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ORIGINAL PAPER

# Endosomal location of dopamine receptors in neuronal cell cytoplasm

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**Abstract** Five subtypes of dopamine receptor exist in two subfamilies: two $D_1$-like ($D_1$ and $D_5$) and three $D_2$-like ($D_2$, $D_3$ and $D_4$). We produced novel monoclonal antibodies against all three $D_2$-like receptors and used them to localize receptors in Ntera-2 (NT-2) cells, the human neuronal precursor cell line. Most of the immunostaining for all three receptors colocalized with mannose-6-phosphate receptor, a marker for late endosomes formed by internalization of the plasma membrane. This result was obtained with antibodies against three different epitopes on the $D_3$ receptor, to rule out the possibility of cross-reaction with another protein, and controls without primary antibody or in the presence of competitor antigen were completely negative. In rat cerebral cortex and hippocampus, some of the dopamine receptor staining was found in similar structures in neuronal cell cytoplasm. Only some of the neurons were positive for dopamine receptors and the pattern was consistent with previously-reported patterns of innervation by dopamine-producing neurons. Endosomal dopamine receptors may provide a useful method for identifying cell bodies of dopamine-responsive neurons to complement methods that detect only active receptors in the neuronal cell membrane.

**Keywords** G protein-coupled receptor · Endosome · NT-2 cells · Ntera-2 · Monoclonal antibody · Brain · Hippocampus · Cerebellum · Cerebral cortex · Mannose-6-phosphate receptor

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## Introduction

Dopamine receptors are members of the G protein-coupled receptor (GPCR) family, which initiate their biological function by coupling to GTP regulatory proteins (G proteins). In humans, five dopamine receptor subtypes, DRD1 (Monsma et al. 1990), DRD2 (Bunzow et al. 1988), DRD3 (Sokoloff et al. 1990), DRD4 (van Tol et al. 1991) and DRD5 (Sunahara et al. 1991) have been identified. The $D_1$-like receptors, DRD1 and DRD5, stimulate adenylyl cyclase (Monsma et al. 1990; Albert et al. 1990) whereas the $D_2$-like receptors, DRD2, DRD3 and DRD4, are coupled to inhibitory G proteins and their stimulation leads to inhibition of adenylyl cyclase (Sokoloff et al. 1990). All five have an extracellular amino terminus, seven transmembrane domains and an intracellular carboxyl terminus. Abnormalities in dopamine neurotransmission are implicated in the aetiology of neurological and psychiatric disorders including schizophrenia (Williams et al. 1998) and Parkinson's disease (Oliveri et al. 2000).

The distribution of $D_2$-like dopamine receptor mRNAs in brain has been studied by in situ hybridization (Sokoloff et al. 1990; Mengod et al. 1989) and radioligand binding studies (Martres et al. 1985; Bouthenet et al. 1987; Camps et al. 1989) have revealed the distribution of biologically-active receptors, including the use of sub-type selective ligands (Levesque et al. 1992; Herroelen et al. 1994; Hall et al. 1996; Bancroft et al. 1998). Most immunohistochemical studies of $D_2$-like receptors so far have used only a single antibody, with the notable exception of two studies of DRD4 that used antibodies against two epitopes (Defagot et al. 1997; Mauger et al. 1998). Use of antibodies against two or more epitopes is the best safeguard against cross-reaction, especially with monoclonal antibodies (Morris 1998). The reported sizes of $D_2$-like dopamine receptors on western blots have varied widely in the literature (36–95 kDa) and cross-reaction with other proteins has not always been ruled out.

In the present study, we have used a new panel of antibodies against three different DRD3 epitopes to determine the cellular and subcellular localization of DRD3 in rat brain and Ntera-2 cells. Additional monoclonal antibodies against specific peptide sequences in DRD2 and DRD4 were used to support and confirm the subcellular localization of $D_2$-like dopamine receptors. We suggest that the presence of $D_2$-like dopamine receptors in cytoplasmic vesicles could provide a useful means to identify the cell bodies of dopamine-responsive neurons, since neuronal extensions with dopamine receptors often project far into other regions of the brain.

