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Immunogold Localization of the Dopamine Transporter: An Ultrastructural Study of the Rat Ventral Tegmental Area

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The dopamine transporter (DAT) plays an important role in the plasmalemmal reuptake of dopamine and, thus, in the termination of normal dopaminergic neurotransmission. DAT is also a major binding site for cocaine and other stimulants, the psychoactive effects of which are associated primarily with the inhibition of dopamine reuptake within mesocorticolimbic dopaminergic neurons. We used electron microscopy with an anti-peptide antiserum directed against the N-terminal domain of DAT to determine the subcellular localization of this transporter in the rat ventral tegmental area (VTA), the region that contains the cell bodies and dendrites of these dopaminergic neurons. We show that in the VTA, almost 95% of the DAT immunogold-labeled profiles are neuronal perikarya and dendrites, and the remainder are unmyelinated axons. Within perikarya and large proximal dendrites, almost all of the DAT immunogold particles are associated with intracellular membranes, including saccules of Golgi and cytoplasmic tubulovesicles. In contrast, within medium- to small-diameter dendrites and unmyelinated axons, most of the DAT gold particles are located on plasma membranes. In dually labeled tissue, peroxidase reaction product for the catecholamine-synthesizing enzyme tyrosine hydroxylase is present in DAT-immunoreactive profiles. These findings suggest that intermediate and distal dendrites are both the primary sites of dopamine reuptake and the principal targets of cocaine and related psychostimulants within dopaminergic neurons in the VTA.

Key words: dopamine; transporter; uptake; ultrastructure; ventral tegmental area; midbrain; mesocorticolimbic; electron microscopy; immunogold; dendritic release; plasma membrane; cocaine; amphetamine; neurotoxicity

Dopaminergic transmission is terminated primarily by the active uptake of dopamine by a specific sodium- and chloride-dependent plasmalemmal dopamine transporter (DAT) (Horn, 1990; Boja et al., 1994; Giros et al., 1996). DAT is also the site of entry of parkinsonism-inducing neurotoxins into dopaminergic neurons (Edwards, 1993; Uhl and Kitayama, 1993), a target of therapeutic agents such as methylphenidate (Nomikos et al., 1990), and the principal "receptor" involved in the reinforcing and addicting properties of cocaine and other drugs of abuse (Ritz et al., 1987; Kuhar et al., 1991; Giros et al., 1996). In particular, the self-administration of psychostimulants has been associated with blockade of DAT within mesocorticolimbic dopaminergic neurons (Koob and Bloom, 1988). These neurons are known to play a critical role in the central regulation of motor and motivational functions (for review, see Le Moal, 1995).

Although most studies of mesocorticolimbic dopaminergic neurons have focused on their striatal and cortical terminals, dopamine is also known to be released from the somata and/or dendrites of these neurons (Bradberry and Roth, 1989; Kalivas et al., 1989; Kalivas and Duffy, 1991; Rice et al., 1994), which are located in the ventral tegmental area (VTA). Accordingly, several lines of evidence have shown that there is functional expression of DAT in this region. Autoradiographic uptake experiments, for example, have demonstrated the presence of sodium-dependent dopamine uptake in the VTA that is sensitive to DAT inhibitors (Beart et al., 1979; Beart and McDonald, 1980). In addition, the local application of DAT-inhibiting drugs in the VTA has been shown to increase extracellular levels of dopamine (Kalivas and Duffy, 1991; Chen and Reith, 1994), leading to inhibition of dopaminergic cell firing in this region (Einhorn et al., 1988). Together, these observations suggest that somatodendritic dopamine transporters in the VTA may play a role in both normal dopaminergic transmission and the responsiveness to cocaine and other stimulants (for review, see Chen and Reith, 1997).

In spite of the evidence for functional dopamine transporters in the VTA, the subcellular localization of DAT in this region has not yet been examined. Experiments using ligand-binding autoradiography (Javitch et al., 1985; Dawson et al., 1986; Mennicken et al., 1992) and light microscopic immunocytochemistry (Ciliax et al., 1995; Freed et al., 1995) have shown that DAT is present in the VTA, but do not provide sufficient resolution to distinguish between plasmalemmal and intracellular sites of DAT expression. In the present study, we therefore used higher resolution electron microscopic immunogold techniques to determine the ultrastructural localization of DAT in the rat VTA. We confirmed the identity of the dopaminergic neurons by dual labeling with immu-

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nogold for DAT and immunoperoxidase for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH), a marker for dopaminergic somata and dendrites in this region (Pickel and Sesack, 1995). As a result, we identify the potential sites of functional expression of DAT within the cell bodies and dendrites of mesocorticolimbic dopaminergic neurons.

MATERIALS AND METHODS

Tissue preparation. The methods for tissue preparation and immunocytochemical labeling were based on those of Leranth and Pickel (1989) as described previously (Nirenberg et al., 1996a). Four adult, male Sprague Dawley rats (250–400 gm) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused through the ascending aorta with 40 ml of heparin (1000 U/ml heparin in 0.15 M NaCl), 50 ml of 3.75% acrolein, and 2% paraformaldehyde in phosphate buffer (PB), pH 7.4. The brains were removed from the calvarium and post-fixed for 30 min in 2% paraformaldehyde. Coronal sections through the midbrain, 30–40 μm in thickness, were cut with a Lancer vibratome and then incubated for 30 min in 1% sodium borohydride in PB. All sections were cryoprotected for 15 min in a solution of 25% sucrose and 3.5% glycerol in 0.05 M PB, frozen rapidly in chlorodifluoromethane followed by liquid nitrogen, and thawed in room temperature PB.

