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Overlapping Intracellular and Differential Synaptic Distributions of Dopamine D1 and Glutamate NMDA Receptors in Rat Nucleus Accumbens

Yuko Hara and Virginia M. Pickel

Dept. Neurology and Neuroscience, Weill Medical College of Cornell University, New York, NY 10021

Abstract

The dopamine D1 receptor (D1R) in the nucleus accumbens (Acb) shell is highly implicated in psychostimulant-evoked locomotor activity and reward, whereas the D1R in the Acb core is more crucial for appetitive instrumental learning. These behavioral effects depend in part on interactions involving glutamatergic NMDA receptors, whose essential NR1 subunit has physical associations with the D1R. To determine the relevant sites for D1R activation and interactions involving NMDA receptors, we examined the electron microscopic immunolabeling of D1R and NR1 C-terminal peptides in rat Acb shell and core. In each Acb subdivision, the D1Rs were located principally on extrasynaptic plasma membranes of dendritic shafts and spines and more rarely associated with cytoplasmic endomembranes. Many D1R-labeled somata and dendrites also contained NR1 immunoreactivity. In comparison with D1R, NR1 immunoreactivity was more often seen in the cytoplasm and near asymmetric synapses on somatodendritic profiles. In these profiles, notable overlapping distributions of D1R and NR1 occurred near endomembranes. The exclusively D1R or D1R and NR1 containing dendrites were most prevalent in the Acb shell, but also present in the Acb core. In each region, NR1 was also detected in axon terminals without D1R, which formed excitatory-type synapses with D1R-labeled dendrites. These results provide ultrastructural evidence that D1Rs in the Acb have subcellular distributions supporting, 1) intracellular co-trafficking with NR1, and 2) modulation of the postsynaptic excitability in spiny neurons affected by presynaptic NMDA receptor activation. The region-specific differences in receptor distributions suggest a major, but not exclusive, involvement of Acb D1R in reward-related processing.

Keywords

appetitive instrumental learning; endomembrane; immunocytochemistry; reward; shell; ultrastructure

Introduction

The dopamine D1 receptor (D1R) in the nucleus accumbens (Acb) is critically involved in attentional processes and conditional motor responses to food and other reinforcers including addictive drugs (Ikemoto et al., 1997; Ikemoto, 2002; Di Chiara et al., 2004; Kelley, 2004). The diversity in function is consistent with the known expression of D1R

mRNA in both the shell and core compartments of the Acb (Weiner et al., 1991; Lu et al., 1998). The shell receives substantial mesolimbic dopaminergic (DA) innervation (Deutch and Cameron, 1992), and is particularly important in reward (Ikemoto et al., 1997; McKinzie et al., 1999; Rodd-Henricks et al., 2002; Anderson et al., 2003). In contrast, the Acb core receives greater DA input from the substantia nigra (Groenewegen et al., 1999). The Acb core is functionally similar to the dorsal striatum, which also receives primarily nigrostriatal DA input and regulates motor function (Zahm, 1992; Groenewegen et al., 1999; Canales and Iversen, 2000). In addition, D1R in the Acb core is important for appetitive instrumental learning (Smith-Roe and Kelley, 2000; Di Ciano et al., 2001; Baldwin et al., 2002).

Glutamate NMDA receptors are also prevalent in the Acb shell and core. Moreover, their activation can affect many of the same functions as those mediated by the D1R, including locomotor activity (Hamilton et al., 1986; Pulvirenti et al., 1994), associative learning (Saul'skaya and Marsden, 1996), and reward (Pulvirenti et al., 1992; Bristow et al., 1994). DA terminals in both the Acb shell and core also converge on single dendrites with glutamatergic terminals derived from the hippocampal formation, medial prefrontal cortex and amygdala (Johnson et al., 1994). Together, these observations suggest important functional associations between D1Rs and NMDA receptors in many of the same neurons in Acb shell and core.

Several lines of evidence support intracellular associations between D1Rs and NMDA receptors. Many D1R-mediated functions are dependent on coincident activation of NMDA receptors (Wu et al., 1993; Adriani et al., 1998; Smith-Roe and Kelley, 2000; David et al., 2004). D1R activation also potentiates NMDA receptor-induced responses in neostriatal slices (Levine et al., 1996) and induces trafficking of NMDA receptors between subcellular compartments (Dunah and Standaert, 2001). Conversely, NMDA receptor activation alters D1R subcellular distributions within striatal neurons (Scott et al., 2002). Moreover, studies in cell lines and hippocampal neurons indicate that the C-terminals of the D1R and the essential NR1 subunit of the NMDA receptor have physical protein-protein interactions (Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004).

