basal ganglia structures, including forebrain striatal regions and the midbrain ventral tegmentum (Fig. 3). Immunolabeling was not visible in the neocortex, diencephalon, caudal brainstem, or cerebellum. Immunolabeled perikarya were detected in the substantia nigra zona compacta and in the ventral tegmental area (Figs. 3*B*, 4*A*). Thick, nonvaricose processes (most likely dendrites) emanating from these cells were also detected within these areas, and in the substantia nigra zona reticulata. Other mesencephalic regions examined in the same sections exhibited little or no detectable D_2-LI (Fig. 3*B*). In forebrain areas, D_2-LI was localized to diffusely distributed punctate processes throughout the dorsal and ventral aspects of the caudate putamen nucleus (striatum), as well as the nucleus accumbens and olfactory tubercles (Fig. 3*A*). Peroxidase immunoreactivity was not readily detectable in cortical regions by light microscopy (Fig. 3*A*). Within the striatum, D_2-LI was heterogeneously distributed within the neuropil (Fig. 4*B,C*), with patches of dense immunoreactivity surrounded by areas of lighter labeling (Fig. 4*B*). A few perikarya were lightly labeled (Fig. 4*B*). No peroxidase product was detected within the striatal white matter or corpus callosum (Figs. 3*A*, 4*B,C*).

When midbrain or forebrain sections were incubated in preimmune serum (not shown) or rat immune serum preadsorbed with the immunizing D_2 peptide (Fig. 4*D*), no peroxidase labeling was detected. Immune serum preadsorbed with nonimmunizing peptides from the D_2 or β_2 adrenergic receptors produced specific labeling of the midbrain and striatum comparable to that seen with immune serum alone (not shown).

Electron microscopic cellular and subcellular localization of D_2-LI: correlation to TH immunoreactivity

Substantia nigra/ventral tegmental area

Within a region encompassing the medial substantia nigra and the paranigral ventral tegmental area, diffuse and light labeling for D_2-LI was seen in perikarya at the electron microscopic level. In these perikarya, the Golgi apparatus and rough endoplasmic reticulum typically lacked detectable D_2-LI (not shown). More intense peroxidase product was seen in the dendritic branches of these cells in tissue either singly labeled for D_2-LI (Fig. 5) or dually labeled for D_2 peptide and TH (Fig. 6). Within dendrites, D_2-LI was most intensely localized to saccules of smooth endoplasmic reticulum and to patches along plasmalemmal surfaces postsynaptic to axon terminals (Fig. 5*B*). Less intense D_2-LI was distributed along nonsynaptic portions of the plasmalemmal surface and in association with microtubules and mitochondrial membranes. Neither myelinated axons nor axon terminals exhibiting D_2-LI were seen in this region. However, small intensely labeled, presumably axonal processes were often observed in bundles of unmyelinated axons (Fig. 5*A*). Occasionally, in a favorable plane of section such as seen in Figure 6*B*, peroxidase labeling could be seen in preterminal portions of axons whose terminal varicosities were devoid of immunoreactivity. Weak D_2-LI was also occasionally localized to astrocytic processes in apposition to immunolabeled dendrites (Fig. 6).

In dually labeled sections through the substantia nigra and ventral tegmental area, numerous dendrites contained both peroxidase immunoreactivity for the D_2 peptide and gold-silver immunolabeling for TH (Fig. 6*A*). Within the same sections, other dendrites were singly labeled for either D_2-LI or TH immunoreactivity. These singly labeled dendrites were frequently observed in close proximity to one another (Fig. 6*B*). Gold-silver particles were localized throughout the cytoplasm of TH-immunoreactive dendrites and were rarely detected in the sur-

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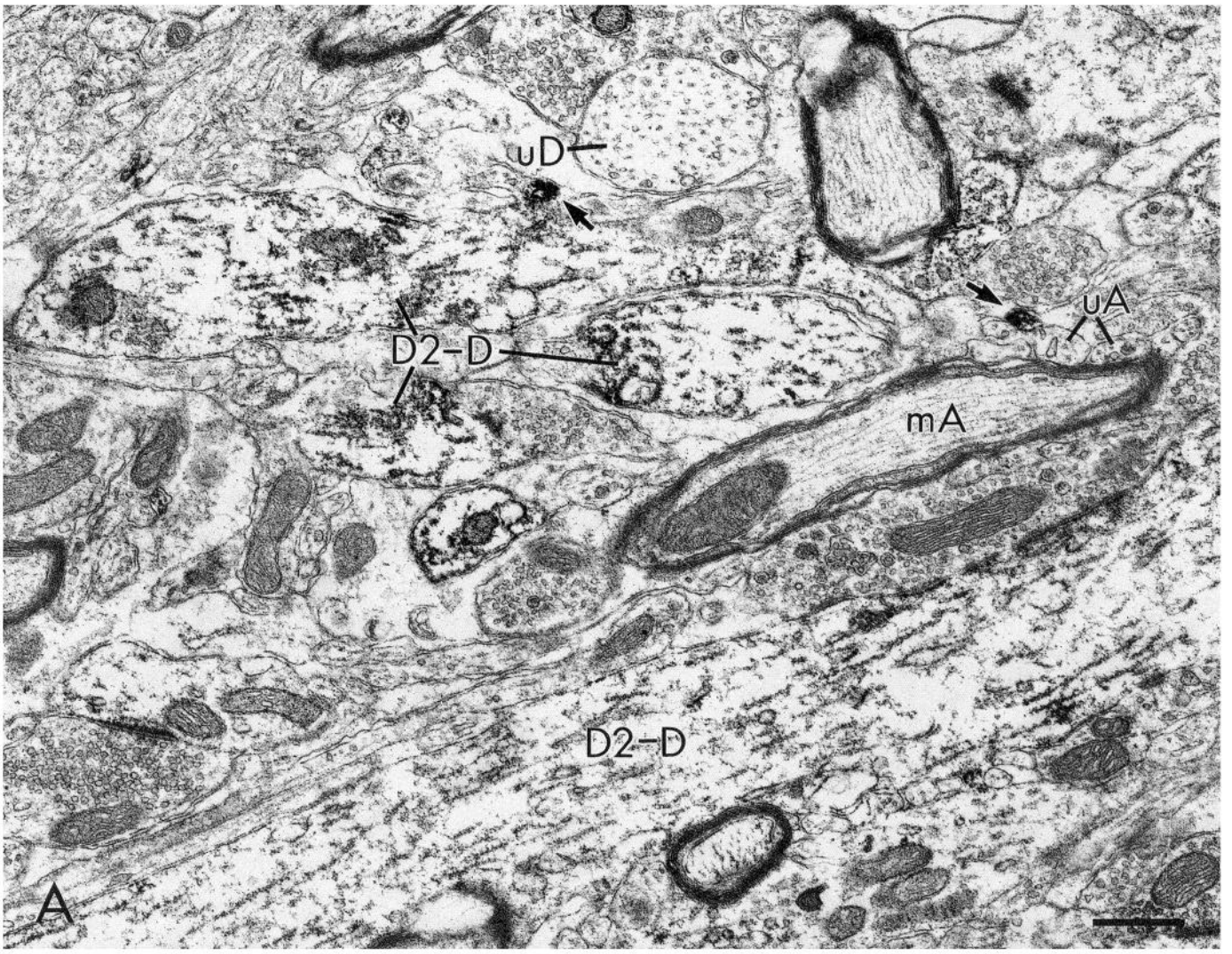
Figure 5. Electron micrographs of D_2-LI in the medial substantia nigra/ventral tegmental area. *A*, At low magnification, numerous dendrites exhibit peroxidase D_2-LI (*D2-D*). Other unlabeled dendrites exhibit no detectable immunoperoxidase reaction product (*uD*). Small, intensely labeled processes are seen in the neuropil (*arrows*), often adjacent to other nonimmunoreactive myelinated (*mA*) and unmyelinated (*uA*) axons. *B*, At high magnification, D_2-LI within dendrites is prominently localized to saccules of smooth endoplasmic reticulum (*ser*) and along the outer membranes of certain mitochondria (*m*). D_2-LI is also localized to the densities postsynaptic to unlabeled axon terminals (*uT*) (*curved arrows*). Nonsynaptic portions of the plasmalemmal surface also show accumulations of immunoreaction product (*straight arrows*). Scale bars: *A*, 0.5 μm; *B*, 0.25 μm.