Antisera. As in previous studies (Nirenberg et al., 1996a; Pickel et al., 1996; Nirenberg and Pickel, 1997), we used two well-characterized antisera: a rabbit polyclonal anti-peptide antiserum directed against the N-terminal domain of rat DAT (Freed et al., 1995; Revay et al., 1996) and a commercially available mouse monoclonal antiserum directed against TH (Incstar, Stillwater, MN). The free-floating tissue sections were incubated overnight at room temperature in 0.1% BSA-Tris-saline (0.9% NaCl in 0.1 M Tris), pH 7.6, to which one of the following had been added: (1) a 1:6000 dilution of the DAT antiserum, for single labeling; (2) a 1:6000 dilution of the DAT antiserum and a 1:10,000 dilution of the TH antiserum, for double labeling; or (3) no primary antiserum, as a negative control.

Immunocytochemical labeling. In tissue that was prepared for dual labeling, the bound TH antiserum was identified using the ABC method (Hsu et al., 1981) as follows. The sections were incubated for 30 min in a 1:400 dilution of biotinylated goat anti-rabbit immunoglobulins in 0.1% BSA, for 30 min in a 1:100 dilution of avidin–biotin peroxidase complex, and then for 6 min in a solution consisting of 22 mg of DAB and 10 μl of 30% hydrogen peroxide in 100 ml 0.1 M Tris-saline, pH 7.6.

All tissue sections were prepared for silver-enhanced immunogold labeling (Chan et al., 1990) as follows. They were incubated for 2 hr in a 1:50 dilution of colloidal gold- (1 nm) conjugated anti-rabbit IgG (Amersham, Arlington Heights, IL), fixed for 10 min in 2% glutaraldehyde in PBS, and reacted for 3–7 min with a silver solution using a light stable intenSEM kit (Amersham).

Electron microscopy. The immunolabeled tissue sections were fixed in 2% osmium tetroxide for 60 min, dehydrated in a series of graded ethanols and propylene oxide, and flat-embedded in Epon 812 between two pieces of Aclar plastic. Ultrathin sections from the lateral parabrachial and paranigral subnuclei of the VTA, sampled from rostrocaudal sections ranging between −5.1 and −5.3 from bregma (Paxinos and Watson, 1986), were collected from the outer surface of the plastic-embedded tissue using an ultramicrotome (Research and Manufacturing, Tucson, AZ). These sections were then counterstained with lead citrate and uranyl acetate and examined with a Philips (Mahwah, NJ) electron microscope. Electron micrographs that were used for illustrations were scanned on a Power Macintosh 8500/150 Computer (Apple Computer, Cupertino, CA) with an AGFA Arcus II scanner (Agfa-Gevaert, Montsel, Belgium) in combination with FotoLook (Agfa-Gevaert) and Photoshop (version 3.0.4, Adobe Systems, Mountain View, CA) software. QuarkX-Press (version 3.32, Quark, Denver, CO) and Adobe Illustrator (version 6.0; Adobe Systems) software were then used to prepare and label the composite figures.

Identification of labeled profiles. Classification of labeled profiles and subcellular organelles was based on the criteria of Peters et al. (1991). Neuronal perikarya were identified by the presence of a nucleus, Golgi apparatus, and rough endoplasmic reticulum (RER). Dendrites were distinguished from unmyelinated axons primarily by their larger diameter and synaptic input from axon terminals. In addition, the dendrites contained more RER, a relative paucity of neurofilaments, and/or a high proportion of uniformly distributed microtubules. Proximal dendrites were distinguished from more distal dendrites by their large size, continuity with neuronal somata, and/or content of RER. Synaptic junctions were defined by the presence of a junctional complex, a restricted zone of parallel membrane appositions with slight enlargement of the extracellular space, a cluster of presynaptic vesicles, and an associated postsynaptic thickening. Puncta adherentia were identified as junctional zones characterized by small, symmetric cytoplasmic densities and an accumulation of extracellular dense material, without any of the other characteristic features of synaptic junctions. Nonsynaptic contacts (appositions) were identified as sites of closely spaced parallel plasma membranes that were not separated by glial processes, but that lacked synaptic junctions. Profiles were considered to be immunogold-labeled if they contained at least two immunogold particles, and were considered to be immunoperoxidase-labeled if they contained a cytoplasmic precipitate that made them appear more electron-dense than morphologically similar unlabeled profiles observed within the same tissue section. An exception was made for unmyelinated axons in which even a single gold particle was considered indicative of immunolabeling, because their small size minimized the likelihood of detection of immunolabeling.

Identification of subcellular organelles. Small synaptic vesicles (SSVs) were identified by their 30–60 nm cross-sectional diameter, round to pleomorphic shape, and electron-lucent lumen. Large dense core vesicles (DCVs) were identified by their size (80–120 nm in cross-sectional diameter) and by the presence of an electron-dense core surrounded by an electron-lucent halo. Tubulovesicles were defined as electron-lucent membranous structures that were irregular in shape and >70 nm in maximal cross-sectional diameter. Subcellular organelles were considered to be immunogold-labeled if they were directly contacted by at least one gold particle and were contained within a profile in which there were two or more gold particles.

