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Internalization of D1 Dopamine Receptor in Striatal Neurons *In Vivo* as Evidence of Activation by Dopamine Agonists

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To investigate how dopamine influences the subcellular localization of the dopamine receptors in the striatal dopaminoceptive neurons, we have used immunohistochemistry to detect D1 dopamine receptors (D1R) after modifications of the dopamine environment. In normal rats, D1R are located mostly extrasynaptically at the plasma membrane of the cell bodies, dendrites, and spines. The intrastriatal injection of the full D1R agonist SKF-82958 and the intraperitoneal injection of the same molecule or of amphetamine (which induces a massive release of dopamine in the striatum) induce modifications of the pattern of D1R immunoreactivity in the dorsal and ventral striatum. Whereas normal rats display homogenous staining of the neuropile with staining of the plasma membrane of the cell bodies, either treatment provokes the appearance of an intense immunoreactivity in the cytoplasm and the proximal dendrites. The labeling pattern is heterogeneous and more intense in the striosomes than in the matrix. Analysis of semithin sections and electron microscopy studies demonstrates a translocation of the labeling from the plasma membrane to endocytic vesicles and endosomes bearing D1R immunoreactivity in the cytoplasm of cell bodies and dendrites. Injection of D1R antagonist (SCH-23390) alone or injection of D1R antagonist, together with amphetamine or SKF-82958, do not provoke modification of the immunoreactivity, as compared with normal rat.

Our results demonstrate that, *in vivo*, the acute activation of dopamine receptors by direct agonists or endogenously released dopamine provokes dramatic modifications of their subcellular distribution in neurons, including internalization in the endosomal compartment in the cytoplasm. This suggests that modifications of the localization of neurotransmitter receptors, including extrasynaptic ones, may be a critical event that contributes to the postsynaptic response *in vivo*.

Key words: dopamine receptors; receptor distribution; internalization; extrasynaptic receptors; immunohistochemistry; striatum; endosomes

The actions of most neurotransmitters are mediated by a large family of G-protein-coupled receptors (Lohse, 1993). In most instances these receptors have a preferential localization at the surface of the plasma membrane of the neurons. Immunohistochemical studies have demonstrated that the receptors either can be located within the body of the postsynaptic specialization and/or can be located extrasynaptically at the surface of the cell bodies and dendrites (Aoki et al., 1989; Baude et al., 1993; Levey et al., 1993; Hersch et al., 1994, 1995; Yung et al., 1995; Caillé et al., 1996). *In vitro* studies with cells expressing naturally occurring receptors or with transfected cells have demonstrated that the activation of these receptors by endogenous or artificial ligands promotes multistep molecular and cellular events contributing to the postsynaptic response and to the metabolism and recycling of the receptors. This includes the activation or inhibition of second messenger systems and the desensitization and recycling of the receptors via phosphorylation–dephosphorylation processes (Benovic et al., 1988; Raposo et al., 1989; Lefkowitz and Caron, 1993; Fonseca et al., 1995; Roettger et al., 1995; Krueger et al., 1997). *In vitro* models show that the latter events involve primarily the internalization of the receptors and complex intracytoplasmic trafficking, which include the formation of endocytic vesicles and endosomes (Fonseca et al., 1995; Roettger et al., 1995; Trogadis et al., 1995; Koenig and Edwardson, 1997). Nevertheless, little is known about the *in vivo* behavior and fate of neuroreceptors and especially about the influence of the environment caused by neurotransmitters and related drugs on the addressing, the localization, and the distribution of receptors. Recent data demonstrate that the evoked release or the direct application of neuropeptides (substance P or neurotensin) or the injection of an opiate agonist promotes *in vivo* dramatic and profound modifications of the localization of the corresponding receptors, including translocation from the plasma membrane, internalization in endosomes, and recycling at the membrane (Faure et al., 1995; Mantyh et al., 1995a,b; Sternini et al., 1996; Lin et al., 1997).

Dopamine is a fast-acting neurotransmitter inducing effects via G-protein-coupled receptors (Seeman and Van Tol, 1994; Sokoloff and Schwartz, 1995; Jaber et al., 1996). Dopamine receptors are direct or indirect targets for many molecules, including neuroleptics, and indirect dopamine agonists, such as amphetamines and cocaine (Jackson and Westlind-Danielson, 1994; Seeman and Van Tol, 1994). Among these receptors the D1 receptor isotype (D1R) mediates dopamine actions via the activation of adenylate cyclase (Gingrich and Caron, 1993; Sokoloff

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and Schwartz, 1995). The striatum, a major site for dopamine action, is densely innervated by dopamine fibers. Immunohistochemical analysis demonstrates that the cell bodies, dendrites, and spines of the postsynaptic striatal neurons display D1R primarily extrasynaptically located at the plasma membrane (Levey et al., 1993; Hersch et al., 1995; Caillé et al., 1996).

To understand the cellular response mediated by dopamine, we have searched whether the dopamine environment may influence the distribution and the localization of D1R in striatal neurons *in vivo* by using immunohistochemistry. We demonstrate here that the injection of the D1R agonist SKF-82958 and the release of dopamine induced by amphetamine provoke dramatic and acute modification of D1R localization, especially its internalization in the cytoplasm of cell bodies and dendrites via endocytosis. Our results demonstrate that modifications of the neurotransmitter environment can influence directly the intracellular trafficking of the corresponding receptor *in vivo*. They also bring morphological evidence that extrasynaptic D1R can react after ligand binding and can undergo internalization and recycling under direct stimulation.

MATERIALS AND METHODS

Animals and tissue preparation

Adult male Wistar rats (250–300 gm; Centre d'élevage Janvier, Le Genest St. Isle, France) were used in this study. They were maintained under standard housing conditions, and experiments were performed in accordance with the guidelines of the French Agriculture and Forestry Ministry (decree 87849, license 01499) and with the approval of Centre National de la Recherche Scientifique. They were treated either by the D1R full agonist SKF-82958 or by amphetamine that induces a massive release of dopamine via action on the dopamine transporter.

Intraperitoneal injection of SKF-82958. Rats ($n = 10$) received a single injection of SKF-82958 (2 mg/kg) (Research Biochemicals, Natick, MA) dissolved in 0.9 gm/l NaCl. After an appropriate survival time (20–60 min), the rats were anesthetized deeply with chloral hydrate and processed for immunohistochemical detection of D1R, as described below. Control experiments included normal rats maintained in the same housing conditions ($n = 2$), rats intraperitoneally injected with saline ($n = 3$; survival time, 40 min), rats having received an injection of the D1R antagonist SCH-23390 (0.5 mg/kg, Research Biochemicals, Natick, MA) dissolved in 20% acetic acid (10 mg/ml) and diluted (0.25 mg/ml) in 0.9 gm/l NaCl ($n = 4$; survival time, 40 min), and rats having received combined injections of SCH-23390 and SKF-82958 ($n = 5$; survival time, 40 min).

Intrastriatal injection of SKF-82958. Rats ($n = 12$) were anesthetized with urethane (1.15 gm/kg) and mounted in a stereotaxic frame. Glass micropipettes (tip external diameter, 30 μm) were filled through the tip by negative pressure and implanted into the dorsal striatum (bregma, + 1.2 mm; lateral, 2.0 mm; depth, 4.2 mm). SKF-82958 (1 mg/ml in saline: NaCl 9 gm/l, KCl 0.2 gm/l, and $CaCl_2$ 1.3 mM) was injected by air pressure (500 nl in 2 min). Animals were allowed to survive from 4 min to 5 hr after the end of the injection. Control injections of the same volume of vehicle solution were made simultaneously in the contralateral striatum.

Intraperitoneal injection of amphetamine. Rats ($n = 11$) received a single injection of 5 mg/kg D-amphetamine sulfate (Coopérative Pharmaceutique Française, Melun, France) and were allowed to survive from 20 min to 4 hr after the end of the injection. Control groups received saline injection ($n = 3$), SCH-23390 injection alone ($n = 4$), or a combined injection of SCH-23390 (0.5 mg/kg) and amphetamine ($n = 3$).