## Methods

### Antibody production

For monoclonal antibodies, Balb/c mice were immunized with peptides conjugated to bovine serum albumin (BSA) through cysteine residues using MBS (3-maleimidobenzoic acid *N*-succinimidyl ester). Suitable human peptide sequences were identified on the basis of specificity for each subtype and antigenicity. The peptides were synthesized by Alta Bioscience (University of Birmingham, UK). Hybridoma fusions with the Sp2/0 mouse myeloma cell line were performed as described previously (Nguyen and Morris 1996). Hybridoma culture supernatants were screened by enzyme linked immunosorbent assay (ELISA) using plates coated with the respective unconjugated peptide. ELISA positive wells were further screened by Western blotting on total Ntera-2 protein extract and total brain (rat and human) extract.

A rabbit polyclonal antiserum raised against the same N-terminal DRD3 peptide was obtained from Calbiochem. A polyclonal antiserum was produced against a DRD3 C-terminal recombinant protein (amino acids 205–400) by immunization of Balb/c mice. The corresponding cDNA was obtained from a full-length human DRD3 construct by PCR and was cloned into pET 32a expression vector (Novagen, Darmstadt, Germany). The expressed protein was purified for immunization by affinity chromatography using His.Bind resin (Novagen, Darmstadt, Germany) and used to immunize Balb/c mice. All animal procedures were performed under UK license.

### Enzyme linked immunosorbent assay

A 96 well PVC microtitre plate (Dynatech, Billingshurst, UK) was coated with 50 µl per well of the peptide at a concentration of 5–10 µg/ml in PBS, and incubated at room temperature for 1 h. The antigen was then removed and the plate was washed 3 times with incubation buffer (IB, PBS + 1% Triton X-100). 300 µl of a 4% solution of skimmed milk powder in IB was added to each well for 30 min at room temperature. After washing 3 times with IB, 50 µl of culture supernatant was added at room temperature for 1 h. The primary antibody was removed and the plate washed 4 times with IB. Horseradish peroxidase-conjugated rabbit anti-mouse Ig (DAKOpatts, Denmark; 50 µl of 1/1,000 in dilution buffer: 0.1% BSA, 1% horse serum and 1% fetal calf serum in IB) was then added and incubated at room temperature for 1 h. The plate was washed 4 times with IB. One hundred and fifty microlitres of substrate solution (0.4 mg/ml *o*-phenylene-diamine in 25 mM phosphate-citrate buffer pH 5.0 with 0.1% $H_2O_2$) was added until the colour developed. The reaction was then stopped with 50 µl of 3 M $H_2SO_4$. Samples were read using a 492 nm filter on a Titertek Multiscan Plate reader.

### Western blotting

Human brain samples were provided by the Medical Research Council Brain Bank, Kings College, London, UK. Total protein extracts of human or rat brain were made by Dounce homogenization in four volumes of SDS extraction buffer (10% SDS, 10% EDTA, 5% 2-mercaptoethanol, 10% glycerol, 50 mM Tris–HCl, pH 7.0) and clarified by centrifugation at 160,000 *g* for 30 min. After SDS-PAGE on 10% polyacrylamide gels, proteins were transferred to nitrocellulose electrophoretically overnight. After blocking for 1 h in 3% skimmed milk powder in incubation buffer (0.05% Triton X-100 in PBS), blots were incubated with mAb (1/100 dilution of culture supernatant in incubation buffer/1% horse serum/1% fetal calf serum/0.1% BSA) for 1 h. After washing 3 times in PBS, blots were incubated with biotinylated anti-mouse Ig and a peroxidase-avidin detection reagent (Vectastain ABC Elite, Vector Laboratories, Peterborough,

UK) according to the manufacturer's instructions. Blots were developed with diaminobenzidine in 25 mM phosphate-citrate buffer (pH 5.0), 0.012% $H_2O_2$.

### Cell culture

The human embryonal carcinoma cell line, Ntera-2 (Andrews et al. 1984), also known as NT-2, was cultured in Dulbecco's MEM with Glutamax-I (Invitrogen, Paisley, UK) and 10% fetal bovine serum (Invitrogen, UK). Cells were seeded onto coverslips at $5 \times 10^5$ cells and, after culture for 48 h, were fixed in 50:50 acetone–methanol for 5 min.