Figure 6. Electron micrographs exhibiting the codistribution of peroxidase D_2-LI and immunogold labeling for TH in the ventral tegmental area. *A*, A dendrite contains both peroxidase D_2-LI, localized primarily to saccules of smooth endoplasmic reticulum (*ser*), and immunogold-silver particles for TH (*D2+TH-D*). A dendrite containing neither immunoperoxidase nor immunogold labeling (*uD*) is seen in the adjacent neuropil. *B*, Another dendrite from a nearby region of the same tissue is singly labeled for the D_2 peptide (*D2-D*), but lacks detectable TH immunoreactivity. The adjacent TH-immunoreactive dendrites (*TH-D*) are, at best, only weakly labeled for the D_2 peptide. Light peroxidase D_2-LI is also seen in the preterminal portion (*open arrow*) of an otherwise unlabeled terminal. In both *A* and *B*, immunoreactive dendrites are surrounded on several sides by astrocytic processes (*asterisks*), some of which may contain weak D_2-LI. Scale bars: *A*, 0.5 μm; *B*, 0.25 μm.

Figure 7. Electron micrographs through the dorsal striatum showing dendritic localization of D_2-LI. *A*, Peroxidase D_2-LI is barely detected in a proximal dendrite, but is highly localized to more distal aspects of the same process. A patchy distribution of D_2-LI is localized to plasmalemmal surfaces that are apposed to an unlabeled axon (*uA*) (*straight arrow*) and to another immunoreactive dendrite (*facing