RESULTS

Immunogold labeling for DAT was seen primarily within neuronal perikarya (Figs. 1*A*, 2) and dendrites (Figs. 1*B*, 2*B*, 3, 4). Of 192 observed DAT labeled profiles, 4% were perikarya and 90% were dendrites. The remaining profiles were unmyelinated axons (data not shown). No labeling was seen in glial cells or other non-neural elements. The observed pattern of DAT immunolabeling was present in sections from the surface of tissue that had been incubated with the DAT primary antiserum, but was absent from sections in which the primary antiserum had been omitted, as well as from those that were sampled from deeper in the tissue, where immunoreagent penetration is poor (Leranth and Pickel, 1989). Immunogold labeling for DAT was selectively localized to cytoplasmic surfaces of intracellular or plasma membranes. In contrast, the peroxidase labeling for TH was more diffusely distributed throughout the cytoplasm of the labeled profiles (Figs. 2-4).

Perikarya

Immunogold labeling for DAT was detected within neuronal perikarya in the VTA (Figs. 1*A*, 2). These perikarya were apposed to (Figs. 1*A*, 2*B*) and, in some cases, received synaptic input from (Fig. 1*A*) unlabeled axon terminals. DAT-labeled perikarya were also contacted by glial processes (Fig. 2*A*) and DAT-labeled (Fig. 2*B*) or unlabeled (Fig. 1*A*) dendrites. In sections that had been processed for dual labeling of DAT and TH, most of the somata that were immunogold-labeled for DAT also contained peroxidase reaction product for TH (Fig. 2*A*,*B*).

Within perikarya, DAT gold particles were localized to intracellular membranes and were not in direct contact with the plasma or nuclear membranes (Fig. 1*A*). When observed at high magnification, these DAT-labeled cytoplasmic organelles consisted of tubulovesicles that were irregular in shape and larger than the SSVs observed in adjacent unlabeled axon terminals. DAT-labeled tubulovesicles were often located near saccules of Golgi (Figs. 1*A*, 2*A*,*B*).

Dendrites

Immunogold labeling for DAT was seen in many dendritic profiles of varying size in the VTA (Figs. 1*B*, 2*B*, 3, 4). Of 172 observed