The regional D1R distribution in the Acb has been established by ligand binding studies (Savasta et al., 1986), and by light microscopic immunocytochemistry (Levey et al., 1993; Yung et al., 1995; Dunah and Standaert, 2001). The essential NR1 subunit of the NMDA receptor has also been localized by electron microscopic immunocytochemistry in the Acb shell (Gracy and Pickel, 1996). However, neither the subcellular distribution of the D1R, nor sites for potential D1R/NR1 interactions have been examined in either the Acb shell or core. Subcellular location is a major determinant of the D1R availability for ligand binding, affecting either postsynaptic excitability or presynaptic transmitter release onto output neurons of the Acb. The intracellular locations of the D1R and NR1 also may provide new insight into D1R-NMDA receptor cross-talk in the Acb. To determine these locations, we used electron microscopic immunocytochemistry to dually localize the C-terminals of D1R and NR1 in the Acb medial shell and core of the rat brain. The results provide the first ultrastructural evidence that D1R and NR1 are co-expressed and have partially overlapping endomembrane distributions in somatodendritic profiles enriched in the Acb shell. They also suggest a modulatory role for NMDA receptors in transmitter release onto dendrites activated through postsynaptic D1Rs.

Materials and Methods

Animals and tissue preparation

The animal protocols in this study strictly adhered to NIH guidelines concerning the Care and Use of Laboratory Animals in Research, and were approved by the Animal Care Committee at Weill Medical College of Cornell University. Adult male Sprague Dawley rats (250–400 g; Taconic Farms, Germantown, NY) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and were perfused via the ascending aorta first with 10 ml of heparin (1000 U/ml), then with 60 mL of 3.75% acrolein and 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4), followed by 200 ml of 2% PFA in PB. The brains were removed and postfixed with 2% PFA in 0.1 M PB for 30 minutes at 4°C, and then cut coronally into 40 µm sections on a Vibratome (Leica, Deerfield, IL). All Acb sections used for this study were selected at 1.60 mm anterior to Bregma (Paxinos and Watson, 1986) and contained both the shell and the core (see Fig. 1). These sections were placed in 1% sodium borohydride in 0.1 M PB for 30 minutes to remove excess aldehydes and then rinsed thoroughly with 0.1 M PB. To enhance the penetration of immunoreagents, the sections were incubated in a cryoprotectant solution (25% sucrose and 3.5% glycerol in 0.05 M PB) for 15 minutes and rapidly freeze-thawed by immersing in liquid chlorodifluoromethane (Freon, Refron Inc., NY) followed by liquid nitrogen and finally in room temperature 0.1 M PB. The sections were then rinsed in 0.1 M Tris-buffered saline (TBS).

Antibodies

A rat monoclonal antibody raised against a 97 amino acid peptide corresponding to the C-terminus of the human D1R was obtained commercially (Clone 1-1-F11 S.E6, Sigma RBI, Saint Louis, MO). The D1R antibody has been well characterized and shown to specifically recognize the antigenic peptide by Western blot, immunoprecipitation and blocking control experiments used for immunocytochemistry (Levey et al., 1993).

Two polyclonal rabbit antisera raised against a 20 (919–938) and 30 (909–938) amino acid peptide corresponding to the C-terminus of rat NR1 were affinity purified (06–311, Upstate, Lake Placid, NY; AB1516, Chemicon International, Temecula, CA). These antisera recognize four of the seven splice variants of NR1. Western blots have shown that both antisera recognize a single band of 120 kDa corresponding to NR1. These anti-NR1 antibodies have no cross-reactivities with other glutamate receptor subunits (Petrálie et al., 1994; Ehlers et al., 1995).

Immunocytochemical labeling

The free-floating sections were incubated in 0.5% bovine serum albumin (BSA) in 0.1 M TBS, pH 7.6, for 30 minutes to block non-specific binding of antisera. Then the sections were incubated for 48 hours at 4°C in the primary antibody solution, which consisted of the affinity purified rabbit polyclonal anti-NR1 (1:25 for immunogold-silver, 1:100 for immunoperoxidase method) and/or rat monoclonal anti-D1R (1:500 for immunoperoxidase, 1:100 for immunogold-silver method) with 0.1% BSA in 0.1 M TBS.

For immunoperoxidase labeling, the sections were rinsed in 0.1 M TBS, incubated first in a 1:400 dilution of biotinylated donkey anti-rat (for D1R immunoperoxidase) or goat anti-rabbit (for NR1 immunoperoxidase) IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 30 minutes, and then washed in 0.1 M TBS. For immunoperoxidase detection, the sections were incubated in avidin-biotin peroxidase complex (Vectastain Elite Kit, Vector Labs, Burlingame, CA) for 30 minutes, washed with 0.1 M TBS, and then placed in 0.022% 3,3'-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and 0.003% hydrogen peroxide in 0.1 M TBS for 6 minutes.

For light microscopy, sections labeled with D1R immunoperoxidase were mounted on gelatin-coated slides, dehydrated, and coverslipped. These sections were examined with a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) and images were taken by a Micropublisher 5.0RTV camera (QImaging, BC, Canada). The image was adjusted for brightness and contrast using Adobe Photoshop (version 7.0.1, Adobe Systems Inc., Mountain View, CA). The image was then imported into PowerPoint, Microsoft Office, 2001, to add lettering and a trapezoid to illustrate the locations in the Acb shell and core from which the electron micrographs were taken.