### Preparation of rat brain sections

A whole brain was removed from an adult Sprague-Dawley rat immediately post-mortem and rinsed in ice-cold isotonic saline for 1 h. It was then mounted and frozen in isopentane cooled in liquid nitrogen and sectioned dorsolaterally into 5 μm frozen sections. The sections were mounted on microscope slides in the same orientation and allowed to air dry.

### Immunofluorescence microscopy

Cells and tissue sections were fixed using 50% acetone/50% methanol. The primary antibody was diluted in PBS/1% horse serum/1% fetal calf serum and incubated on the slide for 1 h at room temperature. After washing with PBS, FITC-conjugated rabbit anti-(mouse Ig) (1/50: Vector Labs, Burlingame, USA) in PBS/1% horse serum/1% fetal calf serum was applied for 30 min. After counterstaining nuclei with ethidium bromide, slides were mounted in 10% glycerol in PBS and photographed using a Leica DMRB epifluorescence photomicroscope with 10× or 63× objectives. For double labelling, fixed cells were incubated sequentially with rabbit antisera and mouse mAb for 1 h, followed by a mixture of FITC-conjugated anti-(mouse Ig) (1/50 dilution) (Vector Labs, Burlingame, CA, USA) and TRITC-conjugated anti-(rabbit Ig) (1/100 dilution) (DAKO, Glostrup, Denmark) for 30 min. Confocal images were captured using a Zeiss LSM510 Meta confocal system.

## Results

### Production of antibodies that recognize 50 kDa dopamine receptors in human brain

A panel of monoclonal antibodies (Table 1) was raised against the three $D_2$-like dopamine receptors using BSA-conjugated, subtype-specific peptides corresponding to the human DRD3 N-terminal domain (ASLSQLSSH-C; two mAbs MANDOP31 and 32), the DRD3 third cytoplasmic domain (RIYVVLKQR-C; three mAbs MANDOP33-35), the DRD2 third cytoplasmic domain (AARRAQELEME-C; two mAbs MANDOP21 and 22) and the DRD4 third extracellular domain (DVRQRDPAVC; one mAb MANDOP41). Hybridoma fusions were screened by ELISA with unconjugated peptide and by Western blotting using total human and rat brain extracts. Figure 1a shows three mAbs against both DRD3 epitopes (MANDOP31, 32 and 35) that recognize a band of the expected size (50 kDa) in a human brain extract. The two mAbs against DRD2 and one against DRD4 also recognized a protein of the same size (Fig. 1b). The DRD2 and N-terminal DRD3 peptide immunogens show no sequence similarity to the other DRD subtype sequences, but the DRD4 peptide does have four of ten amino acids identical to DRD3 only, so the possibility of cross-reaction with DRD3 cannot be entirely ruled out. A C-terminal DRD3 fragment (amino acids 205–400) expressed from a pET32 plasmid and purified by affinity chromatography was used to produce a polyclonal antiserum that will recognize a third DRD3 epitope for confirmation of specificity. Subcellular fractionation was carried out on total Ntera-2 cell extract by a series of high-speed centrifugations. The 50 kDa protein recognized by MANDOP35 was absent from cytosolic fractions until extracted by Triton X-100, consistent with membrane-associated DRD3 (Fig. 1c).

**Table 1** A panel of monoclonal antibodies against different epitopes of each of the $D_2$-like dopamine receptors

| Antibody name | Clone number | Dopamine receptor | Antigen (amino acids) |
|---|---|---|---|
| MANDOP 31 | 1D7 | DRD3 | N-terminus (2–10) |
| MANDOP 32 | 3F1 | DRD3 | N-terminus (2–10) |
| MANDOP 33 | 3B7 | DRD3 | 3rd Cytoplasmic domain (210–218) |
| MANDOP 34 | 4D10 | DRD3 | 3rd Cytoplasmic domain (210–218) |
| MANDOP 35 | 9F4 | DRD3 | 3rd Cytoplasmic domain (210–218) |
| MANDOP 21 | 3B9 | DRD2 | 3rd Cytoplasmic domain (272–282) |
| MANDOP 22 | 3D9 | DRD2 | 3rd Cytoplasmic domain (272–282) |
| MANDOP 41 | 2B9 | DRD4 | 3rd Extracellular domain (176–185) |

### Most of the $D_2$-like dopamine receptors in cultured Ntera-2 cells are found in endosomal vesicles in the cytoplasm

Ntera-2 cells exhibit properties of a committed neuronal precursor at an early stage of development (Andrews et al.