arrowheads*). Note the absence of peroxidase product at plasmalemmal surfaces in contact with unlabeled terminals (*curved arrows*). In more distal portions of the dendrite, D_2-LI is most intensely associated with smooth endoplasmic reticulum (*ser*) and microtubules (*mt*). *B*, Dense peroxidase D_2-LI is detected within distal dendrites, particularly in association with the smooth endoplasmic reticulum (*ser*) and spines (*S*). Unlabeled spines (*uS*) and dendrites (*uD*) are visible in the surrounding neuropil. The immunolabeled dendrites exhibit close membrane appositions with labeled and unlabeled dendrites (*facing arrowheads*). *C*, Within spines, peroxidase reaction product for D_2-LI is densely associated with the spine apparatus (*sa*) and densities postsynaptic to unlabeled axon terminals (*uT*) (*curved arrows*). The postsynaptic density at the *top* of the micrograph appears more intensely labeled than that in the *lower right*. Scale bars: *A*, 0.5 μm; *B* and *C*, 0.25 μm.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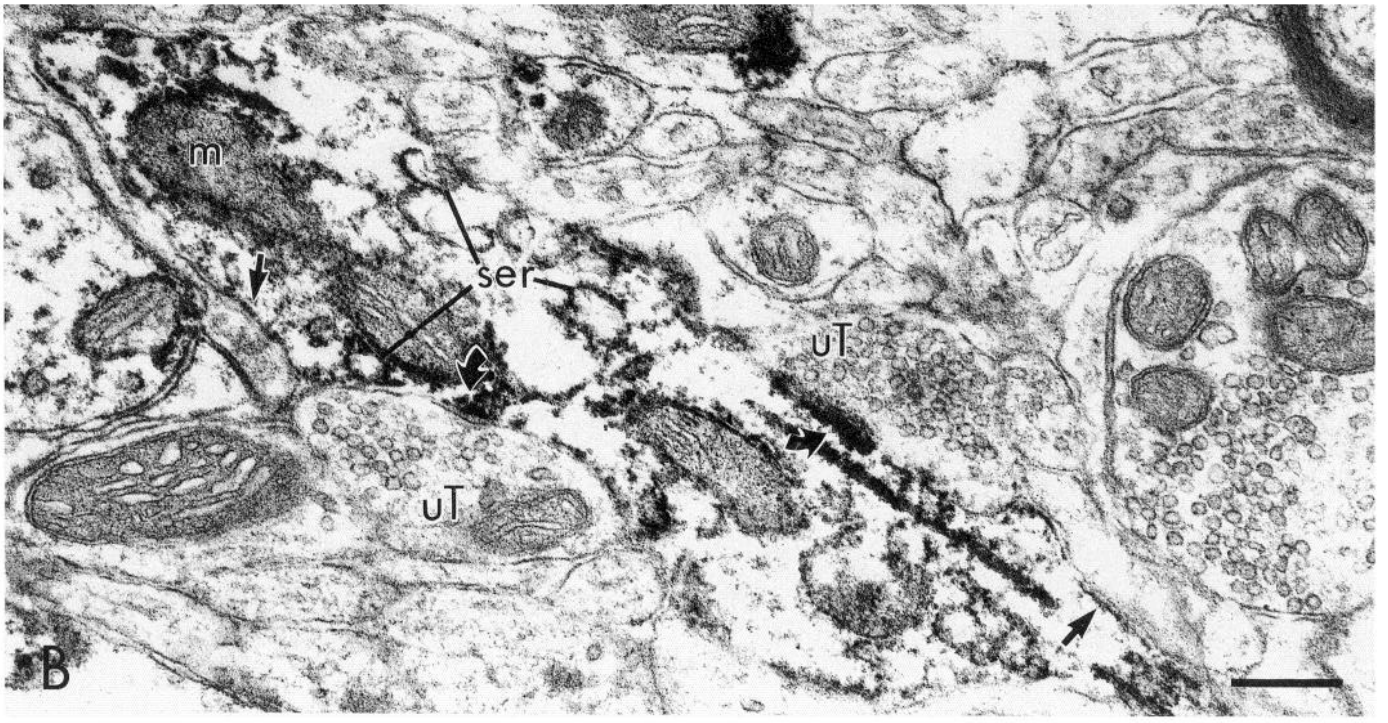
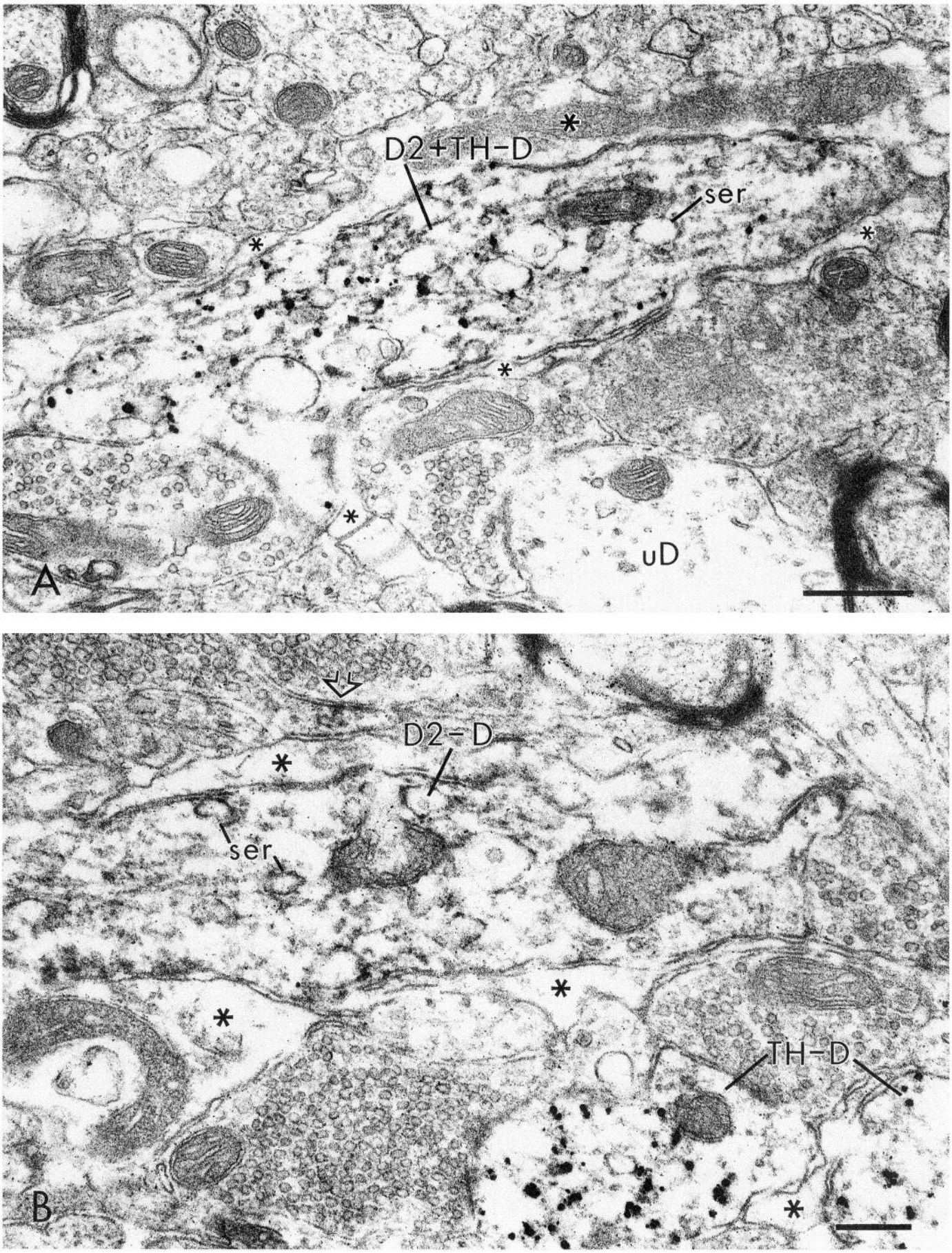
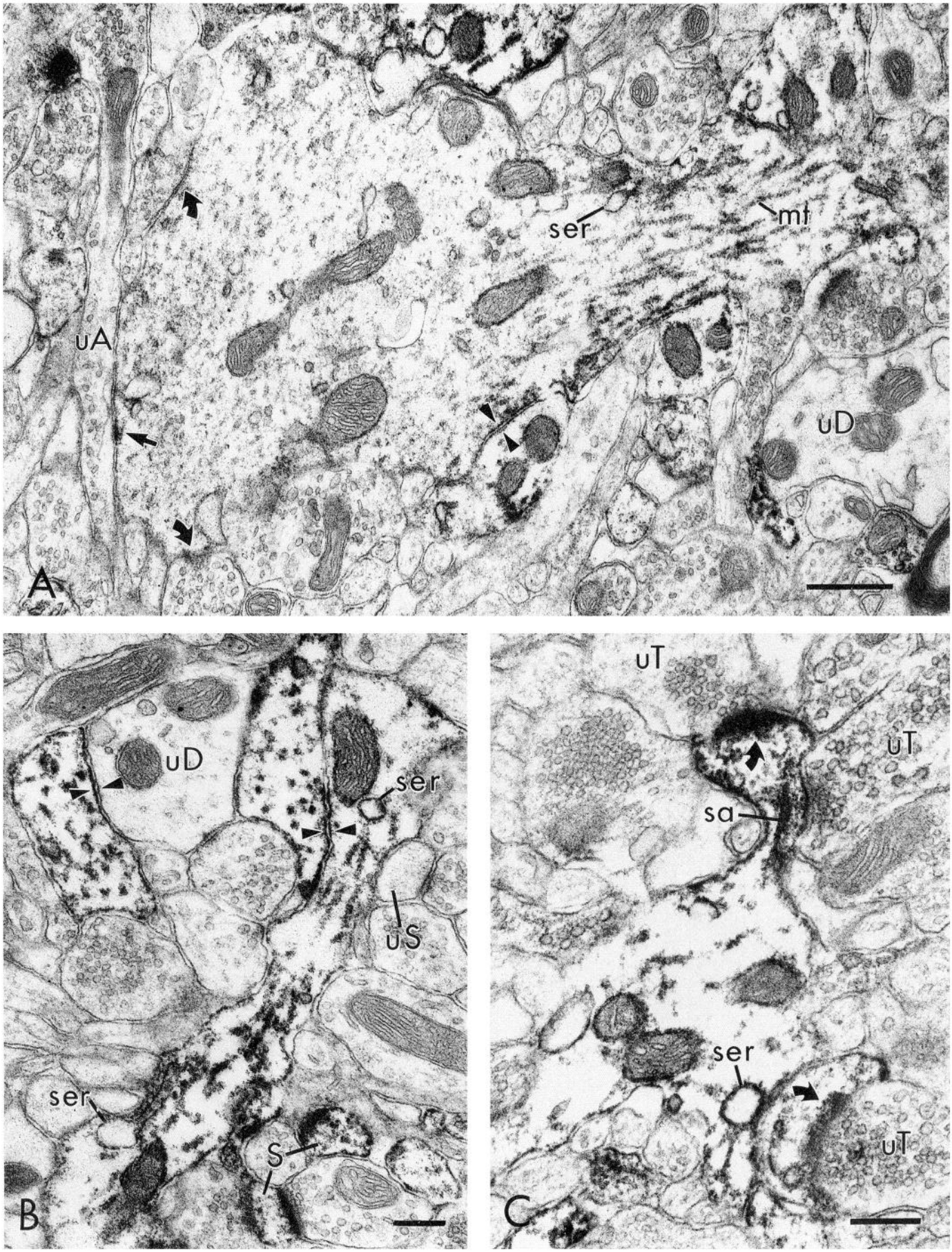
D2-D
A


m
ser
uT
uT
B


D2+TH-D
ser
uD
A
D2-D
ser
TH-D
B


ser
mt
uA
uD
A
uD
ser
uS
ser
S
B
uT
uT
sa
ser
uT
C

rounding neuropil. The subcellular distribution of D_2-LI did not differ from that observed in singly labeled tissue.