For immunogold-silver labeling, the sections were rinsed in 0.01 M PBS, pH 7.4, blocked in 0.8% BSA and 0.1% gelatin in PBS for 10 minutes, and subsequently incubated for 2 hours in a 1:50 dilution of donkey anti-rabbit (for NR1 immunogold) or goat anti-rat (for D1R immunogold) IgG conjugated with 1 nm colloidal gold (Amersham, Arlington Heights, IL). The sections were rinsed several times with 0.1 M TBS, then placed in 2% glutaraldehyde for 10 minutes to ensure the adherence of the bound colloidal gold. The gold particles were silver-enhanced using the IntenS-EM kit (Amersham) for 7 minutes at room temperature. The sections were fixed in 2% osmium tetroxide in 0.1 M PB for 60 minutes, followed by dehydration with a graded series of ethanols and propylene oxide. These sections then were incubated overnight in 1:1 mixture of propylene oxide and Epon (Electron Microscopy Science, Fort Washington, PA). The sections were transferred to 100% Epon for 2–3 hours and then flat-embedded between sheets of Aclar plastic. Areas containing both the Acb shell and core, immediately medial to the anterior commissure, were cut into ultra-thin (60 nm) sections with a diamond knife (Diatome, Fort Washington, PA) using an ultratome (NOVA LKV-Productor AB, Bromma, Sweden). Thin sections from the outer surface region were collected on 400-mesh copper grids (Electron Microscopy Sciences). These sections were counterstained with 5% uranyl acetate for 20 minutes followed by Reynold's lead citrate (Reynolds, 1963) for 5 minutes. After thoroughly washed and air-dried, the ultra-thin sections were examined at 60 kV with a Philips CM10 transmission electron microscope (FEL, Hillsboro, OR). The microscopic images were captured using AMT Advantage HR/HR-B CCD Camera System (Advanced Microscopy Techniques, Danvers, MA).

Electron microscopic analysis

To assess the distributions of D1R and NR1 immunoreactivities, data analysis was performed on ultra-thin sections collected near the surface of the tissue at the interface with the embedding material. The profiles containing D1R and/or NR1 immunoreactivity were classified as either neuronal (dendrites, spines, axons and axon terminals) or glial based on criteria from Peters et al. (1991). Immunoperoxidase labeling was regarded as positive when the electron-dense reaction product in individual profiles was greater than that seen in other morphologically similar profiles in the neuropil. Profiles were considered to be selectively immunogold-labeled even when they contained only one gold particle to avoid false negative labeling of smaller profiles. This approach of immunogold quantification was validated by examining the spurious gold particles distributed in tissue elements such as myelin, which is not known to express NR1. In 25525.7 μm^2 of Acb shell and core examined, there were 889 myelinated axons, of which only 7 had a single NR1 immunogold particle on the myelin. We also determined the average distance between two immunogold particles within longitudinally-cut dendritic profiles. The mean distance between these particles was 1018.75 nm (± 196.87 , $n=28$), whereas thin sections were 60–70 nm thickness.

About 100 micrographs at 13,500X magnification were obtained from each rat (one vibratome section from each of three rats) and each subregion. A total area of 12,065 μm^2 was examined in the Acb shell and 13,460 μm^2 in the core. Data were plotted as the mean numbers of D1R-, NR1- or dual-labeled profiles within 3400 μm^2 examined in each animal and subregion. One way analysis of variance (ANOVA) in StatView (SAS Institute, Cary,

NC) was used for statistical comparisons between the Acb shell and core in D1R and NR1 distributions. All shell and core comparisons were performed using D1R immunoperoxidase and NR1 immunogold labeling. Additional sections from one other rat were examined using reversed secondary immunomarkers (immunogold for D1R and immunoperoxidase for NR1) to confirm that D1R and NR1 have similar labeling patterns regardless of the immunomarkers used. MCID Elite software (version 6.0, Imaging Research Inc., Ontario, Canada) was used to measure the cross-sectional diameters of dendritic profiles in the Acb shell and core. For preparation of microscopic illustrations, Adobe Photoshop software (version 7.0.1, Adobe Systems Inc., Mountain View, CA) was used to enhance contrast and sharpness of the digital images. These images were then imported into PowerPoint, Microsoft Office, 2001 to make composite plates and add lettering.