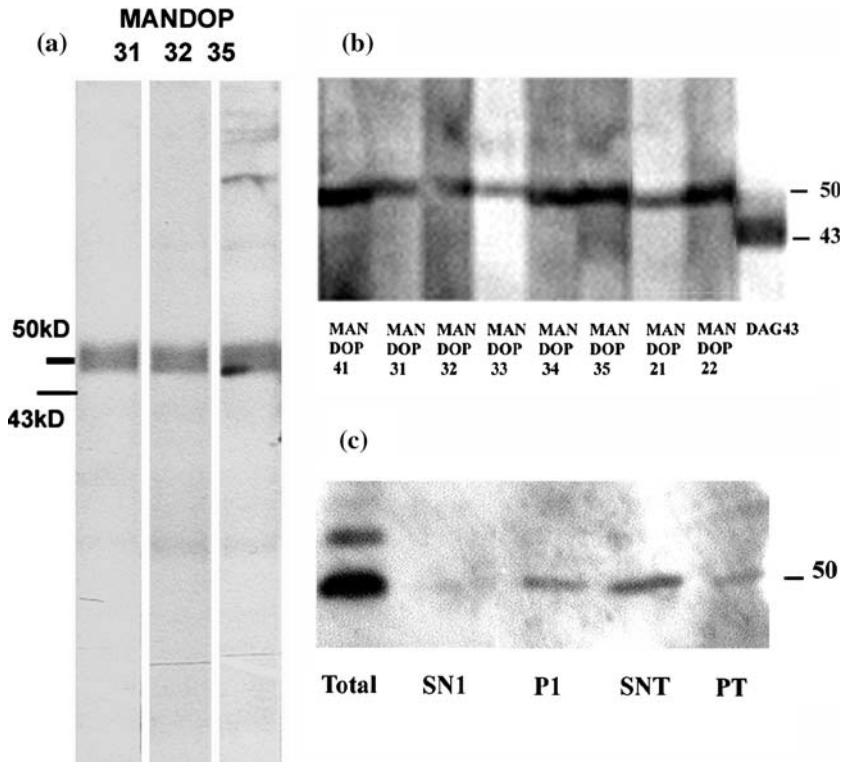


**Fig. 1** Monoclonal antibodies against $D_2$-like dopamine receptors recognize proteins of 50 kDa that are associated with membranes. (**a**) Western blots of human brain cortex extract were developed with three dopamine D3 receptor antibodies at 1/100 dilution of culture supernatant, two mAbs against the N-terminus and one against the 3rd cytoplasmic domain. A control antibody against 43 kDa beta-dystroglycan [DAG43] (Pereboev et al. 2001) was used as a size marker. (**b**) Western blots of a different human brain cortex extract developed with all DRD2, DRD3 and DRD4 antibodies at 1/100 dilution of culture supernatant. (**c**) Subcellular fractionation of Ntera-2 cells by western blotting. Cell pellets were rinsed in PBS, harvested and homogenised in nine volumes of RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5) using a Dounce homogenizer. The suspension was incubated on ice for 15 min and sonicated before centrifuging at 160,000 *g* for 15 min. The supernatant was removed (cytosol, SN1) and the pellet, which contains all membrane fractions, was resuspended in RSB/1% w/v Triton (P1). The suspension was incubated on ice for 15 min, sonicated and centrifuged as described above. The supernatant (Triton-extractable membrane proteins; SNT) was removed and the pellet was resuspended in RSB buffer (PT). All fractions were boiled with an equal volume of 2× SDS sample buffer [2% SDS, 10% 2-mercaptoethanol, 125 mM Tris–HCl (pH 6.8)] for Western blotting with MANDOP35 mAb against DRD3 (1/100)