Tissue preparation. After an appropriate survival time, the animals were processed for tissue preparation. Rats were anesthetized deeply with chloral hydrate and perfused transcardially with 50–100 ml of 0.9% NaCl and 400 ml of fixative (2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed, stored in 2% paraformaldehyde overnight, and cut into 50 μm frontal sections with a vibratome. The sections were collected, cryoprotected in PBS solution (0.01 M, pH 7.4) containing 30% saccharose, and freeze-thawed in isopentane to improve the penetration of immunoreagents. Then the sections were preincubated for 1 hr in PBS with 0.2% BSAc (Aurion, the Netherlands) (PBS–BSAc); at room temperature before treatment with the D1R antiserum.

Immunohistochemical detection of D1R

D1R was detected at the light and electron microscopic level by using a polyclonal D1R antiserum produced and characterized as previously described (Caillé et al., 1995). This antiserum was generated against a fusion protein, including the C-terminal intracytoplasmic part of the receptor. Controls for specificity were performed in previous studies and in the present one. They demonstrated the specificity of the antiserum via Western blot and specific localization of the immunoreactivity in areas and neurons known specifically to express the D1R in rat and human, especially in good correlation with *in situ* hybridization studies and binding analysis (Caillé et al., 1995, 1996; Brana et al., 1996). The specificity of the immunolabelings also was attested by the absence of signal when primary antibody was omitted, when preimmune serum was used, and when D1R antiserum was preadsorbed with the fusion protein.

Detection of D1R on vibratome sections by immunoperoxidase method with tyramide signal amplification. Vibratome sections were incubated in D1R antiserum diluted 1:10,000–1:20,000 in PBS–BSAc for 15–48 hr at 4°C. Then the sections were washed (3× PBS) and incubated for 1 hr at room temperature in biotinylated goat anti-rabbit IgG (1:200 in PBS–BSAc, Amersham-UK, Little Chalfont, UK). After being washed (3× PBS), nonspecific binding sites were blocked by incubation for 30 min at room temperature in TNB (0.1 M Tris-HCl and 0.15 M NaCl) containing 0.5% DuPont blocking reagent (TSA indirect kit, DuPont NEN, Wilmington, DE) and incubated in streptavidin–horseradish peroxidase (SA–HRP) 1:500 in TNB for 30 min at room temperature. The sections were washed three times for 10 min each in TNT buffer (0.1 M Tris-HCl and 0.15 M NaCl with 0.05% Tween 20) and then incubated for 3–10 min in biotinyl tyramide (1:50 in amplification diluent) (TSA indirect kit, DuPont NEN). After being washed (3× TNT), the sections were incubated again with SA–HRP (1:500 in TNB) for 30 min at room temperature and rinsed again (3× TNT). Peroxidase activity was revealed with 3,3′-diaminobenzidine (DAB; 0.05% in Tris buffer, pH 7.6; Sigma, Poole, UK) in the presence of hydrogen peroxide (0.01%). The reaction was stopped by several washes in Tris buffer. The vibratome sections were mounted on glass slides, dehydrated in graded ethanols, and mounted in Eukitt for light microscopic observation.

Detection of D1R in semithin sections with the avidin–biotin method. Vibratome sections were immunostained by the avidin–biotin peroxidase method (ABC; Vectastain-Elite, Vector Laboratories, Burlingame, CA), as previously described (Caillé et al., 1995). The sections were incubated for 48 hr at 4°C with D1R antiserum diluted 1:1000 in PBS–BSAc. After being washed (3× PBS), they were incubated for 1 hr at room temperature in biotinylated goat anti-rabbit IgG (1:200 in PBS–BSAc). After three rinses in PBS, sections were incubated in the ABC complex (0.5% in PBS) for 1 hr. The D1R immunoreactive sites were revealed by incubation in H_2O_2–DAB solution, as described above. After several washes in Tris buffer, selected areas of interest (dorsal striatum) were post-fixed in osmium tetroxide (1% in PBS) for 30 min, washed in PBS, dehydrated in graded ethanol, and transferred to propylene oxide. The sections were preimpregnated with a 1:1 mixture of Araldite and propylene oxide for 1 hr, impregnated with Araldite overnight, and flat-embedded in Araldite. Semithin sections (1–2 μm) were cut with a Reichert ultracut S (Leica, Nusslock, Germany), collected on glass slides, dried, and mounted in Eukitt.

Detection of D1R by immunogold method at the ultrastructural level. Vibratome sections were incubated in D1R antiserum, as described in the immunoperoxidase procedure. After being washed twice in PBS–BSAc and twice in PBS–BSAc supplemented with 0.1% fish gelatin (Aurion) (PBS–BSAc–gelatin), sections were incubated for 24 hr at room temperature in goat anti-rabbit IgG conjugated to ultrasmall colloidal gold particles (0.8 nm; Aurion) diluted in PBS–BSAc–gelatin (1:50). After several washes (3× PBS–BSAc–gelatin, 3× PBS, and 3× 2% sodium acetate), the immunogold signal was intensified by using a silver enhancement kit (Aurion). The reaction was performed in the dark for 15–30 min at room temperature and stopped by two washes in 2% sodium acetate. Then the signal was intensified and stabilized (Trembleau et al., 1994) by immersion of the sections in gold chloride (0.05% in distilled water) for 10 min at 4°C and then in sodium thiosulfate (0.3% in distilled water, two times for 10 min at 4°C). After several washes in PBS, the sections were processed for electron microscopy. They were post-fixed in osmium tetroxide and embedded in Araldite, as described above. Ultrathin sections of immunogold-treated material were cut. They were

collected on copper grids, contrasted with uranyl acetate and lead citrate, and observed with a Phillips CM 10 electron microscope (Phillips Electronic Instruments, Mahwah, NJ).

Quantitative analysis of variations of D1R immunoreactivity. Variations in D1R immunoreactivity were measured at the ultrastructural level by using plates of immunolabeled cell bodies and dendritic shafts of control and SKF-82958-treated rats. Morphometric analysis was performed by using Metamorph software (Universal Imaging, Paris, France). The measures were performed in sections from control animals ($n = 3$; 10 cell bodies and 26 dendrites), from animals ($n = 3$) having received systemic injection of SKF-82958 (19 cell bodies and 20 dendrites), and from animals ($n = 3$) having received intrastriatal injection of SKF-82958 (19 cell bodies and 20 dendrites). Gold immunoparticles present at the plasma membrane were identified and counted, and the results were expressed as the number of immunoparticles per 100 μm of membrane in dendrites and cell bodies (length of analyzed membrane was ~1500 μm for cell bodies and 1000 μm for dendrites for each group). Immunopositive endosomes and endocytic vesicles were identified and counted in the cytoplasm, and the results were expressed as the number of endosomes per 100 μm^2 (surface of analyzed cytoplasm was ~12,000 μm^2 for cell bodies and 2000 μm^2 for dendrites for each group). Details of analyses and measures are described in Figure 6.

RESULTS

Localization of D1R immunoreactivity in normal animals

The analysis of D1R immunoreactivity in normal animals and in animals injected with vehicle solution demonstrated the presence of D1R at the membrane of the cell bodies and dendrites of striatal neurons, as previously described (Caillé et al., 1996). An overview of the immunolabeling in 50-μm-thick vibratome sections demonstrated an intense and homogeneous labeling of the neuropile in the striatum with few, faintly immunoreactive cell bodies (Fig. 1*A,B*). The labeling generally appeared more intense in the nucleus accumbens. The detailed analysis of vibratome and of semithin sections obtained after embedding in Araldite (Fig. 1*B,C*) demonstrated that the large majority of immunoreactivity was located at the membrane of the dendrites and cell bodies. Such localization was confirmed by ultrastructural examination that showed immunoreactivity as gold particles located at the inner side of the plasma membrane of the cell bodies, dendrites, and spines (Fig. 1*D,E*). The large majority of the immunoreactivity appeared extrasynaptically located. In spines, gold particles generally were located at the edge of asymmetrical synapses. Intracytoplasmic immunoreactivity was present in low abundance; it was restricted mostly to a few vesicles with the morphological features of endosomes. The endoplasmic reticulum cisternae and the Golgi apparatus contained very little immunoreactivity.