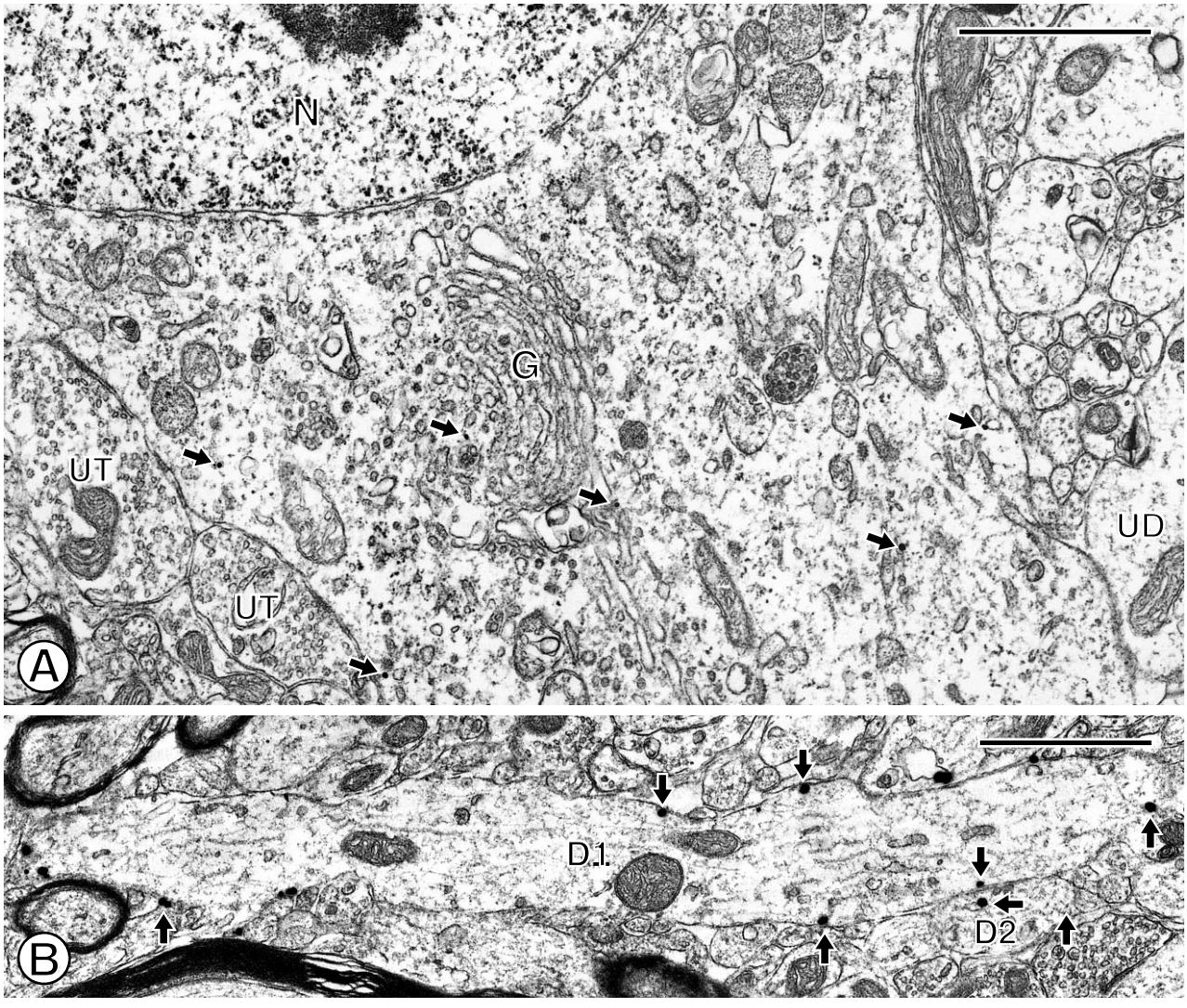

Figure 1. Differential localization of DAT in a perikaryon and dendrites. The tissue was labeled with immunogold-silver for DAT. *A*, DAT immunogold labeling is localized to intracellular membranes (*arrows*), but is not detected along the plasma membrane of a neuronal perikaryon. Some of the DAT gold particles are located near the Golgi apparatus (*G*). The DAT-containing perikaryon is contacted by unlabeled axon terminals (*UT*) and an unlabeled dendrite (*UD*). *B*, DAT immunogold labeling (*arrows*) is located primarily on the plasma membranes of two apposed dendrites (*D1*, *D2*). *N*, Nucleus. Scale bars, 1 μm.

DAT-labeled dendrites, 2 were apposed to DAT-labeled perikarya (Fig. 2*B*) and 25 were apposed to other DAT-immunoreactive dendrites (Figs. 1*B*, 3*A*, 4). The dendro-dendritic appositions were characterized by apposed membranes that were sometimes joined by puncta adherentia (Fig. 4*A*). Neither aggregates of vesicles nor synaptic specializations were typically seen at these junctions. DAT-containing dendrites were also apposed to (Figs. 2*B*, 3*C*) and, in some cases, received symmetric or asymmetric synapses from axon terminals that lacked detectable labeling for DAT (Figs. 2*B*, 4*A*).

In dually labeled tissue, most of the dendrites in which there was immunogold labeling for DAT also contained peroxidase labeling for TH. There was considerable variability, however, in the density of the TH reaction product in these dendrites (Figs. 3, 4). Appositions were also frequently observed between DAT-labeled dendrites containing different densities of peroxidase reaction product for TH (Fig. 4*A*). The density of the TH reaction product did not appear to correlate with the subcellular localization (e.g., internal vs plasma membranes) or number of DAT gold particles within these dendrites.

Within large, proximal dendrites, immunogold labeling for DAT was intracellular, where it was associated with electron-lucent vesicles and tubulovesicles (Figs. 2*B*, 4*B*). As in DAT-containing perikarya, these organelles were usually >70 nm in diameter and thus larger than typical synaptic vesicles. In contrast, in more distal dendrites, most of the DAT gold particles were localized to the plasma membrane (Figs. 1*B*, 3, 4*A*). In one example in which a dendrite was cut in longitudinal section, a wider portion of the dendrite containing intracellular DAT labeling was seen in continuity with a narrower (presumably more distal) portion of the dendrite with more prominent plasmalemmal DAT labeling (Fig. 3*A*). The plasmalemmal immunogold DAT labeling was observed near appositions with glial cells (Figs. 3*B*,*C*, 4), unlabeled axons (Figs. 3*B*,*C*, 4*B*), unlabeled terminals (Fig. 3*C*), and DAT-labeled (Fig. 4*A*) or unlabeled (Fig. 3*A*) dendrites.