Striatum

The cellular and subcellular distribution of D_2-LI within medial and dorsolateral regions of the striatum resembled that seen in the midbrain. Dendrites were the most intensely immunoreactive neuronal elements, exhibiting dense peroxidase product in association with smooth endoplasmic reticulum, some microtubules, and plasmalemmal surfaces, particularly in regions of synaptic specializations (Fig. 7). The greatest density of D_2-LI was detected at distal portions of the dendritic tree, with maximal labeling occurring in dendritic spines (Fig. 7). The spine apparatus, when visible within the plane of section, was usually associated with dense D_2-LI (Fig. 7*C*). Immunoreactive dendrites often exhibited close membrane appositions with other dendrites that either contained or lacked D_2-LI (Fig. 7*B*).

The subcellular distribution of D_2-LI within axons was heterogeneous, with peroxidase product densely localized along the plasmalemmal surface of unmyelinated axons (Fig. 8*A*), some of which were continuous with vesicle-filled varicosities (Fig. 8*D*). Furthermore, D_2-LI was localized to some, but not all synaptic vesicles, and these were frequently located near one plasmalemmal surface (Fig. 8). These D_2 peptide–labeled terminals were only seen near the surface of the tissue. D_2-LI was rarely detected in myelinated axons.

The majority of D_2 peptide–labeled terminals were small and lacked detectable synaptic junctions in the single sections analyzed. However, some terminals exhibiting D_2-LI formed punctate symmetric synapses with distal dendrites or the necks of spines (Fig. 8*A,C*). These synapses were identified by their parallel apposed, electron-dense membranes and intercleft filaments. Postsynaptic densities were either thin or absent at these junctions. In a few instances, terminals forming asymmetric synapses with spines showed detectable D_2-LI (Fig. 8*B*). The dendritic targets of D_2 peptide–immunoreactive terminals were typically unlabeled, although a few exhibited D_2-LI (Fig. 8*C*).

In striatal sections, gold-silver immunoreactivity for TH was localized exclusively to axon terminals, the majority of which did not contain peroxidase labeling for D_2-LI (Figs. 8*C*, 9) and lacked recognizable junctions in single sections (Figs. 8*C,D*; 9). Occasionally, terminals contained immunoreactivity for both TH and D_2 peptide (Fig. 8*D*); these also were typically nonjunctional when viewed in single sections. TH-immunoreactive terminals sometimes contacted dendritic targets containing detectable D_2-LI. In most cases, the targets were the heads or necks of spines (Fig. 9). Classically defined synaptic junctions were usually not observed between these elements when viewed in single or a limited number of serial sections. Close membrane appositions were also observed between TH-immunoreactive terminals and the unlabeled terminals forming asymmetric synapses on D_2 peptide–immunoreactive spines (Fig. 9*C*).

Discussion

D_2-LI was intensely localized to (1) brain regions containing dopamine neurons and their processes; (2) plasmalemmal surfaces and organelles of proximal and distal dendrites, unmyelinated axons, and axon terminals; (3) midbrain and striatal processes that colocalized TH, or were postsynaptic to TH-immunoreactive axon terminals; and (4) dendrites in the ventral midbrain and striatal axon terminals that exhibited no detectable TH-labeling. These multiple locations of the D_2 receptor peptide, and their relation to TH-immunoreactive processes, is shown diagrammatically in Figure 10. The immunocytochemical electron microscopic localization of a D_2 receptor-like protein provides a sensitive means for determining the potential cellular and subcellular elements expressing these proteins in dopamine pathways.

Antiserum specificity

Several lines of evidence suggest that the immunoreactive species presently identified by anti-peptide antiserum in fixed tissue may be the dopamine D_2 receptor. (1) The selective immunolabeling of cultured CHO cells permanently transfected with the cDNA for the rat dopamine D_2 receptor, but not untransfected cells, suggests that the dopamine D_2 receptor was specifically recognized. The reduction of immunostaining by peptide preadsorption also supports specificity of the antibody for the immunizing D_2 peptide sequence. (2) The direct correlation between the light microscopic distribution of D_2-LI in fixed rat brain tissue and both autoradiographic detection of D_2 receptors (Boyson et al., 1986; Bouthenet et al., 1987; Mansour et al., 1990) and immunocytochemical localization of receptors using other anti-peptide antisera (Ariano et al., 1993) further suggests recognition of a dopamine receptor of the D_2 subtype. (3) The colocalization of immunoreactive markers for the D_2 receptor peptide and TH indicates that the antiserum recognizes an antigen contained in dopamine neurons and their processes. (4) The complete absence of immunoreactivity in fixed brain tissue incubated in antiserum preadsorbed with the D_2 peptide indicates that naturally occurring or adjuvant-induced antibodies in the polyclonal antiserum did not contribute to the immunostaining.

In order to acknowledge that other proteins structurally similar to the D_2 receptor peptide might additionally be recognized,