Localization of D1R immunoreactivity after intraperitoneal injection of SKF-82958

Injection of SKF-82958 provoked dramatic modifications of the aspect and localization of D1R immunoreactivity. Vibratome sections showed heterogeneous immunolabeling, with a stronger signal in areas that displayed the aspect and location of striosomes (Fig. 2*A*), such as the pericallosal striosome. Numerous cell bodies present throughout the dorsal and ventral striatum were intensely immunoreactive in the cytoplasm and the proximal dendrites (Fig. 2*B–E*). Higher signal in the areas that may correspond to the striosomes was attributable to a higher density of immunoreactive fibers and cell bodies (Fig. 2*A*). Cell bodies were also numerous and intensely reactive in the nucleus accumbens. The analysis of semithin sections showed that the appearance of immunoreactive cell bodies was attributable to an accumulation of intracytoplasmic deposits with D1R immunoreactivity, with a sharp decrease or a disappearance of the immunolabeling located at the membrane of the neurons (Fig. 2*B*). Similar aspects were also visible in proximal dendrites, as compared with normal rats (Fig. 2*B*). The ultrastructural analysis confirmed this pattern and demonstrated that the intracytoplasmic immunoreactivity appeared to be associated mostly with the cytoplasmic side of membranes, limiting vacuoles that displayed morphological features of the endosomal compartment (Figs. 3, 4*A,B*). These endosomes had homogenous and clear content, had a vesicular or tubulovesicular aspect, and were limited by an irregular membrane. They appeared to be located frequently at the periphery of the cytoplasm at the vicinity of the plasma membrane (Figs. 3*B–D*, 4). Occasionally, the presence of endocytic vesicles associated with the plasma membrane was detected in cell bodies and dendrites (Fig. 3*C,D*). Such modifications were observed at all times in the largest part of immunoreactive neurons, with a maximal effect at 20 and 40 min (Fig. 2*C–E*). Electron dense vacuoles that may correspond to lysosomes did not display immunoreactivity. Golgi apparatus and endoplasmic reticulum did not show modifications, as compared with normal rats. The examination of dendritic spines suggested that there was no modification of the immunolabeling for the receptors located at the periphery of synaptic clefts. Quantification of D1R immunoreactivity at the ultrastructural level confirmed the sharp modifications of the receptor compartmentation in the cell bodies and dendrites (see Fig. 6). Detailed counting showed that there was an important decline in plasma membrane immunoreactivity (Fig. 6*A*; 3.3 times and 3.5 times less in cell bodies and dendrites, respectively) and a parallel increase in the density of endosomal structures bearing D1R immunoreactivity in the cytoplasm (Fig. 6*B*; 4.9 times and 3.9 times more in cell bodies and dendrites, respectively). Control (saline-injected) animals and animals injected with the D1 antagonist SCH-23390 did not demonstrate modifications, as compared with normal rats (data not shown). Simultaneous injection of SCH-23390 with SKF-82958 sharply decreased or abolished the modifications induced by the injection of SKF-82958 alone (see Fig. 2*F*).

Localization of D1R immunoreactivity after intrastriatal injection of SKF-82958

Immunohistochemical analysis performed after direct intrastriatal injection of SKF-82958 demonstrated the same general features as those that occurred after intraperitoneal injections (Fig. 5). Careful examination of the sections showed that the neurons and dendrites located at the vicinity of the site of injection appeared well preserved and showed changes in the localization of the D1R immunoreactivity at the light and electron microscopic level identical to those observed after intraperitoneal injection (Figs. 4*C*, 5*A–F*). These immunoreactive neurons were detectable as early as 4 min after injection and were very intensely reactive 10, 30, and 60 min after injection (Fig. 5*A,B*). Intracytoplasmic localization of the receptor was still detectable after 5 hr, but with a lower intensity (Fig. 5*C*). Quantification at the ultrastructural level also confirmed modifications of D1R distribution that paralleled the ones observed after intraperitoneal injection of SKF-82958 (3.5 times and 3.9 times less immunopositive gold particles at the plasma membrane of the cell bodies and dendrites, respectively; 5.7 and 4.8 times more endosomal structures bearing D1R immunoreactivity in the cell bodies and dendrites, respectively) (Fig. 6). The injection of the vehicle solution alone in the