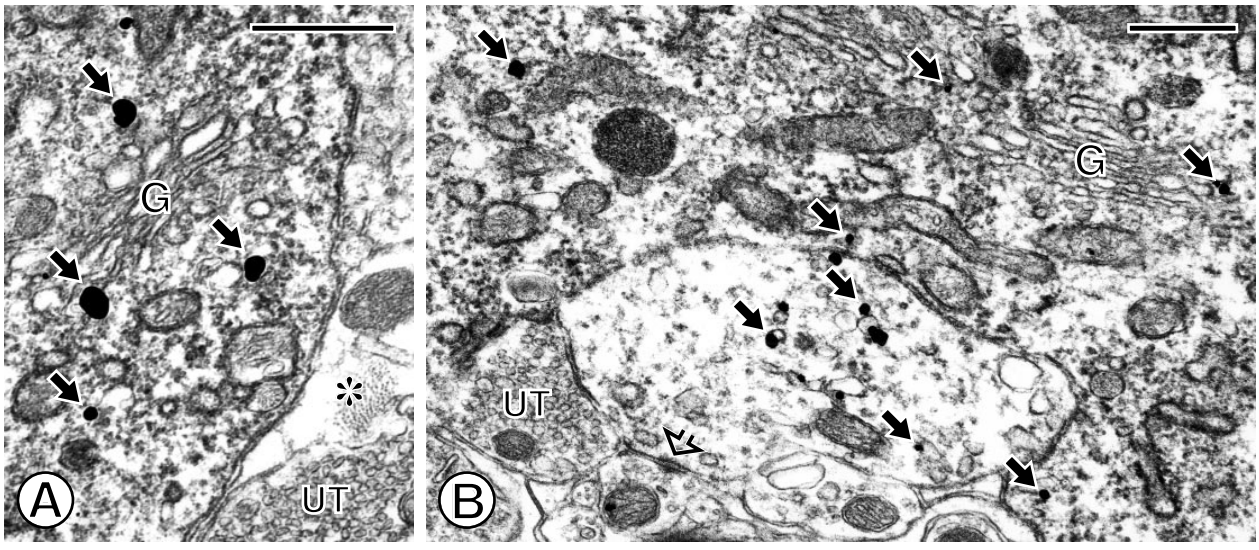

Figure 2. Intracellular DAT labeling in TH-immunoreactive perikarya and large dendrites. The tissue was dually labeled with immunogold for DAT and immunoperoxidase for TH. *A*, DAT immunogold labeling (*arrows*) is seen near the Golgi apparatus (*G*) of a neuronal perikaryon that also contains diffuse peroxidase reaction product for TH. The dually labeled cell is contacted by an unlabeled astrocyte (*asterisk*). *UT*, Unlabeled terminal. *B*, DAT immunogold labeling (*arrows*) and peroxidase labeling for TH are co-localized within the cytoplasm of a perikaryon. Some of the DAT immunogold particles (*arrows*) are located near the Golgi apparatus (*G*). The dually labeled perikaryon is contacted by a large dendrite that contains both intracellular DAT gold labeling (*arrows*) and light peroxidase reaction product for TH. The DAT-labeled perikaryon and dendrite are contacted by an unlabeled terminal (*UT*). The labeled dendrite also receives a symmetric synapse (*open arrow*) from another unlabeled terminal. Scale bars, 0.5 μm.

Clusters of SSVs were seen within the cytoplasm of only 3/172 of the DAT-containing dendrites (Fig. 4*B*). These clusters of vesicles were observed both near and at varying distances from the plasma membrane. The clusters of SSVs did not contain detectable immunogold labeling for DAT and were only observed once within a distance of <0.1 μm from a DAT-immunolabeled plasma membrane (Fig. 4*B*). DCVs were also occasionally detected within DAT-containing dendrites, but were not observed near plasma membranes nor did they contain detectable DAT immunoreactivity (Fig. 4*B*).

Unmyelinated axons

DAT immunogold labeling was infrequently detected in unmyelinated axons, which were small (0.1–0.2 μm) and typically occurred in bundles with other unlabeled axons (data not shown). These axons contained only isolated vesicles and never formed synaptic specializations characteristically associated with axon terminals (Peters et al., 1991). In dually labeled tissue, most of these axons contained peroxidase reaction product for TH. Within these axons, immunogold labeling for DAT was principally located on plasma membranes but was also sometimes associated with electron-lucent cytoplasmic vesicles or tubulovesicles.

DISCUSSION

We have shown that in the rat VTA, plasmalemmal labeling for DAT occurs primarily in TH-immunoreactive, medium- and small-diameter dendrites, and only rarely within small unmyelinated axons. In contrast, DAT labeling was not detected along the plasma membranes of dopaminergic somata and proximal dendrites in this region. These findings suggest that within dopaminergic neurons in the VTA, the intermediate and distal dendrites are both the primary sites of plasmalemmal dopamine transport and the principal targets of cocaine and other stimulants.

Localization of DAT to dopaminergic neurons

The morphology and synaptic associations of DAT-labeled perikarya and dendrites were consistent with the known features of the dopaminergic neurons in the VTA (Domesick et al., 1983; Bayer and Pickel, 1990, 1991). In addition, in dually labeled tissue, most DAT-immunoreactive neurons also contained reaction product for TH, a selective marker for dopaminergic perikarya and dendrites in this region (Pickel and Sesack, 1995). The absence of detectable TH labeling in some DAT-labeled neurons presumably reflects both the known heterogeneity of TH immunoreactivity within dopaminergic neurons in the VTA (Bayer and Pickel, 1990, 1991) and the fact that the TH antiserum was used at a high dilution so as to maximize the detection and subcellular resolution of the DAT gold labeling (Leranth and Pickel, 1989). Immunolabeling for DAT was not detected within glia, suggesting that the glial uptake of dopamine (Kimelberg and Katz, 1986) is mediated by transporters other than DAT (Russ et al., 1996). Together, these findings suggest that DAT is primarily or exclusively expressed in dopaminergic neurons in the VTA. Within these neurons, the presence of DAT labeling on cytoplasmic surfaces of intracellular and plasma membranes is consistent with proposed topological models, which predict that this transporter has an intracellular N-terminal domain (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991).