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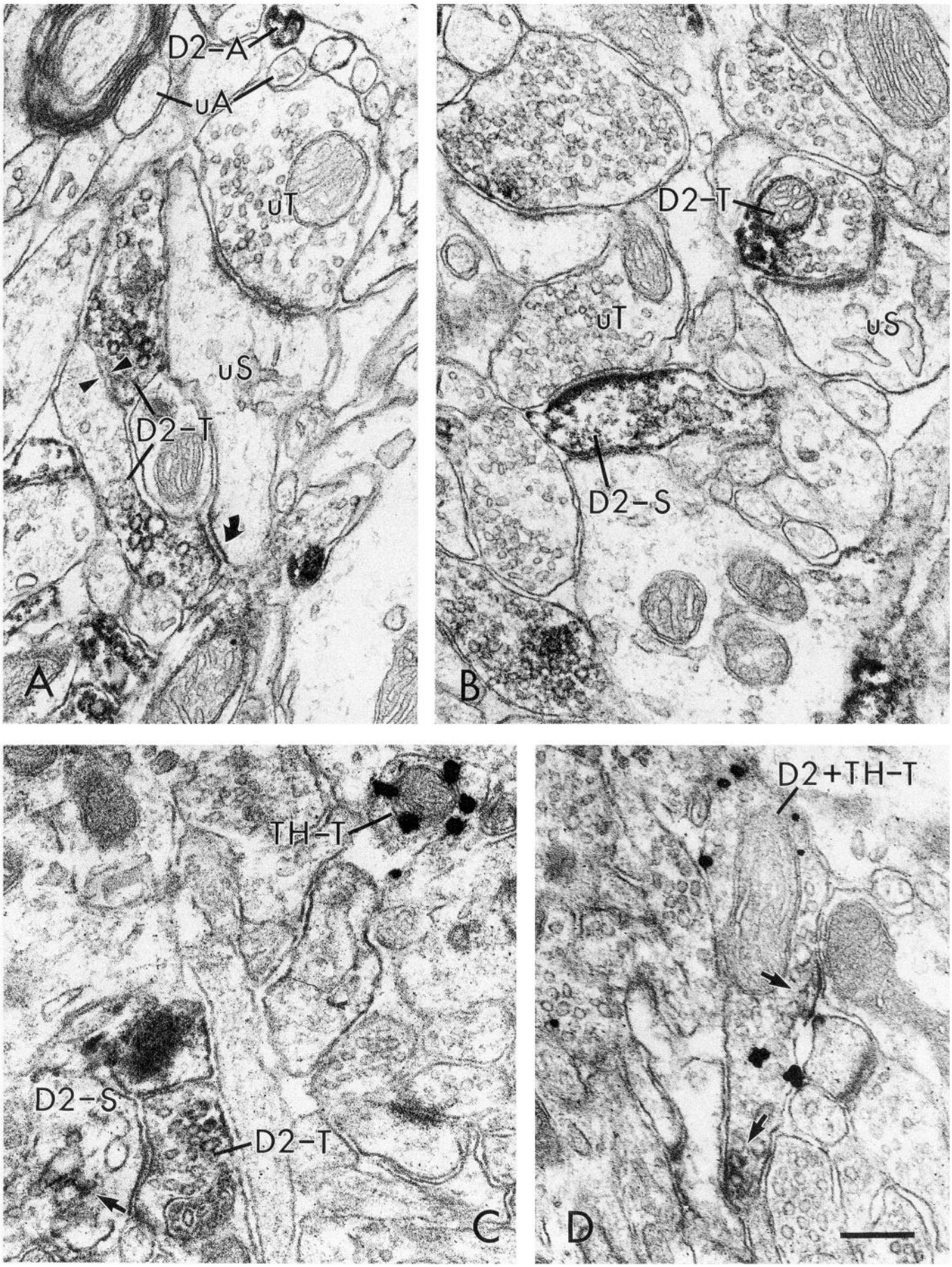
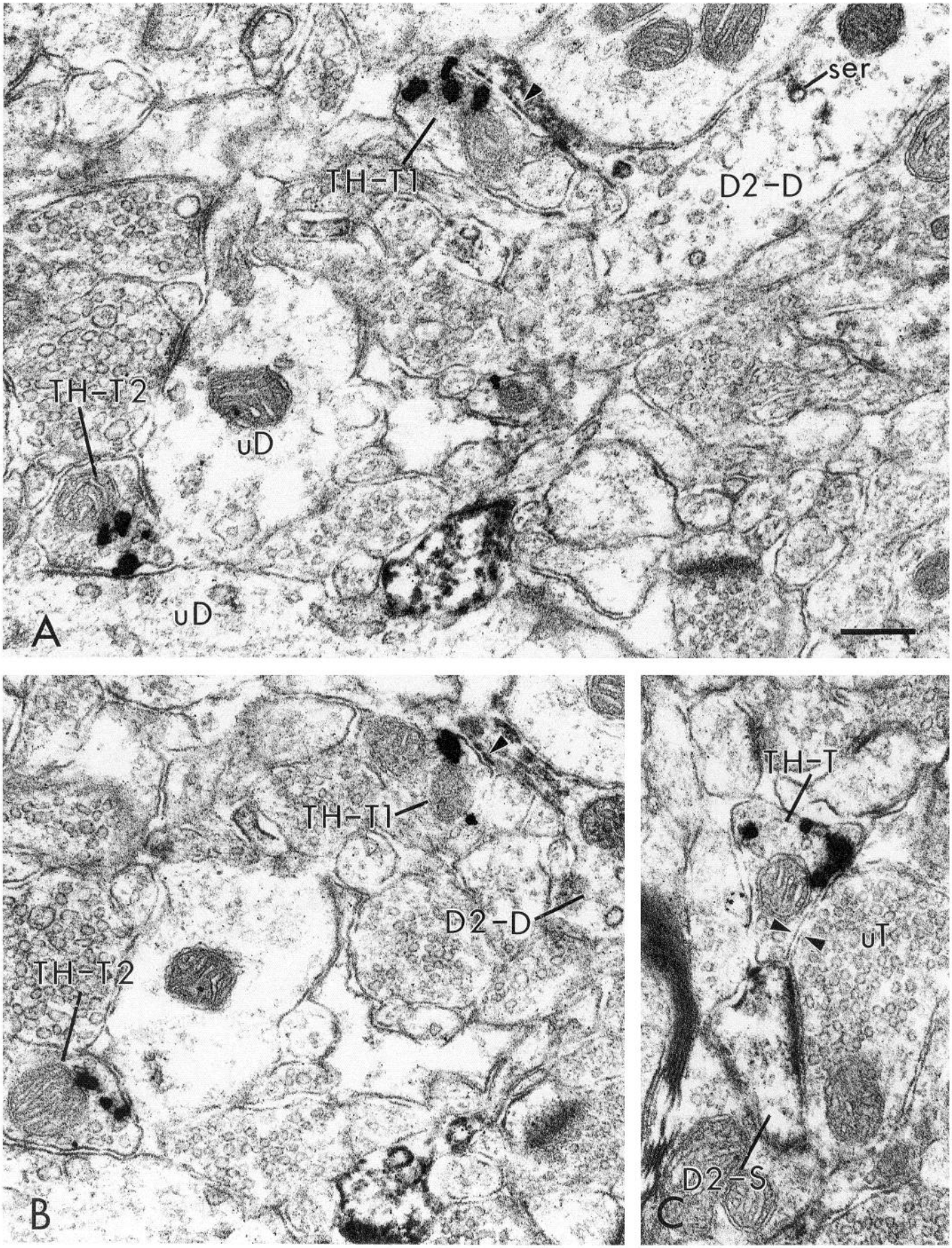
Figure 8. Electron micrographs of striatal sections exhibiting D_2-LI localized to axon terminals. *A,* Two terminals exhibiting peroxidase D_2-LI (*D2-T*) contact an unlabeled dendritic spine (*uS*). The spine also receives an asymmetric synapse from a nonimmunoreactive terminal (*uT*). One D_2 peptide–immunoreactive terminal contacts the head of the spine without forming a recognized junction, while the second immunolabeled terminal forms a symmetric synapse on the spine neck (*curved arrow*). The two immunoreactive terminals are also in close apposition to one another (*facing arrowheads*). Note the heterogeneous distribution of peroxidase reaction product within these terminals. A bundle of labeled (*D2-A*) and unlabeled (*uA*) unmyelinated axons is seen in the upper portion of the micrograph. *B,* Localization of a patch of intense peroxidase D_2-LI in a terminal forming an asymmetric junction (*D2-T*) on an unlabeled spine (*uS*) is depicted. An adjacent unlabeled terminal (*uT*) forms a synapse on a spine containing D_2-LI (*D2-S*). *C,* In tissue dually labeled for D_2 peptide and TH, an axon terminal possessing peroxidase D_2-LI (*D2-T*), but not immunogold-silver labeling for TH, forms a symmetric synapse on a spine (*D2-S*) that contains D_2-LI in association with the spine apparatus (*arrow*). A process within the same field contains gold-silver particles, indicating TH immunoreactivity (*TH-T*), but does not show detectable levels of peroxidase labeling. *D,* An axon varicosity (*D2+TH-T*) exhibits both gold-silver immunoreactivity for TH and patches of peroxidase D_2-LI (*arrows*). The lower, more intensely stained patch of D_2-LI is associated with the plasmalemmal surface and a few small, clear vesicles. Scale bar, 0.25 μm.