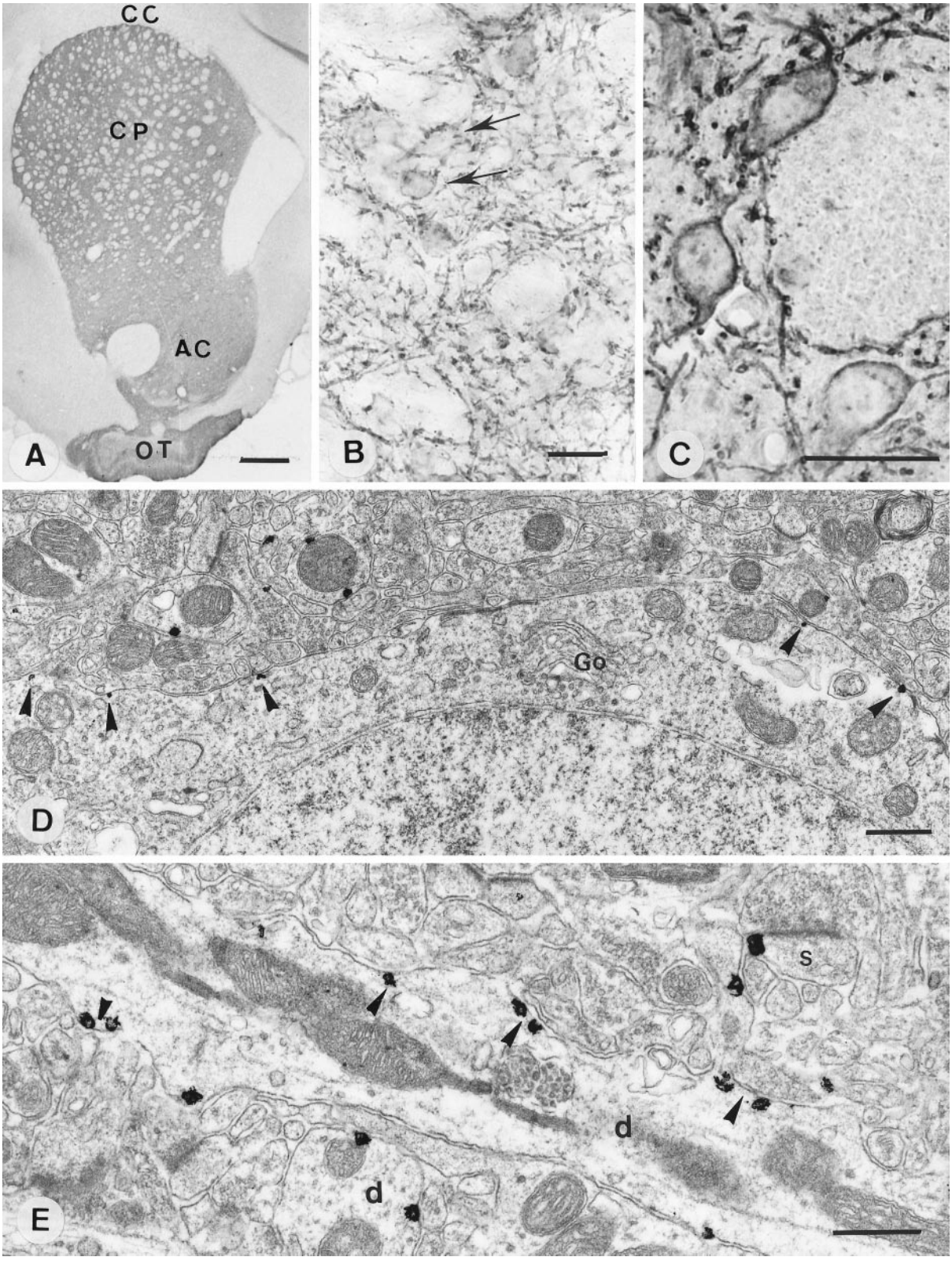

Figure 1. Immunohistochemical detection of D1R in normal rats: light and electron microscopy. *A* shows a general view of the striatum (vibratome section). Immunoreactivity is distributed homogeneously in the neuropile throughout the dorsal and the ventral striatum. *B* and *C* show high magnification in vibratome (*B*) and semithin (*C*) sections. The labeling is located essentially along the membrane of the cell bodies (*arrows*) and in the neuropile as a thin deposit. *D*, *E*, Electron microscopy after the immunogold technique. *D* shows part of an immunoreactive cell body. D1R immunoreactivity is located as gold particles dispersed along the plasma membrane (*arrowheads*); note the absence of intracytoplasmic staining in this neuron. *E* shows a sagittal section of a dendritic shaft with gold particles restricted to the plasma membrane (*arrowheads*). *CC*, Corpus callosum; *CP*, caudate putamen nucleus; *AC*, accumbens nucleus; *OT*, olfactory tubercle; *Go*, Golgi apparatus; *d*, dendrite; *s*, spine. Scale bars: *A*, 250 μm; *B*, *C*, 20 μm; *D*, *E*, 0.5 μm.

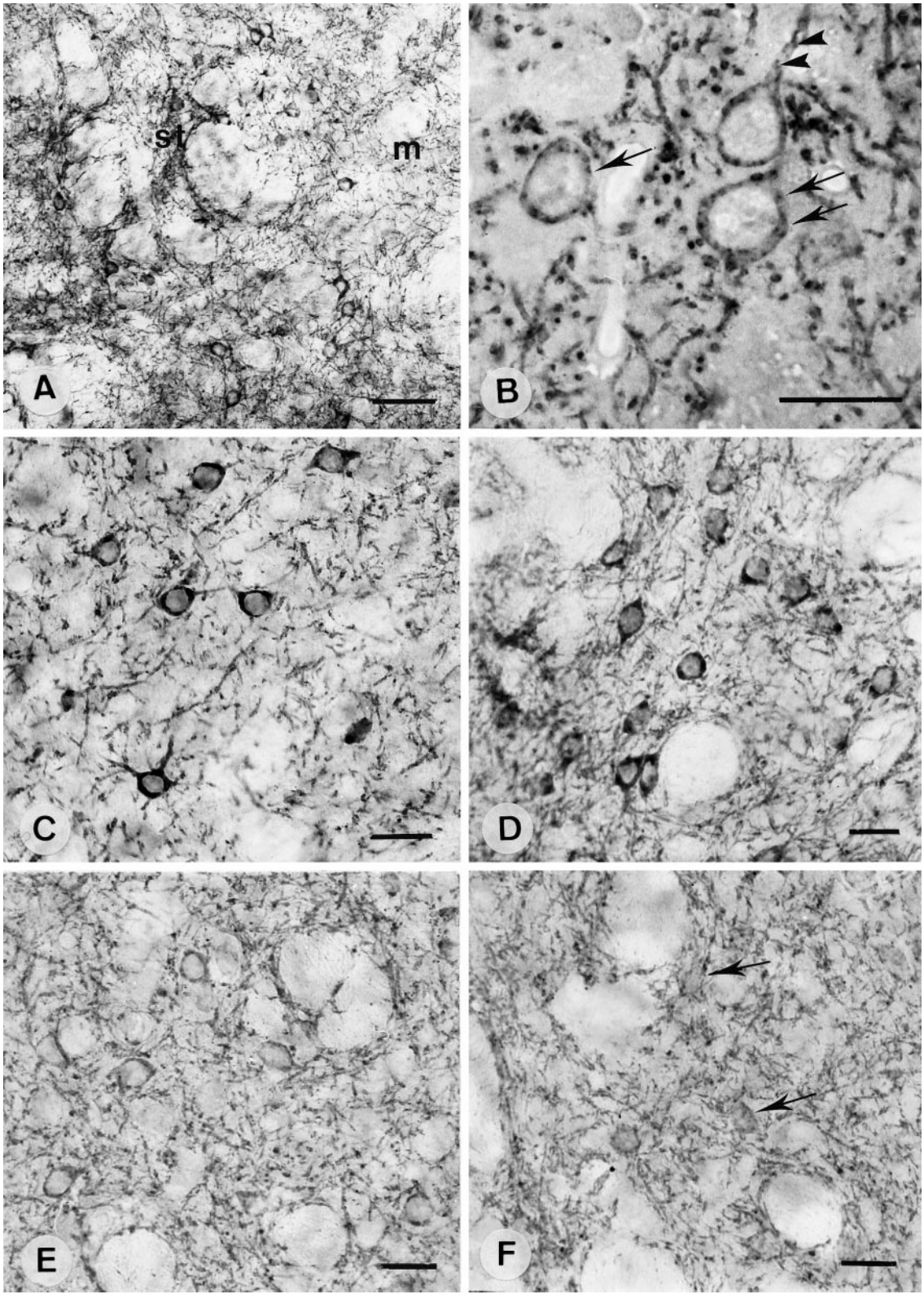

Figure 2. Immunohistochemical detection of D1R after intraperitoneal injection of SKF-82958: light microscopy. *A* and *B* show details of the striatum 40 min after injection. In *A* (vibratome section), the neuropile labeling is heterogeneous, and numerous intensely immunoreactive cell bodies are present. Areas of high signal intensity may correspond to striosomes. In *B* (semithin section), the neuronal immunoreactivity appears mostly as an accumulation of dots located in the cytoplasm (*arrows*). The *arrowheads* point to immunoreactivity inside a dendrite. *C–E* show the striatum at 20 and 40 min and at 1 hr after injection (vibratome sections). The immunoreactive neurons display intense labeling in the cytoplasm and proximal dendrites in *C* and *D*. *E* shows that the signal has decreased after 1 hr. Immunoreactivity of the neuropile is less intense in *C–E* than in the normal or saline-injected rat. *F* (vibratome section) shows the striatum after a combined injection of D1R antagonist SCH-23390 and SKF-82958. The labeling appears identical to that observed in the normal or saline-injected rat in Figure 1*B* (*arrows*). *st*, Striosome; *m*, matrix. Scale bars in *A–F*, 20 μm.