Perikarya and proximal dendrites: sites for DAT synthesis, recycling, and membrane trafficking

Within perikarya and large, proximal dendrites, DAT labeling was exclusively detected within saccules of Golgi and other intracellular membranes. This suggests that the somata and proximal dendrites of mesocorticolimbic dopaminergic neurons are sites of DAT synthesis, recycling, and/or trafficking to and from the cell surface (Parton et al., 1992; Bradbury and Bridges, 1994). The absence of detectable labeling for DAT on the plasma membranes

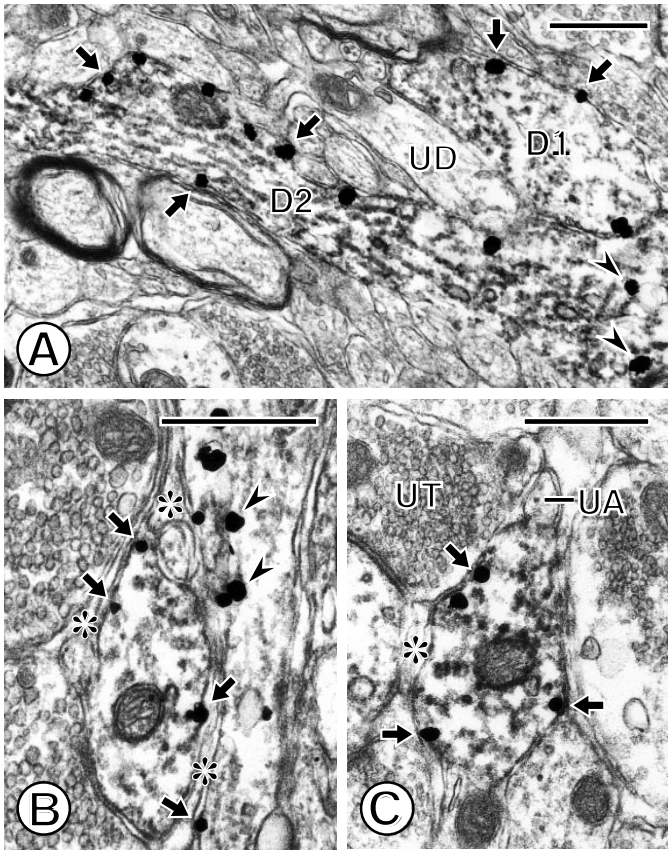

Figure 3. Prominent DAT labeling on plasma membranes of TH-immunoreactive dendrites. The tissue was dually labeled with immunogold for DAT and immunoperoxidase for TH. *A*, Immunogold labeling for DAT (*arrows*) is seen within two apposed dendrites (*D1*, *D2*), both of which also contain peroxidase reaction product for TH. Most of the immunogold particles for DAT are located on or near the dendritic plasma membranes (*arrows*). In the wider portion of the lower dendrite, however, several DAT gold particles are localized to intracellular membranes (*arrowheads*). The dually labeled dendrites are also apposed to an unlabeled dendrite (*UD*). *B*, Two dendrites are dually labeled with immunogold particles for DAT (*arrows*) and electron-dense peroxidase reaction product for TH. Gold particles are seen along the plasma membranes of both dendrites (*closed arrows*) near their contacts with unlabeled astrocytes (*asterisks*). In the dendrite on the *right*, several of the DAT gold particles contact intracellular membranes (*arrowheads*). *C*, Immunogold labeling for DAT (*arrows*) is seen on the plasma membrane of a dendrite that also contains intense peroxidase labeling for TH. The plasmalemmal DAT gold labeling is near contacts with an unlabeled axon terminal (*UT*) and an unlabeled astrocyte (*asterisk*). *UA*, Unlabeled axon. Scale bars, 0.5 μm.

of dopaminergic perikarya and proximal dendrites, however, suggests that plasmalemmal dopamine transport and stimulant binding are unlikely to occur at these sites.

Most of the DAT-labeled intracellular membranes in perikarya and dendrites had the morphological features of smooth endoplasmic reticulum (Broadwell and Cataldo, 1983; Peters et al., 1991). We have referred to these organelles as tubulovesicles, however, to indicate that they might potentially consist of morphologically similar but biochemically distinct intracellular organelles (Nirenberg et al., 1996b; Nirenberg and Pickel, 1997). In midbrain dopaminergic dendrites, tubulovesicles can store monoamines (Hattori et al., 1979; Mercer et al., 1979) and contain the vesicular monoamine transporter-2 (Nirenberg et al., 1996b; Nirenberg and Pickel, 1997), suggesting that they are the major sites of somatodendritic storage and possible release of dopamine. Thus, the presence of DAT immunoreactivity within tubulovesicular membranes suggests a potential role for DAT in regulating intracellular dopamine storage pools within midbrain somata and dendrites (Nirenberg et al., 1996a). Additional studies are necessary, however, to determine whether these DAT-immunoreactive tubulovesicles are functional sites of DAT expression.

Intermediate and distal dendrites: major sites for dopamine transport and psychostimulant binding

Plasmalemmal DAT labeling was most frequently observed on medium- to small-diameter dendrites, suggesting that these dendrites are the principal sites of functional DAT expression in the VTA. The DAT that is expressed on dendritic plasma membranes thus presumably plays a major role in regulating extracellular dopamine concentrations in this region. Within these neurons, the magnitude, and perhaps even the direction, of dopamine transport by dendritic DATs would depend on the membrane potential as well as on the plasmalemmal concentration gradients for dopamine and co-transported ions (for review, see Levi and Raiteri, 1993; Hitri et al., 1994; Bannon et al., 1995).