D2-A
uA
uT
uS
D2-T
A
uT
D2-T
uS
D2-S
B
TH-T
D2-S
D2-T
C
D2+TH-T
D


ser
TH-T1
D2-D
TH-T2
uD
uD
A
TH-T1
D2-D
TH-T2
B
TH-T
uT
D2-S
C

the antigenic species immunolabeled by anti-peptide antiserum in fixed brain tissue is herein referred to as a "D_2 receptor-like" protein. In this regard, Western blot analysis of brain homogenates using the anti-D_2 peptide antiserum failed to label proteins in the molecular weight range (Amlaiky and Caron, 1986) of the D_2 receptor. In one instance, the anti-D_2 peptide antiserum did weakly label a 69 kDa protein in striatal and cerebellar membrane fractions. We believe that this staining, which was seen in only one of four experiments, represents nonspecific labeling resulting from the excessive antibody concentrations and incubation conditions used in this experiment. Furthermore, we do not believe that this protein is labeled immunocytochemically in fixed tissue, as comparable antibody dilutions were never used for these studies, and peroxidase product was never detected within the cerebellum. Thus, the anti-D_2 peptide antiserum appears to label an antigen in fixed tissue that is below threshold for recognition by the Western blot assay. This difference may reflect both the low levels of total tissue protein contributed by the dopamine D_2 receptor (Strange, 1990) and the preferential recognition by the antiserum of a fixed form of the antigen.

Additional studies will be needed to assess whether the D_2 peptide antiserum cross-reacts with other dopamine receptor subtypes. However, the present results suggest that such cross-reactivity is unlikely. First, amino acid sequences homologous to the D_2 peptide fragment used for immunization do not occur in the published sequences of D_{1A}, D_{1B} (D_5), D_3, or D_4 receptors (Civelli et al., 1991; Sibley and Monsma, 1992). Second, immunoperoxidase product was not detected in axon terminals in the substantia nigra/ventral tegmental area, the major site of D_1 receptor localization in this region (Mansour et al., 1990; Fremeau et al., 1991; Meador-Woodruff et al., 1991; Weiner et al., 1991; Huang et al., 1992). Third, an increasing dorsal to ventral density of immunoreactivity in forebrain targets was not observed, as would be expected if this antiserum also recognized D_3 receptors confined to limbic structures (Sokoloff et al., 1990; Bouthenet et al., 1991). Finally, peroxidase immunoreactivity did not exhibit greater density in cortex, compared to striatum, as has been reported from Northern blot analysis of D_4 receptor probe (Van Tol et al., 1991).

Light microscopic immunocytochemical localization

The localization of D_2 peptide–immunolabeled perikarya in the zona compacta of the substantia nigra and the ventral tegmental area, as well as the distribution of immunoreactive processes throughout the dorsal and ventral extent of the striatum, matches the regions of heaviest immunolabeling seen with other anti-D_2 peptide antisera (Ariano et al., 1993) and furthermore, corresponds to the distribution of immunolabeling for TH or dopamine (Pickel et al., 1975; Geffard et al., 1984; Decavel et al., 1987). The present observations also agree in essence with the results of autoradiographic studies describing the localization of dopamine receptor ligands (Boyson et al., 1986; Bouthenet et al., 1987; Mansour et al., 1990), although a systematic comparison of all brain regions was not attempted. The limited light microscopic immunolabeling of cortex by the D_2 peptide antiserum may reflect the low level of D_2 receptor expressed in cortical tissue (Bouthenet et al., 1987, 1991; Mansour et al., 1990; Weiner et al., 1991). However, D_2-LI was detectable by electron microscopy in the prefrontal cortex (S. R. Sesack and V. M. Pickel, unpublished observations), which is densely innervated by dopamine fibers (Van Eden et al., 1987). Thus, while immunocytochemical localization of D_2 peptide at the light microscopic level may be less sensitive than autoradiographic methods, it appears to be suitable for electron microscopic localization.

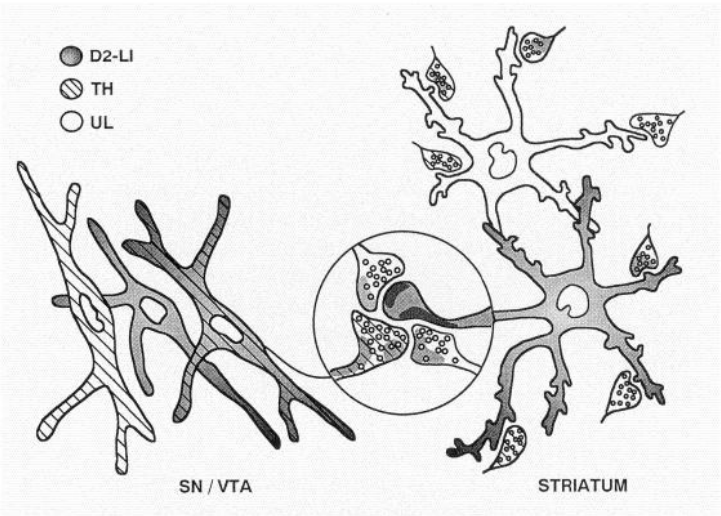


Figure 10. Schematic diagram illustrating the cellular localization of immunoreactivities for the dopamine D_2 receptor peptide and TH in the midbrain and striatum. The distribution of D_2-LI is represented by *graded shading*, and TH localization is shown by *hatching*. All other processes were not identified in terms of their receptor or transmitter content and are unlabeled (*UL*). Many dendrites in the substantia nigra and ventral tegmental area (*SN/VTA*) exhibit both D_2 receptor peptide and TH labeling, although some dendrites contain only one of these markers. Perikarya and the most proximal dendrites typically contain only low levels of D_2-LI. In the striatum, the greatest density of D_2-LI occurs in spines (*inset*) and distal dendritic processes, with considerably lighter labeling evident in more proximal processes and perikarya. Only a population of spines and dendrites in the striatum exhibit detectable D_2-LI. Some of these labeled processes are contacted by terminals containing immunoreactivity for TH. A heterogeneous distribution of D_2-LI is also detectable in the preterminal and varicose portions of axon terminals. Most of these terminals make thin, symmetric synapses on spines or distal dendrites, or fail to form synaptic junctions in single planes of section. A few D_2 peptide–immunoreactive terminals are identified as catecholaminergic (presumably dopaminergic) on the basis of their TH immunoreactivity (*inset*). Other unmyelinated axons (not shown) and terminal varicosities without TH immunoreactivity also contain D_2-LI. Some of these labeled terminals from asymmetric axospinous synapses, while others, like the TH axons, primarily contact the necks of spines (*inset*).