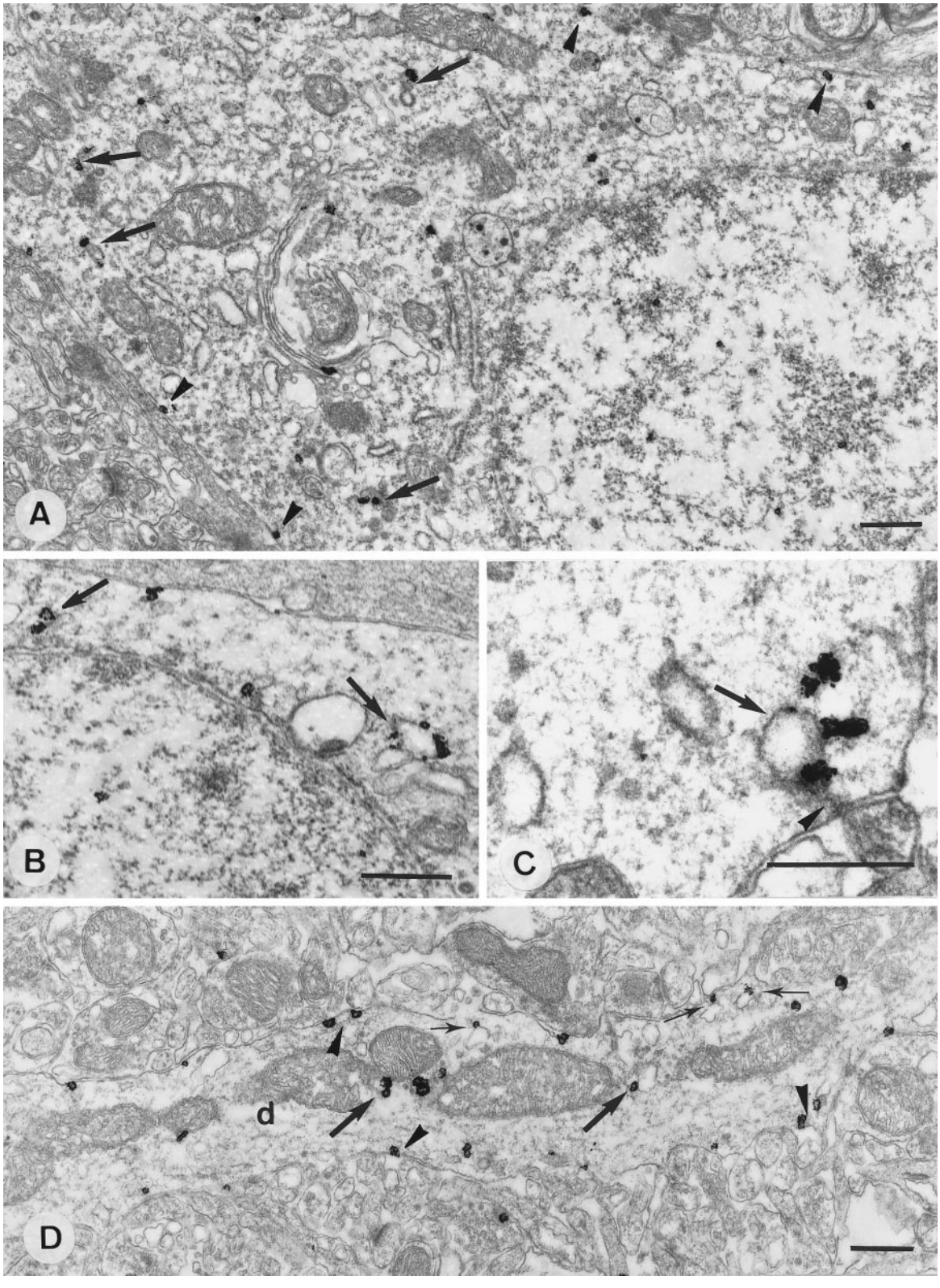

Figure 3. Immunohistochemical detection of D1R after intraperitoneal injection of SKF-82958 (40 min): electron microscopy after immunogold technique. *A* shows detail of a cell body. Part of the immunoreactivity is still at the plasma membrane (*arrowheads*), but there is an accumulation of gold particles in the cytoplasm (*arrows*). *B* and *C* show details of the cytoplasmic labeling; it is restricted mostly at the periphery of vesicles that have morphological features of endosomes inside the cytoplasm (*arrows* in *B*) or of endocytic vesicles (*arrow* in *C*) located at the immediate vicinity of the plasma membrane; the *arrowhead* in *C* points to the neck linking the plasma membrane to the endocytic vesicle. *D* shows a dendrite. Part of the immunoreactive material is located at the membrane (*arrowheads*), but many gold particles are present inside the dendritic shaft (*large arrows*). The *thin arrows* point to endocytic vesicles containing DR1 immunoreactivity at the vicinity of the plasma membrane. *d*, Dendrite. Scale bars, 0.5 μm.

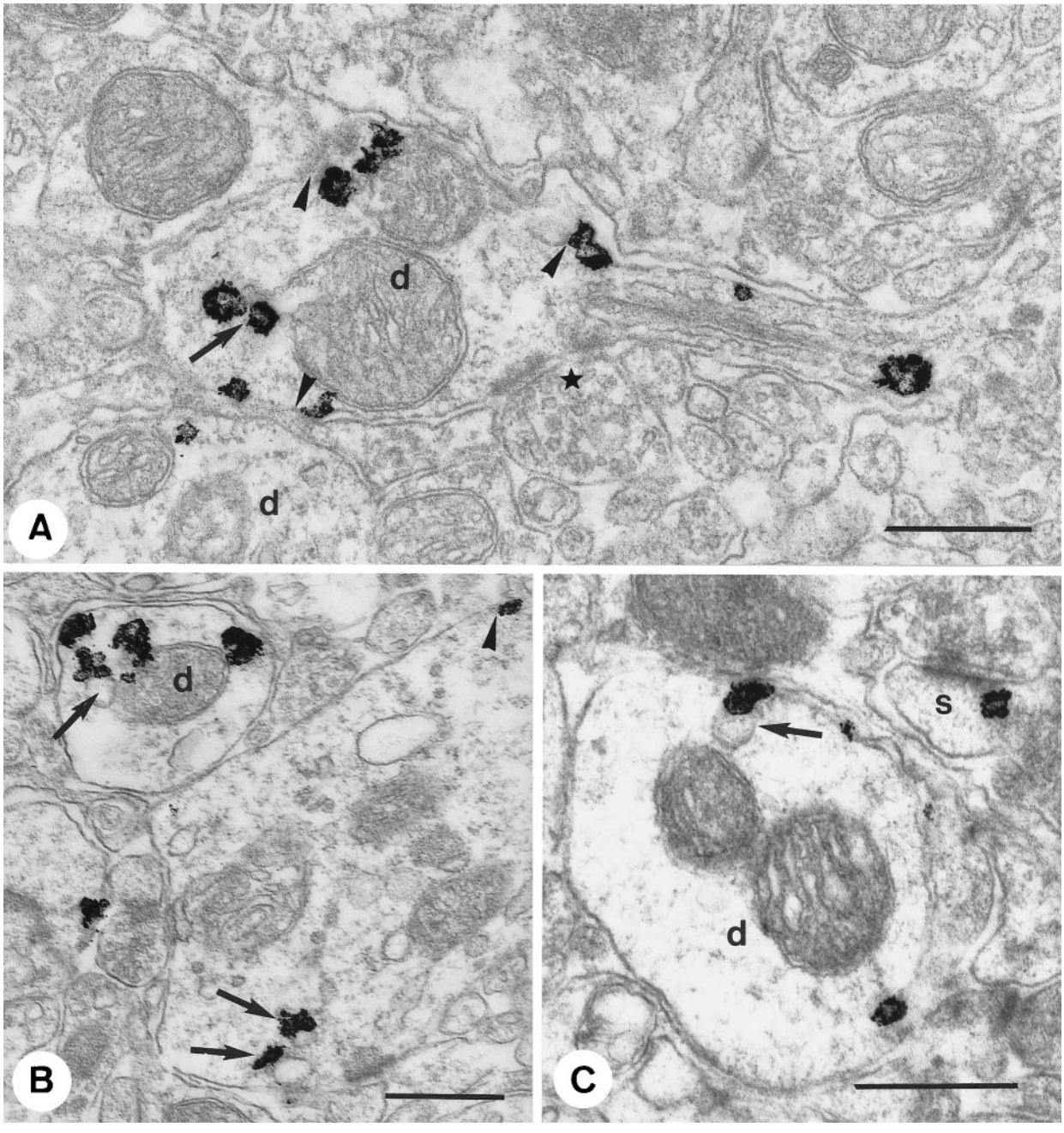

Figure 4. Immunohistochemical detection of D1R after injection of SKF-82958: electron microscopy after immunogold technique and details of dendrites. *A* shows the transversal section of a dendrite with a dendritic spine, with a synapse (*star*) located at the neck of the spine. Sections demonstrate various aspects of the receptor at the membrane (*arrowheads*) and of the internalization of the receptor with especially early phases of the formation of the endocytic vesicles and endosomes in *B* and *C* (*arrows*). *A*, At 40 min after intraperitoneal injection. *B*, At 40 min after intraperitoneal injection. *C*, At 10 min after intrastriatal injection. *d*, Dendrite; s, dendritic spine. Scale bars, 0.5 μm.

contralateral striatum did not provoke any modification of D1R immunoreactivity (data not shown).