Results

Intense D1R immunoperoxidase labeling was observed by light microscopy in the Acb and the overlying caudate-putamen nucleus (Fig. 1). The striatal D1R labeling was non-homogeneous and appeared considerably more abundant in the Acb shell compared with the core (Fig. 1). Electron microscopy revealed the presence of D1R immunoreactivity in many somatodendritic and a few axonal profiles using either peroxidase or gold labeling methods in each of the Acb subregions. In the dendritic profiles, the D1R labeling had a prominent non-synaptic plasmalemmal distribution. NR1 immunoreactivity was often seen in D1R-containing dendrites or their afferent terminals, but differed from D1R in having a considerably greater cytoplasmic and synaptic distribution. There were no observed regional differences between the Acb shell and core in the subcellular distributions of NR1 in somatodendritic or axonal compartments. There were, however, significant regional differences in the number of D1R and D1R-NR1 dual-labeled dendritic profiles.

D1R and NR1 localization in somata and large dendrites

In somata, D1R immunogold particles were sparse and almost exclusively located in portions of the cytoplasm distant from the plasma membrane. These particles were often associated with endomembranous structures such as the Golgi lamellae and smooth endoplasmic reticulum (Fig. 2A).

In sections that were processed for dual labeling, D1R immunogold particles within the Acb somata occasionally contacted aggregates of NR1 immunoperoxidase reaction product. The overlapping distributions were most evident near endomembranes of the Golgi complex (Fig. 2A). Similarly, in large dendrites (diameter of 2 μ m or larger), D1R and NR1 immunoreactivities were superimposed along the endomembranes in the central portion of the cytoplasm (Fig. 2B). Dually labeled somata contained a round nucleus characteristic of striatal spiny projection neurons (DiFiglia et al., 1980). This is consistent with findings in the dendritic spines, where D1R and NR1 were also co-expressed.

D1R and NR1 distributions in medium- small dendrites and dendritic spines

D1R-labeled medium-small (<1 μ m) diameter dendrites and dendritic spines were prevalent in both Acb shell and core. Approximately 70% (382/549) of D1R immunogold particles were located on the plasma membrane. Although most dendritic spines and some shafts labeled for D1R received asymmetric, excitatory-type synapses, the gold particles were rarely detected within or near the asymmetric postsynaptic specialization. Instead, these particles were more often located at points on the plasma membrane contacted by small unmyelinated axons, or axon terminals with poorly defined membrane specializations (Fig. 3A). Some D1R-labeled dendrites were apposed to other dendrites. In these profiles, immunogold particles were observed on the plasmalemma contacting either another D1R-

labeled dendrite (Fig. 3B) or unlabeled dendrites (data not shown). In dendritic spines, D1R gold particles were particularly abundant on the plasma membrane along the spine necks, often apposed by axonal or glial profiles (Fig. 4A). In spine heads, D1R gold particles were present most frequently on the extrasynaptic plasma membrane (Fig. 4B, 4C).

The NR1 immunogold or peroxidase labeling also was commonly seen in dendrites and dendritic spines. In contrast with D1R, however, a considerably lower percentage of NR1 gold particles in dendrites were associated with the plasma membrane (27.0%; 119/440); and the majority was located within the cytoplasm. In addition, the NR1 compared with D1R was more often seen within or near asymmetric synapses on dendritic spines.

Within D1R- and NR1- dually labeled dendritic profiles, the subcellular distributions of these receptors partially coincided. In common dendrites and dendritic spines, D1R immunoperoxidase labeling was found along the spine neck and parts of the spine head, whereas NR1 immunogold particles were usually located in the spine head, across from an asymmetric excitatory-type synapse (Fig. 5A). Some dually labeled spines showed highly invaginated or perforated asymmetric synapses, typically associated with synaptic plasticity (Geinisman et al., 1989; Nikonenko et al., 2002). In these profiles, NR1 immunogold particles were located in the cytoplasm near the postsynaptic membrane specialization, while D1R immunoperoxidase products were distributed diffusely throughout the spine (Fig. 5B).

The dually labeled dendritic spines received asymmetric synapses from unlabeled axon terminals that contained either all small clear synaptic vesicles or a mixture of small clear and large dense-core vesicles (Fig. 5C). The D1R immunogold particles in these dual-labeled dendritic spines were located on the extrasynaptic membrane or in the cytosol of the spine head, while NR1 immunoperoxidase reaction products were concentrated at the postsynaptic density (Fig. 5C).

Axonal D1R distribution

In contrast with somata and dendrites, axon terminals rarely contained detectable labeling for D1R or D1R and NR1. Of all D1R labeled profiles, only 2.5% (38/1522) and 1.7% (20/1169) were axon terminals in the Acb shell and core, respectively. Axon terminals containing D1R immunogold particles either made symmetric inhibitory-type synapses (Fig. 6A) or asymmetric synapses (Fig. 6B). D1R immunogold particles were located in the cytosol, near small clear vesicles (Fig. 6A), or occasionally near the extrasynaptic plasma membrane of axon terminals (Fig. 6B).

Axonal NR1 distribution and input to D1R-labeled dendrites

In the Acb shell, 22% (205/932) of all NR1-labeled profiles were axon terminals and 24% (224/917) were axon terminals in the Acb core. Although only one or two NR1 immunogold particles were found in each profile, these particles were discretely located on the axonal plasma membrane. In contrast, no gold-silver deposits were seen over myelin or other tissue elements without known NMDA receptors within the immediate neuropil showing plasmalemmal NR1 immunogold in the axon terminals.