1984) and have been shown by reverse transcriptase-polymerase chain reaction and functional studies to express DRD2 and DRD3 dopamine receptors (Hurlbert et al. 1999; Sodja et al. 2002). Using immunofluorescence microscopy, all dopamine receptors were found almost exclusively in large cytoplasmic vesicles in Ntera-2 cells (Fig. 2a). No such staining was seen in HeLa cells or human skin fibroblasts. To show that this staining pattern was not due to a cross-reaction, double label immunofluorescence microscopy was carried out using a mouse monoclonal antibody against the DRD3 third cytoplasmic domain (MANDOP34), and a commercial rabbit polyclonal antiserum against the DRD3 N-terminus and their co-localization was complete (Fig. 2b). All three monoclonal DRD3 epitopes gave the same result and there was no staining in the absence of primary antibody (inset: Fig. 2a). Antibody staining of vesicles was also prevented by the peptide antigen (see Fig. 3). A double label using the same commercial rabbit antiserum against DRD3 and a commercial mouse monoclonal antibody against mannose-6-phosphate receptor, a marker for late endosomes, showed co-localization (Fig. 2c), suggesting that the cytoplasmic vesicles are a late stage in the pathway for receptor internalization and turnover via lysosomes.

## Some dopamine receptors were detected at the expected plasma membrane sites

One would normally expect to find $D_2$-like dopamine receptors in the plasma membrane, close to synapses with dopamine-producing neurons, and Fig. 2d shows a section of rat basal ganglia with DRD3 staining of dendrite-like processes. Similar staining was observed in other areas of the brain, including cortical layer V of cerebral cortex, where dopaminergic innervation is also expected (not shown). Identification and proper characterization of different dendritic processes is a complex neuroscience study beyond the scope of this paper, but we show Fig. 2d to