←

Figure 9. Electron micrographs from the dorsal striatum showing the codistribution of peroxidase D_2-LI and immunogold labeling for TH. *A* and *B*, An axon terminal shown in serial section contains immunogold-silver labeling for TH (*TH-T1*) and is in close apposition (*arrowhead*) to the neck of a spine exhibiting dense D_2-LI. The parent dendrite (*D2-D*) also exhibits D_2-LI in association with the smooth endoplasmic reticulum (*ser*). The TH-immunolabeled terminal in the lower portion of the micrograph (*TH-T2*) forms symmetric synapses on two unlabeled dendrites (*uD*). *C*, A TH-immunoreactive terminal (*TH-T*) contacts a D_2 peptide–immunoreactive spine (*D2-S*), without forming a recognizable junction. The spine receives synaptic input from an unlabeled terminal (*uT*) that is in close apposition to the TH-T (*facing arrowheads*). Scale bar, 0.25 μm.

Ultrastructural localization of D_2-LI

Subcellular distribution

Heterogeneities in the density of subcellular D_2 peptide labeling suggest that there is some selectivity of organelle- and membrane-associated peroxidase product. For example, not all vacuoles of smooth endoplasmic reticulum, synaptic vesicle membranes, or regions of plasmalemmal surface exhibited D_2 peptide labeling in otherwise immunoreactive processes. Thus, the presence of antigen may not be uniform in these structures. The observation of immunolabeling primarily on the intracellular membrane surface, and not within the synaptic cleft or extracellular space, is consistent with detection of an intracellular protein, or an intracellular loop of an integral membrane protein (Aoki et al., 1989). The present description of subcellular localization of D_2-LI may be complicated, to some degree, by the diffusibility of peroxidase reaction product (Novikoff et al., 1972). Future studies using less diffusible immunogold techniques will be needed to verify the present results and to characterize further the loci of antigen in transit or inserted into the plasma membrane.

Substantia nigra/ventral tegmental area

In the medial substantia nigra and ventral tegmental area, the localization of dense immunolabeling in dendrites, and not axon terminals, is consistent with the localization of D_2 peptide in dopamine neurons that possess few recurrent collaterals (Bayer and Pickel, 1990). As mentioned previously, these results also suggest that the D_2 peptide antiserum does not recognize D_1 receptors that have been shown to reside on axons and terminals (Huang et al., 1992), presumably descending from the striatum and ventral forebrain (Mansour et al., 1990; Fremeau et al., 1991; Meador-Woodruff et al., 1991; Weiner et al., 1991). The finding that D_2-LI was intensely localized to small processes that were frequently surrounded by other unmyelinated axons suggests that extrinsically or intrinsically directed axons may express a D_2 receptor-like protein. Sparse peroxidase product for D_2 peptide in astrocytic processes also is consistent with the localization of dopamine receptors, which have been described on astroglial cells (Murphy and Pearce, 1987; Hansson and Rönnbäck, 1988).

Perikarya and large proximal dendrites exhibited only weakly detectable D_2-LI, even at the tissue surface where antibody penetration was maximal. This finding implies that the antigen recognized by the D_2 peptide antiserum reaches detectable levels only in more distal portions of dendrites, and/or is detectable only at a late posttranslational stage. The latter suggestion is consistent with the subcellular distribution of D_2-LI to the smooth endoplasmic reticulum, and not the rough endoplasmic reticulum or the Golgi apparatus. The labeled saccules of smooth endoplasmic reticulum also may reflect internalized receptor from the plasmalemmal surface (Broadwell et al., 1980; Holtzman, 1992).

The observation that numerous D_2 peptide–immunoreactive dendrites in the midbrain also contained immunogold labeling for TH, an exclusive marker for dopamine neurons in this region (Swanson and Hartman, 1975), clearly demonstrates that the D_2 peptide antiserum recognizes an antigen contained in the dopaminergic cell group. This finding is consistent with the localization of a D_2 autoreceptor, which dopamine neurons have been shown to express on the basis of physiological, biochemical, and anatomical evidence (Aghajanian and Bunney, 1977; Skirboll et al., 1979; White and Wang, 1984a; Lacey et al., 1987; Wolf and Roth, 1987; Morelli et al., 1988; Le Moine and Bloch, 1991). In some instances, TH-labeled dendrites did not contain detectable D_2-LI, consistent with reports that some dopamine neurons are devoid of, or express only low levels of, D_2 receptors (Chiodo et al., 1984; White and Wang, 1984b; Le Moine and Bloch, 1991). Alternatively, subpopulations of dopamine neurons may express D_3 (Sokoloff et al., 1990; Bouthenet et al., 1991), or some other, as yet uncharacterized, variant of D_2 receptors.

Occasionally, dendrites in the substantia nigra/ventral tegmental area contained D_2-LI but lacked immunogold reaction product for TH. Frequently these dendrites were immediately adjacent to processes exhibiting intense TH immunoreactivity. One explanation for this finding is that the D_2 peptide may be expressed in some nondopamine neurons, consistent with physiological accounts of dopamine's local effects in the midbrain (Ruffieux and Schultz, 1980; Cheramy et al., 1981; Waszczak and Walters, 1986). Alternatively, these dendrites may arise from neurons reported to express high levels of D_2 mRNA, but low levels of TH immunoreactivity (Le Moine and Bloch, 1991).

Before alternative explanations for D_2 peptide localization can be further explored, limitations in the sensitivity of dual labeling immunocytochemical methods must be considered. To some extent, an underestimation of dually labeled dendrites may have resulted from inequivalent sensitivities of the immunoperoxidase and immunogold methods (Chan et al., 1990). In addition, we have noted that some loss of label occurs with combined primary antibody incubation, compared to single antibody labeling at the same dilution (Sesack and Pickel, unpublished observations). Finally, the ability to detect low levels of peroxidase reaction product may also be limited by heavy metal staining. In this regard, initial studies with unstained tissue from the midbrain indicate that D_2-LI is detectable in most TH-labeled dendrites. Future studies directed toward a more quantitative assessment of the codistribution of D_2 peptide and TH are in progress.