The axon terminals containing NR1 were presynaptic to D1R-labeled (Fig. 7A–C), or unlabeled dendritic spines. The NR1 immunogold particles were often located on the plasma membrane, away from the excitatory-type asymmetric synapse (Fig. 7A–C). This pattern of an NR1-containing axon terminal forming an asymmetric synapse on a D1R-labeled dendrite was seen in both Acb shell (Fig. 7A) and core (Fig. 7B, 7C). These synaptic associations were also evident when the immunomarkers were reversed to D1R immunogold and NR1 immunoperoxidase (data not shown).

Quantitative regional comparisons of D1R and NR1 receptor distributions

Dendrites and dendritic spines comprised almost 90% (1365/1522) of all D1R-labeled profiles in the Acb shell, and 86% (1003/1169) of these in the Acb core. In equal areas (10,200 μm^2) of neuropil, however, the Acb shell contained significantly more D1R-labeled dendrites compared to the core (843 in shell, 452 in core; $p < 0.01$; Fig. 8). Also, D1R-labeled dendrites in the Acb shell had larger dendritic diameter than those in the core (0.613 μm in shell, 0.569 μm in core; $p < 0.05$). No significant differences between the shell and core were seen in the numbers of D1R-labeled spines or axon terminals (Fig. 8). Small unmyelinated axons and glial processes made up less than 2% of the total D1R-labeled profiles and were not included in the graphs.

NR1 immunogold labeling also was usually seen in Acb dendritic profiles, but these profiles were equally prevalent in shell and core (58.5%, 545/932 in shell; 57.3%, 525/917 in core). Similar to D1R-labeled dendrites, NR1-labeled dendrites in the Acb shell had larger diameters than those in the core (0.769 μm in shell, 0.657 μm in core; $p < 0.01$). There were no significant differences between the Acb shell and core in the numbers of NR1-labeled dendritic spines or axon terminals (Fig. 8).

In the Acb shell, 56% (282/503) of all NR1 immunogold-labeled dendritic profiles contained D1R immunoperoxidase, and 28.6% (282/986) of all D1R immunoperoxidase-labeled dendritic profiles contained NR1 immunogold. In the Acb core, only 31.3% (148/472) of all NR1 immunogold-labeled dendritic profiles contained D1R immunoperoxidase, and 23.5% (148/628) of all D1R immunoperoxidase-labeled dendritic profiles contained NR1 immunogold. When the frequency of D1R- and NR1-dual labeling was examined in equal areas (10,200 μm^2) of neuropil, the Acb shell contained significantly more dual-labeled dendrites compared to the core (237 in shell, 100 in core; $p < 0.01$; Fig. 8).

Discussion

Our results provide the first ultrastructural evidence that D1Rs in rat Acb are located on extrasynaptic plasma membranes of many somatodendritic profiles in which the D1R has a partially overlapping intracellular distribution with NR1 (Fig. 9). In addition, we show that although D1Rs are sometimes present in axon terminals, these terminals rarely co-express NR1. NR1, however, is seen in terminals presynaptic to spiny dendrites containing the D1R (Fig. 9). Together, these findings suggest that both intracellular and synaptic associations contribute to the functional association between D1 and NMDA receptor systems in the Acb. The observed regional similarities and differences in D1R and NR1 distributions suggest a global impact of the D1R and NMDA receptor associations on an output from Acb, with the greatest effect on behaviors associated with the Acb shell.

Methodological considerations

The antibodies to D1R and NR1 that were used in this study have been well characterized and show high specificities in the Acb and other brain areas known to express these proteins (Weiner et al., 1991; Levey et al., 1993; Petralia et al., 1994; Gracy and Pickel, 1995; Lu et al., 1995; Gracy and Pickel, 1996). The Acb shell and core were examined with both combinations of immunomarkers (immunogold or immunoperoxidase) for D1R and NR1, and the same distributions were observed. Thus, the described immunoreactivities in the present study are thought to represent the distributions of D1R and NR1 in the Acb.

Profiles were considered as immuno-labeled when they contained even one gold particle, since background labeling was minimal. Furthermore, the average distance between two NR1 immunogold particles within longitudinally-cut dendrites was considerably greater than the thickness of ultrathin sections, reducing the frequencies of NR1 gold particles being

present within many transversely-cut dendritic profiles. Our method of quantifying profiles with one or more immunogold particles has been used when characterizing other receptors in the Acb (Pickel et al., 2004). Moreover, several studies in the striatum show that even one gold particle in a small profile can represent a greater density of labeling than multiple particles in a larger structure (Wang and Pickel, 2001; Wang and Pickel, 2002). The low frequencies of NR1 immunogold detection within a single plane of section, however, may have contributed to an underestimation of NR1 single- and D1R/NR1 dual-labeled profiles.