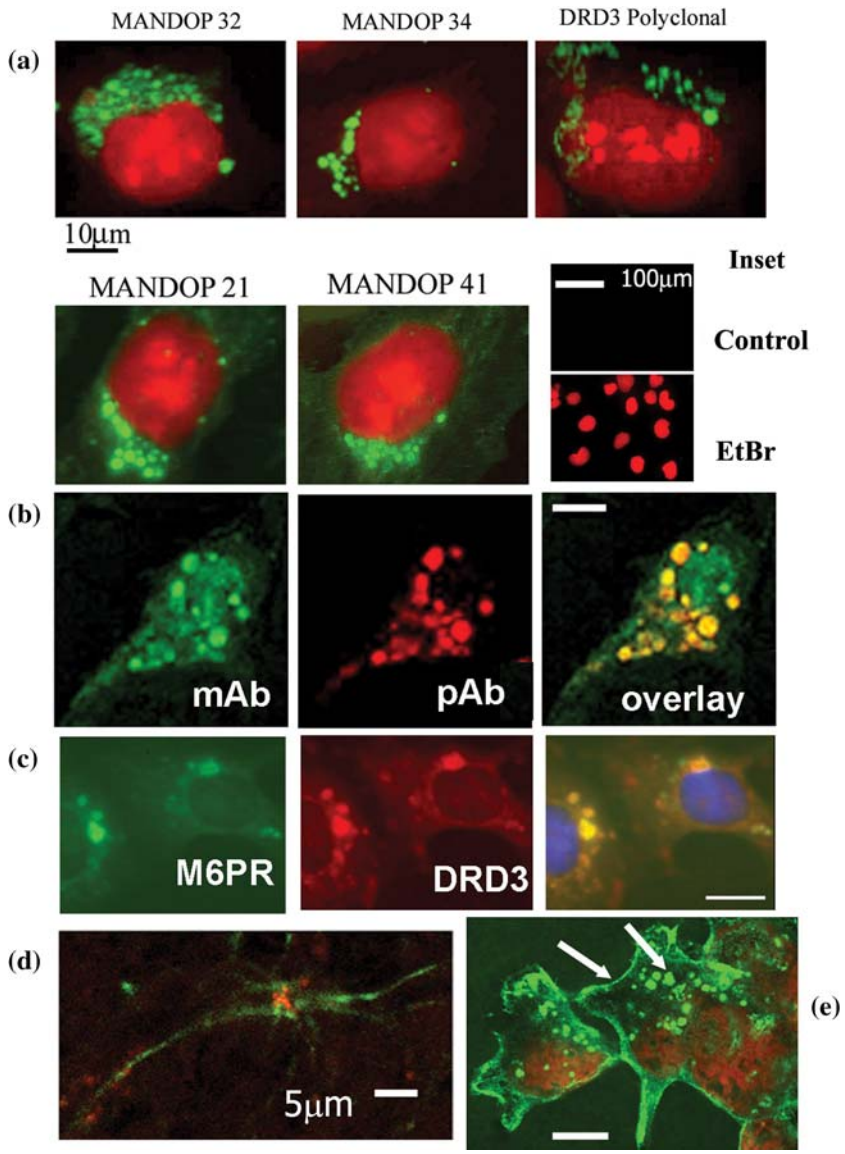


**Fig. 2** Localization of $D_2$-like dopamine receptors in endosomes in Ntera-2 cells and on dendritic processes in rat brain. All size *bars* are 10 microns, except where indicated. (**a**) Each receptor (*green* indicates FITC conjugated anti-mouse Ig) is localized in cytoplasmic vesicles, often adjacent to the nucleus (*red* indicates ethidium bromide, 1 μg/ml). The control experiment (*inset*) shows that DRD3 fluorescence was completely absent when the primary mAb was omitted. (**b**) DRD3 distribution in Ntera-2 cells was examined by double-labelling with a commercial rabbit polyclonal antibody (*red* indicates TRITC conjugated anti-rabbit Ig) against the DRD3 N-terminus (Calbiochem) (1/500) and a DRD3 monoclonal antibody (*green*) against the 3rd cytoplasmic domain (MANDOP 34) (1/4). The overlay shows good colocalization (*yellow*). (**c**) Ntera-2 cells were double-labelled with the same rabbit antiserum against DRD3 (1/500: *red*) and a mouse monoclonal antibody against mannose-6-phosphate receptor [Ab2733 (2G11) Abcam, Cambridge, UK: 1/200: *green*]. In the overlay, nuclei were counterstained using DAPI (1 μg/ml: *blue*). (**d**) Sections from rat basal ganglia were stained with MANDOP34 (1/4) and FITC conjugated anti-mouse (1/50) and counterstained with ethidium bromide (1 μg/ml). DRD3 receptors are localized in thin processes. This staining was absent in controls in which primary mAb was replaced with culture medium (not shown). These photographs were taken with a Zeiss LSM510 Meta confocal system. (**e**) DRD3 staining of both plasma membrane (*left white arrow*) and internal vesicles (*right white arrow*) in Ntera-2 cells. Staining as in (**d**)

emphasize that our mAbs may detect dopamine receptors in their functional location in plasma membranes, as well as in endosomes. Indeed, DRD3 receptors are sometimes detectable in the plasma membrane of Ntera-2 cells (Fig. 2e), although cell culture appears to favour location in endosomes.

## Endosome-like staining by dopamine receptor antibodies can be used to identify specific subsets of rat brain neurons

Figure 3a shows that all three receptors are also present as cytoplasmic vesicles in neurons of cortical layers V and VI