Localization of D1R immunoreactivity after intraperitoneal injection of amphetamine

Intraperitoneal injection of amphetamine provoked modifications of D1R immunoreactivity at the light and ultrastructural level that appeared similar to those previously described after the injection of SKF-82958 (Fig. 7). These modifications were maximal 20 and 40 min after injection (Fig. 7*C,D*) and were still detectable after 90 min with a lower intensity (Fig. 7*E*). Immunoreactivity after 4 hr was the same as for the controls. Injection of SCH-23390, together with amphetamine, strongly reduced or abolished modifications of the immunolabeling (data not shown).

DISCUSSION

Analysis of the modifications of D1R distribution after DR stimulation

The results of the present study demonstrate that D1R naturally expressed at the surface of dopaminoceptive neurons in the CNS can undergo regulated internalization and sequestration in the

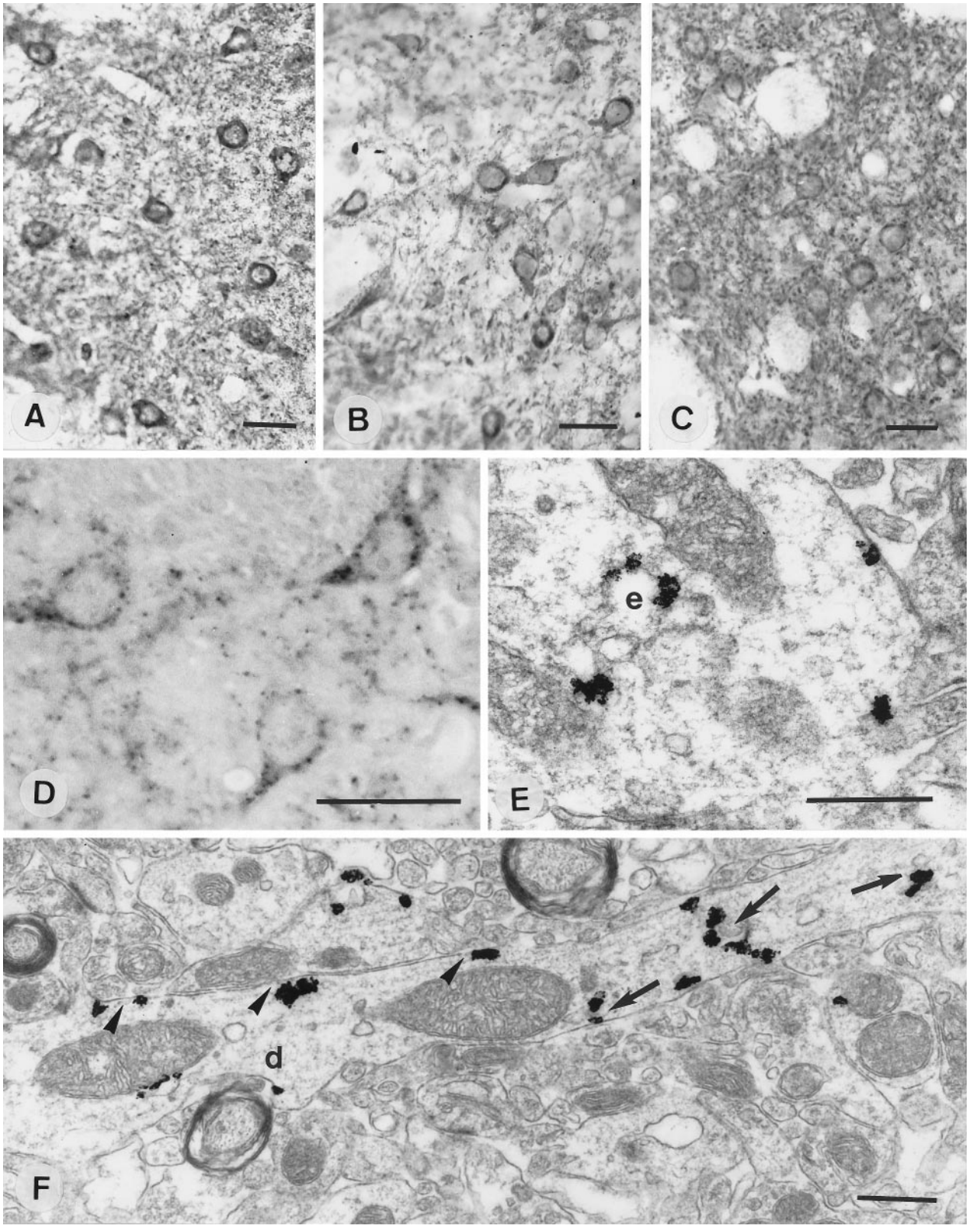

Figure 5. Immunohistochemical detection of D1R after intrastriatal injection of SKF-82958. *A–C* (vibratome sections) show the time course of the D1R immunoreactivity at 30 min (*A*), 1 hr (*B*), and 5 hr (*C*) after SKF-82958 injection. All views have been taken at the same distance from the injection site. The neurons are highly labeled at short times and stay immunoreactive after 5 hr. *D* shows details of neurons in semithin section with immunoreactivity internalized in the cytoplasm (10 min after injection). *E*, *F*, Electron microscopy after immunogold technique demonstrates the presence of immunoreactive endosomes in a cell body (*E*) and in a dendrite (*F*, *arrows*) at 10 min after injection. *e*, Endosome; *d*, dendrite. Scale bars: *A–D*, 20 μm; *E, F*, 0.5 μm.