D1R distributions in dendrites and dendritic spines

The D1R labeling was most prominently located on the plasma membrane of dendrites and dendritic spines in both the Acb shell and core. Since D1Rs show agonist-induced internalization (Dumartin et al., 1998), the prevalence of D1R labeling on the plasma membrane suggests low levels of dopamine release under basal conditions. Alternatively, activated D1Rs may be rapidly recycled to the cell surface. This may depend in part on concurrent NMDA receptor activation, which can increase plasmalemmal insertion of D1Rs (Scott et al., 2002).

The D1R on dendrites and dendritic spines was often apposed by small axonal profiles. This is consistent with studies suggesting that dopamine is released from small 20 axons and terminals with poorly defined synapses (Groves et al., 1994; Pickel et al., 1996). The predominant distribution of dopamine transporters in small axons also supports the idea that dopamine release and reuptake occur extensively at extrasynaptic axonal locations (Nirenberg et al., 1997; Jansson et al., 1999). Physiological concentrations of dopamine are present in extrasynaptic sites in the Acb (Parsons and Justice, 1992). Therefore, the D1R distribution on the plasmalemma of dendritic profiles apposing small axons is consistent with extrasynaptic DA transmission in the Acb. Such transmission might also be facilitated by the compartmentalization produced by glial profiles. This is suggested by observed contacts between astrocytic processes and D1R-labeled plasma membranes of dendrites in the present study.

Some D1R immunogold particles were found in two apposing dendrites, both of which resembled those of spiny neurons that are mainly GABAergic (Meredith, 1999). The D1R plays a role in synchronization of neuronal networks through electrical or other types of coupling between Acb dendrites (O'Donnell and Grace, 1993). The activation of D1Rs on surfaces of apposed dendrites would require diffusion of dopamine from more distant axons. Such diffusion may be facilitated by bursting activity of DA neurons in response to relevant sensory activation (Overton and Clark, 1997). DA modulation of connexin proteins at gap junctions of Acb shell may contribute to emotional and locomotor responses (Dere et al., 2003).

Overlapping endomembrane and differential synaptic distributions of D1R and NR1 in dendritic profiles

Our results provide ultrastructural evidence that D1R and NR1 have partially overlapping endomembrane distributions. This observation suggests that the previously reported protein-protein interactions between the D1R and NR1 in *in vitro* systems (Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004) also occurs in intracellular compartments of Acb neurons. The specific association of D1R and NR1 with some of the same dendritic endomembranes also may, however, reflect commonalities in receptor trafficking without necessarily having physical associations. In comparison with D1Rs and other G protein-coupled receptors, considerably less is known about the NMDA receptor subunits (Brady and Limbird, 2002; Wenthold et al., 2003). Conceivably, prior activation of D1R could

markedly inhibit NMDA-mediated currents due to intracellular associations that diminish the availability of essential NR1 subunits at the dendritic surface.

In contrast with dendritic endomembranes, D1R and NR1 had largely differential distributions in dendritic spines. The D1R was located on extrasynaptic plasma membranes of the spine neck, while NR1 was located near the asymmetric excitatory-type post-synaptic density in the spine head. These are strategic locations, because DA inputs terminate on the spine neck, while glutamatergic inputs terminate on spine heads in the Acb (Sesack et al., 2003). These distributions suggest that dopamine and glutamate differentially activating the D1 and NMDA receptors are principally derived from different terminals. The presence of D1R and NR1 in common spines receiving asymmetric perforated synapses suggest that both D1R and NMDA receptors may be involved in plasticity (Geinisman et al., 1989; Nikonenko et al., 2002). These dual-labeled Acb spines receiving perforated synapses may play a particularly important role in reward and appetitive instrumental learning mediated through Acb shell and core (Ikemoto et al., 1997; Smith-Roe and Kelley, 2000; Floresco et al., 2001).

Axonal D1R and NR1 distributions

D1R labeling was detected in axon terminals including those that made asymmetric or symmetric synapses. We have shown previously that D2 receptors are located presynaptically in corticostriatal terminals forming asymmetric excitatory-type synapses with spine heads (Wang and Pickel, 2002) and in striatal GABAergic terminals (Delle Donne et al., 1997). Moreover, D1- and D2-like receptors are co-localized in presynaptic varicosities of both the dorsal striatum and Acb (Wong et al., 1999). Thus, at least some of the D1R-labeled terminals may include those that also contain D2 receptors. The results confirm and extend prior studies showing that D1R activation can affect the presynaptic release of both glutamate and GABA (Ding et al., 2003; Guzman et al., 2003; Paspalas and Goldman-Rakic, 2005).