The expression of DAT on plasma membranes of intermediate and distal dendrites in the VTA also suggests that these dendrites are major targets for cocaine and other psychostimulants in this region. The blockade of DAT would be expected to increase the extracellular concentrations of dopamine near these dendrites, thereby allowing greater diffusion distances for dopamine throughout the VTA (Einhorn et al., 1988; Bradberry and Roth, 1989). This increase in extracellular dopamine might permit high levels of dopamine to reach distant dopamine receptors that do not normally receive physiologically relevant concentrations of this transmitter (Koob and Bloom, 1988).

Dopaminergic axons: minor sites for plasmalemmal dopamine transport and psychostimulant binding

Only a few unmyelinated axons and no axon terminals contained detectable DAT immunogold labeling. These axons were dually labeled for DAT and TH and are likely to originate from both local (A10) and extrinsic (A8 and A9) dopaminergic cell groups (Deutch et al., 1988). The presence of DAT labeling on plasma membranes in these axons suggests that they may also represent functional sites of DAT expression in the VTA (Kalivas and Duffy, 1991). The sparseness of the DAT-labeled axons and absence of DAT labeling in axon terminals, however, indicate that axons probably play a relatively minor role both in psychostimulant binding and in regulating extracellular dopamine concentrations in this region.

Relationship to sites of dopamine release

Although somatodendritic release of dopamine is known to occur from midbrain dopaminergic neurons (Geffen et al., 1976; Kalivas et al., 1989; Bernardini et al., 1991; Zhang et al., 1994), we only once observed plasmalemmal DAT labeling near clusters of dendritic SSVs. This suggests that there is either extensive extracellular diffusion of dopamine after its release from dendritic SSVs or that these vesicles are not the primary organelles involved in dopamine release in this region. Earlier studies have shown that midbrain dopaminergic dendrites contain few SSVs regardless of the relationship of these vesicles to dopamine uptake sites (Wilson et al., 1977; Groves