Striatum

Dendrites. In the medial and dorsolateral aspects of the striatum, the predominant localization of D_2-LI to spines and distal dendrites matches the preferential distribution of synapses formed by dopamine terminals on the most distal portions of medium spiny neurons (Bouyer et al., 1984; Freund et al., 1984). These findings further suggest that the antigen recognized by the D_2 peptide antiserum may reflect receptive sites for dopamine, although the distribution of dopamine receptors may not always parallel dopamine synaptic inputs. The observation that numerous dendritic processes in the striatum contained no detectable D_2-LI may be consistent with the segregation of dopamine receptor subtypes to different populations of striatal neurons (Gerfen et al., 1990). Alternatively, a restricted localization of D_2 peptide immunoreactivity to the necks of some spines also would give the impression of nonlabeling whenever continuity between spines and dendrites was not seen within the plane of section. Since some striatal cells may express multiple dopamine receptor classes (Meador-Woodruff et al., 1991; Surmeier et al., 1992), future dual labeling immunocytochemical (and/or *in situ* hybridization) studies are needed to examine further the cellular and subcellular expression of multiple receptive sites for dopamine.

The potential functional significance, if any, of D_2-LI at sites

of plasmalemmal apposition between striatal dendrites is not known. However, it is interesting to note that electrical and dye-coupling of cells in striatum have been described in physiological studies (Cepeda et al., 1989), and that dopamine appears to reduce the resistance of these presumed intercellular channels through a D_2 receptor action (Onn et al., 1992).

Axons and axon terminals. The predominant features of striatal axons and terminals containing D_2-LI are similar to those described for dopamine axons: lack of appreciable axon myelination, small size of varicosities, content of primarily small clear vesicles, frequent nonjunctional contacts in single sections, and occasional formation of thin, symmetric synapses on spines and distal dendrites (Bouyer et al., 1984; Freund et al., 1984). The dopaminergic nature of some D_2 peptide–immunoreactive varicosities was further suggested by their colocalization of TH, which is primarily contained in dopamine, as opposed to norepinephrine, terminals in this region (Swanson and Hartman, 1975). However, our dual labeling studies suggest that the varicose portion of most TH-labeled terminals contained little, if any, D_2-LI, having instead a more restricted distribution of receptors on preterminal axons. Thus, our results are consistent with the ultrastructural localization of dopamine nerve terminal autoreceptors (Wolf and Roth, 1987) but suggest a more discrete localization than previously appreciated. However, given the limitations of the dual labeling method outlined in the previous section, it must also be considered that the codistribution of D_2 peptide and TH immunolabeling was underestimated in the present study.

Our findings are also consistent with the presence of a D_2 receptor-like protein on nondopamine axon terminals having morphological characteristics that are similar to dopamine varicosities. For example, striatal cholinergic interneurons express mRNA for dopamine D_2 receptors (Le Moine et al., 1990), and these receptors appear to regulate ACh release (Fujiwara et al., 1987; Wedzony et al., 1988; Drukarch et al., 1989). Furthermore, terminals immunoreactive for the synthetic enzyme choline acetyltransferase (ChAT) have been observed (Pickel and Chan, 1990) to form axonal and dendritic associations similar to those presently described for D_2 peptide–immunoreactive varicosities. However, as yet, there is no convincing evidence for direct synaptic input from dopamine terminals onto cholinergic cells (Pickel and Chan, 1990), or onto any class of terminals in the striatum (cf. Bouyer et al., 1984; Freund et al., 1984; Pickel and Chan, 1990; Sesack and Pickel, 1992). Thus, if D_2 receptors are indeed present on axons (and/or dendrites) of cholinergic interneurons, they may be functionally activated by dopamine diffusing from terminal release sites (Wightman and Zimmerman, 1990). Additional dual labeling studies will be needed to determine whether some D_2 peptide–immunoreactive terminals derive from aspiny cholinergic interneurons, from GABAergic projection neurons (Girault et al., 1986), or from extrinsic afferent sources (Chesselet, 1984).

The present results also show D_2 receptor peptide localization on a few terminals forming asymmetric synapses on spines, particularly in tissue pretreated by rapid freeze-thaw to enhance antibody penetration. Thus, our results are consistent with the localization of D_2 heteroreceptors on the class of corticostriatal and thalamostriatal terminals forming asymmetric axospinous synapses (Bouyer et al., 1984; Freund et al., 1984; Dube et al., 1988). However, the infrequent detection of immunoreactivity in this terminal class, or in heavily myelinated axons, cannot presently account for the magnitude of physiological and biochemical evidence favoring D_2 receptor regulation of excitability and glutamate release from these terminals (Mercuri et al., 1985; Kornhuber and Kornhuber, 1986; Yang and Mogenson, 1986; Maura et al., 1988; Garcia-Munoz et al., 1991). Our findings are supported by similar ultrastructural observations with other D_2 peptide antibodies (M. A. Ariano, personal communication) and agree with radioligand binding studies reporting difficulty in detecting appreciable D_2 receptor distribution on corticostriatal terminals (Trugman et al., 1986; Joyce and Marshall, 1987). The heterogeneous localization of immunoreactivity for the D_2 peptide within these terminals may account for the infrequent detection in single planes of section. Furthermore, the observation of D_2-LI in numerous unmyelinated, non-TH-labeled axons suggests that functional sites for dopamine reception may reside along preterminal portions of axons at a distance from the synaptic specialization. Alternatively, terminals mediating an excitatory, presumably glutamatergic, postsynaptic action may express an as yet unidentified variant of dopamine receptors. The resolution of this controversy may depend upon further refinements of receptor characterization or anatomical localization methods.

Conclusions and implications for future studies

The light and electron microscopic distribution of D_2-LI is consistent with the localization of a dopamine D_2 receptor-like protein. This antigen is contained in both dopamine (TH-containing) and nondopamine midbrain neurons, suggesting that dendritically released dopamine in this region may have physiological actions on nondopamine cells, in addition to its proposed role in autoreceptor regulation of dopamine neuron excitability. The finding that not all TH-immunoreactive cells express readily detectable D_2-LI is consistent with a low level of autoreceptor expression in some dopamine neurons, making dopamine cell groups heterogeneous with regard to autoreceptor regulation.

In striatal tissue, the expression of D_2 peptide by only a population of spines and dendrites is consistent with at least partial segregation of D_1 and D_2 receptors to separate striatal neurons. The localization of D_2 peptide to nonjunctional axon terminals that also contain immunoreactivity for TH, or to those forming primarily symmetric synapses on spines or distal dendrites, suggests that many presynaptic D_2 receptors in the striatum represent dopamine autoreceptors. However, the possibility remains that some presynaptic D_2-LI represents heteroreceptors for dopamine on nondopamine terminals, supporting pharmacological evidence for dopamine-mediated regulation of transmitter release in this brain region. Future ultrastructural studies localizing dopamine receptor peptides, neurotransmitters, and neuronal tract-tracing agents are needed to clarify further the functional significance of receptor localization for mechanisms of dopamine nervous transmission.

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