NR1 immunoreactivity was found in many axon terminals, none of which expressed D1R. The co-expression of D1R in dendrites, but not in axon terminals suggests important differences in the dendritic and axonal targeting of these receptors. The NR1-labeled terminals were morphologically heterogeneous and may have included many containing glutamate, but distinct from those that express D1R. Presynaptic actions mediated through NMDA receptors may play a role in facilitating calcium entry and glutamate release (Breukel et al., 1998; Woodhall et al., 2001). Other NR1 terminals resembled those of DA axons previously shown to sometimes express NR1 (Gracy and Pickel, 1996). Presynaptic NMDA receptors are also known to regulate dopamine synthesis and release (Chowdhury and Fillenz, 1991; Krebs et al., 1991).

We observed NR1 labeling in some of the terminals providing input to D1R-containing dendrites. The NR1-labeled terminals presynaptic to D1R-immunoreactive dendrites were morphologically heterogeneous and may have included those containing either glutamate or dopamine (see above). Thus, changes in the release of these neurotransmitters may be an important synaptic mechanism for modulation of the output of a specific subpopulation of Acb spiny neurons that express D1Rs. D1R mRNA is co-expressed with protachykinin and prodynorphin in the Acb neurons (Curran and Watson, 1995; Lu et al., 1998). Together, these results suggest that NMDA receptor activation may alter the release of tachykinin and/or dynorphin through indirect actions on afferent terminals.

Regional comparison of Acb D1R and NR1 distribution

We observed a higher density of D1R- or D1R/NR1-labeled dendrites in the Acb shell compared with the core. This is consistent with known enrichment of D1R mRNA levels in the Acb shell (Lu et al., 1998) and suggests that functional and physical interactions between D1R and NMDA receptors may have greater impact on the Acb reward circuitry. This does not negate the importance of these associations within the Acb core, where there is major involvement of both D1 and NMDA receptors in conditional motor behaviors (Kelley et al., 1997; Smith-Roe and Kelley, 2000; Di Ciano et al., 2001). The regional similarities in the types of associations between D1R and NR1 in the Acb suggest their generalized relevance for the many adaptive behaviors ascribed to these receptor systems.

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Figure 1.

Light micrograph showing immunoperoxidase labeling of D1R in the caudate-putamen nucleus (CPN) and the region of the Acb shell and core that was sampled for electron microscopy. In this half coronal section through the rat brain, the Acb D1R immunoperoxidase labeling is unevenly distributed and especially dense in the shell subregion. Ultrathin sections for electron microscopy were collected from the Acb shell and core directly medial to the anterior commissure (ac) at level 1.6mm anterior to Bregma (Paxinos and Watson, 1986). Each section included both core and shell as indicated by the trapezoid. The myelinated axons in the anterior commissure and the base of the trapezoid were used for orientation in the analysis of electron microscopic images. cc, corpus callosum; CTX, cortex; lv, lateral ventricle. Corner arrows indicate the dorsal (d) and medial (m) brain regions. Scale bar, 500 μ m.

Figure 2.

Electron micrographs from Acb shell showing somatodendritic co-localization of D1R and NR1. **A.** In a soma, D1R immunogold particles (arrowheads) are present near the Golgi (G) and endomembranes (em), where there is partial overlap with diffuse NR1 immunoperoxidase product (arrow). **B.** Within a dually labeled large proximal dendrite (D1&NR1-De), one D1R immunogold particle (arrowhead) superimposes NR1 immunoperoxidase labeling (arrow) near membranous organelles in the central portion of the cytoplasm. Scale bar, 0.5 μm .

Figure 3.

Plasmalemmal D1R immunogold labeling in dendritic profiles within the Acb shell. **A.** D1R immunogold particles (arrowheads) in a dendrite (D1-De) and spine (D1-Sp) are located at the plasma membrane apposed by unlabeled small unmyelinated axons (ua1&2) and axon terminals (ut1&2). The spine receives an asymmetric synapse (curved arrow) from an unlabeled terminal (ut3). **B.** D1R immunogold particles (arrowheads) in a dendrite (D1-De1) are located on the plasma membrane apposed by another D1R-labeled dendrite (D1-De2). Both dendrites are apposed by a single unlabeled terminal (ut), which shows a symmetric synapse (dotted arrow) exclusively with D1-De1. Scale bar, 0.5 μm .

Figure 4.

D1R immunogold labeling in dendritic spines in Acb shell (A, B) and core (C). **A.** D1R gold particles (arrowheads) are located along the plasma membrane in the neck of a dendritic spine (D1-Sp) apposed by unlabeled axons (ua1&2) and a small glial process (asterisk). The dendrite also shows an obliquely sectioned synapse from an unlabeled terminal (ut) that appears to be asymmetric (curved arrow). **B.** D1R gold particles (arrowheads) are seen on or near the extrasynaptic plasmalemma of a dendritic spine (D1-Sp). The immunogold particles are apposed by a glial process (asterisk). The spine receives input from an unlabeled terminal (ut) making an asymmetric synapse (curved arrow). **C.** One D1R gold particle (arrowhead) is located on the perisynaptic membrane of a dendritic spine (D1-Sp), while the

others are on the extrasynaptic membrane. The gold particles are apposed by an unlabeled axon (ua) and a glial process (asterisk). The spine receives an asymmetric synapse (curved arrow) from an unlabeled terminal (ut). sa, spine apparatus. Scale bar, 0.5 μ m.