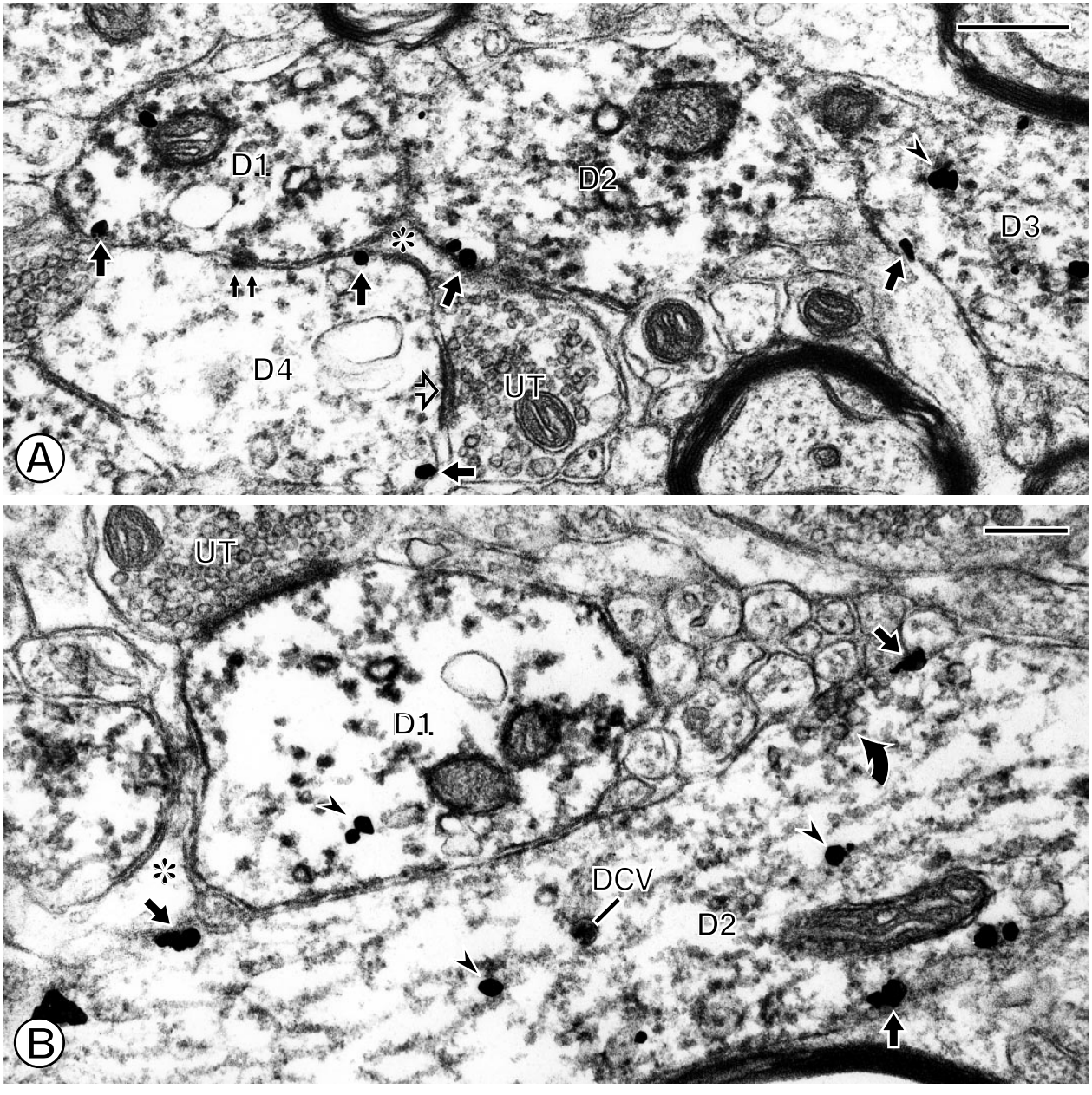

Figure 4. Appositions between DAT-labeled dendrites. The tissue was dually labeled with immunogold for DAT and immunoperoxidase for TH. *A*, Immunogold labeling for DAT is seen within four apposed dendrites (*D1*, *D2*, *D3*, *D4*). Most DAT gold particles are located on plasma membranes (*large closed arrows*), but some are also seen on intracellular membranes (*arrowheads*). Three of the DAT-immunoreactive dendrites (D1, D2, D3) contain intense peroxidase reaction product for TH, but the fourth (D4) contains only light TH labeling. This lightly TH-labeled dendrite (D4) receives a symmetric synapse (*open arrow*) from an unlabeled axon terminal (*UT*). A punctum adherens (*small arrows*) is also seen between two of the DAT-containing dendrites (D1, D4), one of which contains intense TH labeling (D1), and one of which is very lightly TH-labeled (D4). *B*, Immunogold labeling for DAT (*straight arrows*) is seen within two apposed dendrites (D1, D2), both of which contain peroxidase reaction product for TH. Many of the DAT immunogold particles are associated with intracellular membranes (*arrowheads*). In the lower dendrite (D2), some DAT gold particles are also located on the plasma membrane (*straight arrows*). The upper dendrite (D1) is contacted by an unlabeled terminal (*UT*). The lower dendrite (D2) contains an unlabeled dense core vesicle (*DCV*) within the cytoplasm and a small aggregate of unlabeled electron-lucent vesicles near the plasma membrane (*curved arrow*). A plasmalemmal DAT gold particle (*straight arrow*) is seen adjacent to the cluster of electron-lucent vesicles. Scale bars, 0.25 μm.

and Linder, 1983; Nirenberg et al., 1995). In fact, it is possible that some of the structures that we and others have referred to as SSVs may consist of tubulovesicular organelles, which can resemble SSVs when observed in cross-section (Ayala, 1994). Given the paucity of SSVs and DCVs in midbrain dopaminergic dendrites observed in both the present study and previous reports, it has been suggested that most of the reserpine-sensitive storage and calcium-dependent somatodendritic release of dopamine involve tubulovesicular organelles (Cuello and Iversen, 1978; Hattori et al., 1979; Mercer et al., 1979; Nirenberg et al., 1996a,b; Nirenberg and Pickel, 1997). Additional studies are necessary to determine the precise subcellu-

lar relationships between the sites of release and reuptake of dopamine in the VTA.

Comparison with the substantia nigra (SN)

In the present study, we used the same DAT antiserum and identical experimental conditions as in our earlier ultrastructural analysis of the SN (Nirenberg et al., 1996a). As a result, we have shown that in both the VTA and the SN, most of the DAT gold particles in dopaminergic dendrites are located on plasma membranes. In contrast, the DAT gold particles that are present in dopaminergic somata are selectively located on intracellular membranes. Thus, although there may be quantitative differences in the expression of DAT in the SN and VTA (Blanchard et al., 1994; Hurd et al., 1994; Haber et al., 1995; Cragg et al., 1997), we did not detect any qualitative differences in the subcellular targeting of DAT to plasma membranes in the two regions.

We did, however, observe important differences in the cellular relationships between DAT-labeled neurons in the VTA and SN. The most notable distinction was in the prevalence of plasmalemmal DAT labeling near appositions between two or more dopaminergic dendrites in the VTA, but not in the SN (Nirenberg et al., 1996a). This difference appears to reflect the fact that there are more numerous appositions between pairs of dopaminergic dendrites in the VTA than in the SN (Nirenberg et al., 1996a). The dendritic appositions are significant in that they are potential sites for dopamine release through exocytosis from storage organelles and/or reversal of DAT (Atwell et al., 1993; Sulzer et al., 1993).

The apposed DAT-labeled dendrites in the VTA often differed with respect to their levels of immunoreactivity for the catecholamine-synthesizing enzyme TH. Interestingly, dopaminergic neurons in the VTA that express different levels of TH immunoreactivity are also known to vary in the types of synaptic inputs they receive (Bayer and Pickel, 1990, 1991). In light of the known voltage- and concentration-dependence of DAT, the heterogeneity of dopaminergic neurons in the VTA may facilitate the uptake of dopamine into dendrites different from those in which it has been synthesized and released. This would represent a novel form of functional coupling between dopaminergic neurons in the VTA, distinct from the electrotonic coupling that has been demonstrated previously in dopaminergic neurons in the SN (Grace and Bunney, 1983) and nucleus accumbens (O'Donnell and Grace, 1993).

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