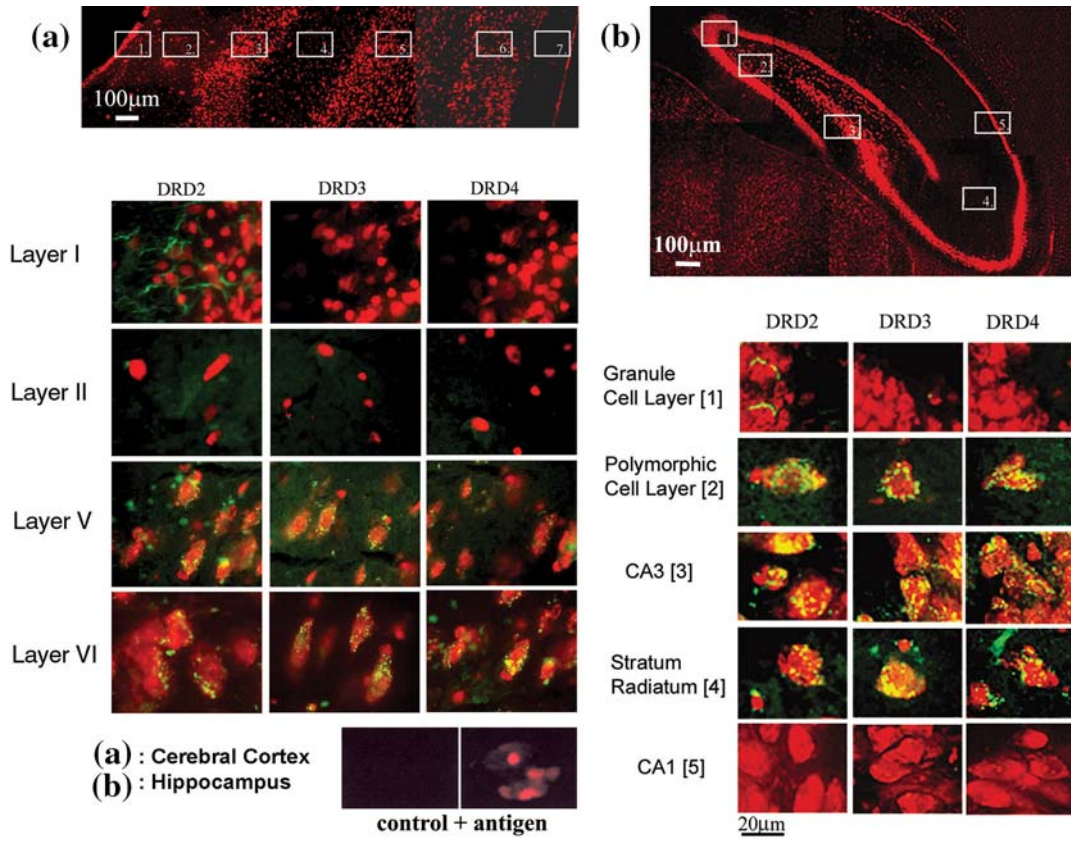


**Fig. 3** Distribution of $D_2$-like dopamine receptors in rat cerebral cortex and hippocampus. Five micrometer serial sections were taken through a whole rat brain. The distribution of all three $D_2$-like dopamine receptors in selected sections was examined using DRD2 (MANDOP 21), DRD3 (MANDOP 34) and DRD4 (MANDOP41) (all 1/4) and FITC conjugated anti-mouse Ig (1/50). Cell nuclei were counterstained with ethidium bromide (1 μg/ml). The ethidium bromide stain was photographed at low power (10× objective) and a montage of the whole section was produced. High power photographs (63× objective) of the regions shown in *boxes* were used to determine the subcellular distribution of $D_2$-like dopamine receptors. (**a**) Cerebral cortex. The *boxes* labelled 1–6 correspond to cortical layers I–VI. Layers III and IV, like layer II, showed no antibody staining for receptors (not shown). The "control + antigen" inset illustrates the prevention of antibody staining by pre-incubation with peptide immunogen; in this case, MANDOP34 (*left*, *green*) on layer V cells in the cortex; ethidium bromide counterstain (*right*, *red*). (**b**) Hippocampus. The montage includes granular neurons of the dentate gyrus and CA1, CA2 and CA3 neurons of the hippocampus. CA3 neurons showed consistent staining of cytoplasmic vesicles for all three receptors, as did occasional neurons in the polymorphic cell layer and within the stratum radiatum and lacunosum regions of the hippocampal formation

of rat cerebral cortex. Antibody staining of the vesicles was prevented by pre-incubation with peptide antigen (see inset for DRD3 control). The DRD2 mAb shows additional staining in layer I, but, with only one mAb available, we cannot rule out cross-reaction with a non-DRD protein. Cross-reaction of MANDOP41 with DRD3 cannot be entirely ruled out. Figure 3b shows a section through the hippocampus and dentate gyrus. Cytoplasmic vesicles containing $D_2$-like dopamine receptors were found in CA3 and other neurons, but were absent from CA1 neurons and from granule cells of the dentate gyrus. Similar endosome-like vesicles were observed in cerebellum (Purkinje neurons) and brain stem (large bipolar neurons) (data not shown). This shows that DRD-positive vesicle staining could be a useful means of identifying the cell bodies of neurons that are making different dopamine receptors.