Figure 5.

D1R and NR1 distributions in common spines in Acb shell. **A.** In a dually labeled spine (D1&NR1-Sp), D1R immunoperoxidase reactivity (straight arrow) is most prominent on the plasmalemma of a process resembling the neck of a spine, originating from a dendrite (De) without detectable labeling. NR1 immunogold aggregate (arrowhead) is located in more distal portions of the spinous process near an asymmetric synapse (curved arrow) from an unlabeled terminal (ut). **B.** NR1 immunogold particles (arrowheads) are located in the cytoplasm within and near the postsynaptic membrane, while D1R immunoperoxidase products are seen diffusely throughout a dually-labeled dendritic spine (D1&NR1-Sp). This spine is receiving a perforated excitatory-type synapse (curved arrow). **C.** D1R immunogold particles (arrowheads) in a dually-labeled dendritic spine (D1&NR1-Sp) are located within the cytoplasm and on extrasynaptic portions of the plasmalemma apposing an unlabeled axonal profile (ua). NR1 immunoperoxidase reaction products are diffusely distributed throughout the cytoplasm and along the dendritic specialization (straight arrow) of an asymmetric synapse (curved arrow). The presynaptic terminal is unlabeled and contains a mixture of small clear and large dense-core vesicles (dcv). us, unlabeled spine. Scale bar, 0.5 μ m.

Figure 6.

D1R immunogold labeling in axon terminals in Acb shell (A) and core (B). **A.** Two immunogold particles (arrowheads) are located in the central portions of an axon terminal (D1-Te). This terminal is joined by a symmetric synapse (dotted arrow) to a dendrite containing one D1R immunogold particle in the cytoplasm near an endomembranous structure (em) presumed to be smooth ER. **B.** D1R immunogold particles (arrowheads) are located on the extrasynaptic plasma membrane of an axon terminal (D1-Te), near appositions with two unlabeled axon terminals (ut1, ut2). Another particle in the same terminal is in the cytosol near the presynaptic specialization at an asymmetric synapse (curved arrow). us, unlabeled spine. scv, small clear vesicle. Scale bar, 0.5 μm.

Figure 7.

D1R immunoperoxidase labeled dendritic spines (D1-Sp) receiving asymmetric synapses (curved arrow) from NR1 immunogold-labeled axon terminals (NR1-Te) in the Acb shell (A) and core (B, C). **A.** NR1 immunogold particles (arrowheads) are located on the extrasynaptic plasma membrane of an axon terminal (NR1-Te). The D1R immunoperoxidase products are prevalent along the extrasynaptic plasmalemma (arrow), but also diffusely distributed within the D1-Sp. **B.** An NR1 immunogold particle (arrowhead) is located on the extrasynaptic plasma membrane of a small axon terminal (NR1-Te). **C.** Two NR1 immunogold particles (arrowheads) are distributed on the extrasynaptic membrane of

an axon terminal (NR1-Te) and another is located in the cytosol near small clear vesicles. us, unlabeled spine. ua, unlabeled axon. Scale bar, 0.5 μm .

Figure 8.

Bar graphs showing the number and types of neuronal profiles immunolabeled for D1R, D1R and NR1, or NR1 in the rat Acb medial shell and core. Data were obtained from one vibratome section from each of three rats. For each rat, an area of at least 3400 μm^2 of neuropil was examined in each subregion, and the labeled profiles were identified and counted. Both D1R and NR1 labeling are observed in somatodendritic and axonal profiles. ANOVA shows that there are significantly more D1R-labeled dendrites in the shell than in the core (* $p < 0.01$). Also, significantly more dendrites are dual-labeled in the Acb shell than in the core (* $p < 0.01$). No other profiles showed significant shell and core differences in number.

Figure 9.

Schematic diagram showing the subcellular distributions of D1R (white isosceles triangles) and NR1 (black chevrons) in a medium spiny Acb neuron. In the soma and proximal dendrite, D1R and NR1 are located in close proximity to each other near endomembranes (em). In a small-medium dendrite, D1R is present on the plasma membrane and NR1 is present in the cytoplasm. A dendritic spine containing both D1R and NR1 is shown on the top. In this spine, NR1 is located near the excitatory-type asymmetric synapse, while D1R is present on the extrasynaptic plasma membrane apposed by an axonal profile (ap). A D1R dendritic spine receiving an excitatory input from an NR1-containing terminal is shown on the left. A dendritic spine expressing D1R alone is shown on the top right. In this spine,

D1R is located on the extrasynaptic plasma membrane, apposed by an axonal profile. A dendritic spine containing NR1 near the excitatory synapse is shown on the bottom right. This drawing does not indicate that all of these spines originate from the same neuron.