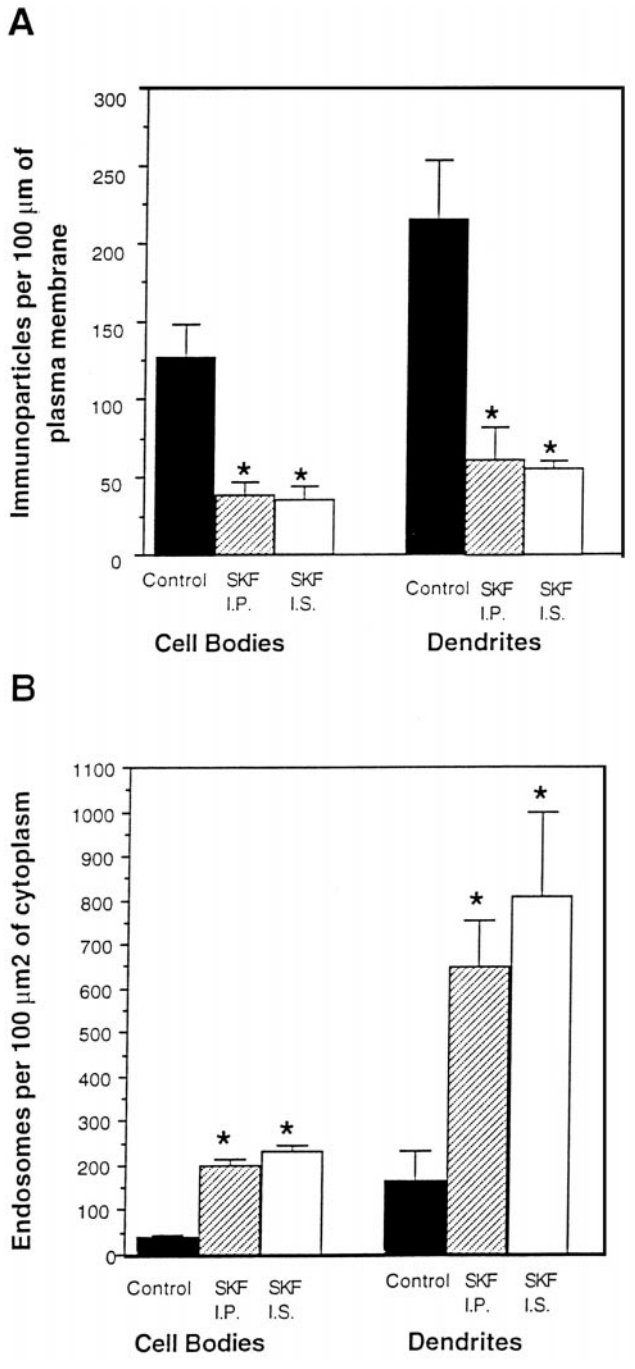

Figure 6. Quantitative analysis of the variations of D1R immunoreactivity in control and SKF-82958-treated rats. Immunoreactive particles and endosomes were counted on micrographs after immunodetection of D1R at the ultrastructural level in control rats, in rats having received intraperitoneal (*I.P.*) injection of SKF-82958 (40 min), and in rats having received intrastriatal (*I.S.*) injection of SKF-82958 (10 min). *A*, Immunoreactive particles (as visible in Figs. 1*D,E*, 3*A*) were counted. *Columns* in *A* correspond to the number of immunoparticles per 100 μm of plasma membrane in cell bodies and dendrites ±SEM. The number of immunoparticles strongly decreases after SKF-82958 injection in cell bodies and dendrites. *B*, Immunoreactive endosomes and endocytic vesicles (as visible in Figs. 3*B–D*, 5*E,F*) were counted. *Columns* in *B* correspond to their density per 100 μm^2 of cytoplasm in cell bodies and dendrites ±SEM. The injection of SKF increases the density of immunoreactive endosomes or endocytic vesicles in cell bodies and dendrites ($^*p < 0.05$; Mann–Whitney nonparametric test).

cytoplasm after *in vivo* activation. Our data show that the same effects are obtained by using a locally or intraperitoneally injected agonist that activates only D1R or by provoking a massive release of endogenous dopamine with amphetamine that activates all dopamine receptor subtypes in the striatum. Light microscopy studies show that short-term action of these molecules dramatically and massively alters the localization and distribution of the D1R immunoreactivity, whatever the treatment. Counting of D1R immunoreactivity shows that D1R immunoreactivity present at the surface of plasma membrane in cell bodies and dendrites of normal rats dramatically drops after intraperitoneal or intrastriatal injection (see Fig. 6). Similarly, the density of endosomes bearing D1R immunoreactivity highly increases in each experimental situation both in cell bodies and dendrites. These modifications of the ultrastructural aspect of the neurons demonstrate that the decrease in plasma membrane immunoreactivity is associated with the increase of the immunoreactive intracytoplasmic vesicular compartment that displays all of the morphological features of the endosomal compartment (Nixon and Cataldo, 1995; Koenig and Edwardson, 1997), especially a clear content, irregular tubulovesicular aspect, and formation of endocytic vesicles from the plasma membrane. Such features correlate with detailed morphological and molecular *in vitro* studies, which demonstrate that stimulation of G-protein-coupled receptors causes a dramatic reorganization of their intracellular distribution, including endocytosis and formation of endosomes (for review, see Koenig and Edwardson, 1997). The translocation of the D1R from the plasma membrane to intracytoplasmic vesicles occurs in the cell bodies and dendrites as also demonstrated *in vivo* for substance P, neurotensin, or opioid receptors (Faure et al., 1995; Mantyh et al., 1995a,b; Sternini et al., 1996). As expected from the *in vitro* mechanisms of the formation of endosomes containing G-protein-coupled receptors (Fonseca et al., 1995; Roettger et al., 1995; Goodman et al., 1996; Krueger et al., 1997), the D1R immunoreactivity (corresponding to the detection of an intracytoplasmic C-terminal region of the molecule; Caillé et al., 1995) is present at the inner side of the plasma membrane in normal animals and is translocated at the membranes of these vesicles in stimulated animals, appearing frequently associated with their cytoplasmic side. Indeed, in the absence of appropriate detection of markers for the various intracytoplasmic compartments, the detailed route of G-protein-coupled receptors endocytosed *in vivo* can be hypothesized only and must await detailed molecular analysis, coupled with immunohistochemical receptor detection. Especially, the present ultrastructural data did not allow us to establish whether these vesicles were associated with clathrin. Nevertheless, identical ultrastructural features strongly suggest that, *in vivo*, the receptors are internalized in vesicles and transferred in the endosomal compartment. The absence of a significant amount of D1R in other vesicular compartments, especially in lysosomes, favors the hypothesis of a recycling of D1R at the plasma membrane after internalization rather than an intracellular degradation. This is supported by the fact that the morphological studies show that D1R immunoreactivity returns to the membrane after stimulation by SKF-82958 or amphetamine. The persistence of a residual intracytoplasmic immunoreactivity 5 hr after intrastriatal injection of SKF-82958 nevertheless suggests that some of the receptor may have a specific intracellular metabolism after internalization. The absence of detectable variations in the aspect and abundance of D1R in the endoplasmic reticulum and Golgi apparatus after stimulation suggests that, under our experimental conditions,

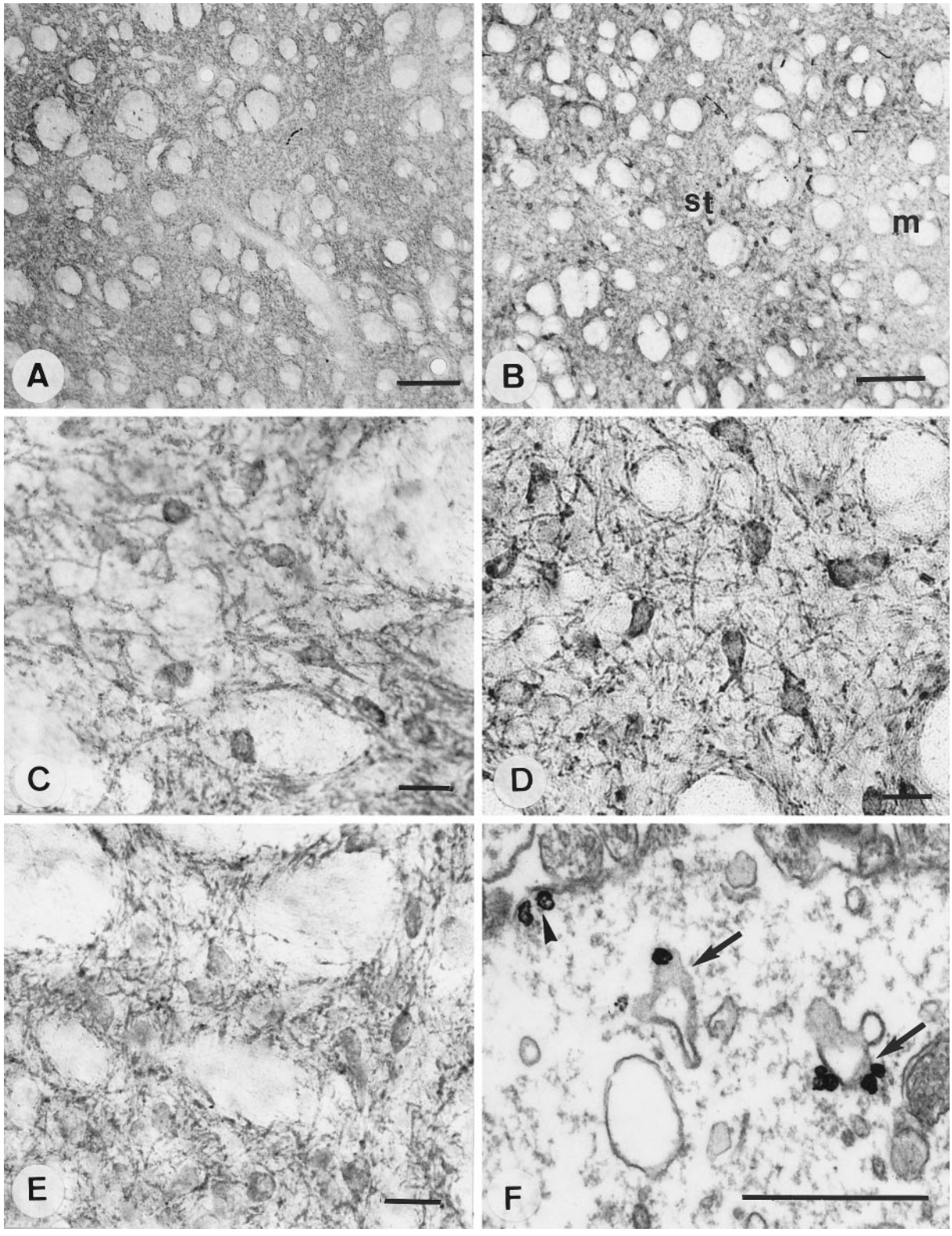

Figure 7. Immunohistochemical detection of D1R after intraperitoneal injection of amphetamine. *A* and *B* show a general view of the dorsal striatum after injection of saline (*A*) or amphetamine (40 min; *B*). As compared with *A*, *B* shows the appearance of heterogeneous labeling with higher signal in areas that may correspond to the striosomes and the presence of numerous cell bodies (vibratome sections). *C–E* show aspects of the immunoreactive cell bodies at 20 min (*C*), 40 min (*D*), and 90 min (*E*) after injection (vibratome section). *F* shows detail of immunoreactive endosomal compartment (*arrow*) in a cell body at the ultrastructural level after immunogold technique. The *arrowhead* points to the receptor at the membrane. *st*, Striosome; *m*, matrix. Scale bars: *A*, *B*, 100 μm; *C–E*, 20 μm; *F*, 0.5 μm.

D1R neosynthesis would not contribute significantly to modifications of D1R compartmentation after stimulation. Nevertheless, we cannot exclude that other modes of stimulation, other time courses, or more detailed ultrastructural study may reveal other cytoplasmic pathways for D1R, including a degradation in lysosomes, as suggested by several *in vitro* studies (Raposo et al., 1989; Nixon and Cataldo, 1995; Roettger et al., 1995). The *in vivo* formation of endosomes triggered by the stimulation of G-protein-coupled receptors is in good correlation with the *in vitro* models showing similar features for D1R, adrenergic, or peptidergic receptors (Fonseca et al., 1995; Ng et al., 1995; Roettger et al., 1995; Trogadis et al., 1995) and with the recent observation of *in vivo* substance P receptor internalization after agonist injection (Mantyh et al., 1995b) or glutamate receptor stimulation (Lin et al., 1997). Nevertheless, in contrast to the observations of Mantyh and colleagues showing the reshaping of dendrites bearing stimulated substance P receptors, we did not observe modifications of the aspects of the dendrites of the dopaminoceptive neurons.

The D1R present at the surface of striatal neurons are mainly extrasynaptic, as demonstrated in previous studies (Hersch et al., 1995; Yung et al., 1995; Caillé et al., 1996). Our results demonstrate that nonsynaptic receptors can respond to *in vivo* stimulation by internalization. It can be expected from these data that the binding of agonists on these nonsynaptic receptors also triggers the cascade of transduction for D1R, including activation of adenylate cyclase. Our data then reinforce the hypothesis that dopamine may act in the striatum on nonsynaptic receptors via diffusion at a distance from release sites (Garris et al., 1994). This also indicates that these nonsynaptic receptors can be, *in vivo*, direct or indirect targets for drugs interacting with dopamine transmission (such as direct dopamine agonists) or for psychostimulants, such as cocaine and amphetamine. The apparent absence of modification of the localization of perisynaptic receptors located in dendritic spines may reflect a distinct metabolism of these receptors or a different time course for internalization. Depending on the availability of efficient antisera, we may explore whether the other dopamine receptors present in the striatum also display modifications of their localization after stimulation.

Functional significance of in vivo internalization

Our data show that localization and subcellular distribution of receptors mediating the effects of a fast-acting neurotransmitter, dopamine, can be altered acutely and dramatically *in vivo* by modifications of the environment of the neuron. The functional significance of these events can be considered on the basis of the previous *in vitro* studies, which show that the internalization of receptors in endocytic vesicles and endosomes is an early event leading to the desensitization of the receptor via phosphorylation and sequestration in the cytoplasm (Lefkowitz and Caron, 1993; Krueger et al., 1997). Although endocytosis and desensitization processes can be dissociated *in vitro* (Ng et al., 1995), they appear to be associated closely in the cascade of cellular and molecular events after *in vitro* agonist stimulation of a G-protein receptor (Lefkowitz and Caron, 1993; Zhang et al., 1998). Our results strongly suggest that modifications of the subcellular distribution of neurotransmitter receptors also might, *in vivo*, be a critical and early element of the postsynaptic response because it is detectable as early as 4 min after agonist injection. This endocytosis may limit or modify the access of a natural or artificial ligand to its receptor and also may contribute to receptor resensitization or downregulation (Zhang et al., 1998). These *in vivo* results must encourage us to investigate experimental and pathological conditions involving dopamine to determine whether modifications of dopamine receptor distribution and localization may contribute to the alteration of dopamine transmission in acute, but also in chronic, situations. It is known that acute and chronic behavioral effects of psychostimulants, such as amphetamine, are linked closely to the early induction of the *c-fos* gene and modifications of the expression of neuropeptide genes in striatal neurons (Young et al., 1991; Cole et al., 1995; Jaber et al., 1995). Most of these mechanisms are mediated via the activation of D1R located on the striatal dopaminoceptive neurons (Young et al., 1991; Drago et al., 1996). These data suggest that the internalization of D1R may be considered not only as an early evidence but also as a major actor of the postsynaptic response. Indeed, these receptors most probably are internalized with their natural or artificial ligands, as demonstrated *in vitro* (Koenig and Edwardson, 1997), and it can be hypothesized that internalization may contribute to regulate receptor availability for extracellular ligands. This internalization is distributed heterogeneously in the striatum, with higher intensity in areas that display the localization and aspect of striosomes. Technical limitations did not allow us to demonstrate unambiguously in this study that heterogeneity in D1R immunoreactivity after SKF-82958 and amphetamine stimulation overlaps with patch/matrix delineation. Nevertheless, the general aspect of the striatum after stimulation strongly favors this hypothesis. These data correlate with the fact that amphetamine injection induces under the same conditions the appearance of c-Fos immunoreactivity (Graybiel et al., 1990), preferentially in the striosome compartment.

Conclusion

Our present data, as well as those described by others, demonstrate acute and massive internalization of postsynaptic neurotransmitter receptors *in vivo* after stimulation (Faure et al., 1995; Mantyh et al., 1995a,b; Sternini et al., 1996; Lin et al., 1997). The present results are the first obtained for a fast-acting classical neurotransmitter and bring new evidence for this concept. The results reinforce the hypothesis that acute or chronic modification of neurotransmitter receptor subcellular distribution may constitute a common event contributing *in vivo* to the postsynaptic response in the CNS under normal, experimental, and pathological conditions.

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