## Discussion

Immunolocalization studies using monoclonal antibodies against the $D_2$-like dopamine receptors have shown an endosomal distribution in the Ntera-2 cell line. This distribution pattern is not a staining artifact, since it is completely dependent on the presence of the primary DRD-specific mAb and is blocked by pre-incubation with antigen. We are confident of this localization for DRD3 since an identical pattern was produced by mAbs against two different epitopes in the N-terminal and third cytoplasmic domains, as well as by an antiserum against the C-terminal region and a commercial rabbit antiserum. This confidence was reinforced (a) by the fact that mAbs against DRD2 (different part of third cytoplasmic domain) and DRD4 (third extracellular domain) stained these vesicles,

suggesting that all three $D_2$-like dopamine receptors are co-distributed in these vesicles in Ntera-2 cells, and (b) by the observation of similar vesicles in rat brain sections. Because unique peptide sequences were chosen as immunogens for each subtype, cross-reaction between $D_2$-like dopamine receptors is hardly possible, except for DRD4 where the possibility of cross-reaction with DRD3 cannot be entirely ruled out. Cross-reaction with some functionally-unrelated protein can be ruled out when antibodies against two or more epitopes are used (i.e. DRD3) but not when only one mAb is available (e.g. DRD2 in Fig. 3). According to current ideas about GPCR trafficking in general (Dohlman et al. 1991), and that of dopamine receptors in particular (Ng et al. 1994), receptors are internalized in "early endosomes" from which they may be recycled to the plasma membrane or targeted for turnover in "late endosomes". It is interesting to note that the dopamine transporter protein, which regulates dopamine receptor activation, is known to be internalized into endosomes by a clathrin-dependent mechanism (Sorkina et al. 2005). Internalization and recycling of GPCRs involves arrestin and a phosphorylation/dephosphorylation cycle (Ng et al. 1994; Ferguson et al. 1996). A number of transfection studies of DRD1 and DRD2 have shown a predominantly cytoplasmic localization for DRD2, while DRD1 is localized in the plasma membranes after transfection (Ng et al. 1994; Vickery and von Zastrow 1999; Prou et al. 2001). Agonist stimulation, however, also results in internalization of DRD1 (Ng et al. 1994; Vickery and von Zastrow 1999). Internalization of the receptor following ligand binding is known to occur for a number of GPCRs, including the $D_2$ type receptors, and this leads to a lowering of surface receptor density (Ferguson 2001; Sun et al. 2003). Agonist-independent internalization of $D_2$ type receptors may be occurring in Ntera-2 cells under the culture conditions used in this study.

Previous immunohistological studies of dopamine receptors in the brain have shown the presence in neuronal cells of DRD1 and 2 (Levey et al. 1993) and DRD4 (Defagot et al. 1997; Wedzony et al. 2000; Rivera et al. 2002) but subcellular distribution of the receptors was not described. Our present antibody studies of rat brain sections have localized $D_2$-like dopamine receptors to specific neuronal cells in the hippocampus and layers V and VI of the cerebral cortex. This distribution is consistent with known dopaminergic afferent systems to the hippocampus (Goldsmith and Joyce 1994) and to cortical pyramidal neurons of layers V and VI (Goldman-Rakic et al. 1989; Gulledge and Jaffe 1998). Unlike Ntera-2 cells in culture, post-mortem rat brain is exposed to very high levels of released dopamine within a few minutes of death (Vulto et al. 1988) and agonist-induced internalization of dopamine receptors can also be a very rapid process (Vickery and von Zastrow 1999; Macey et al. 2004). It is possible that receptors have been internalized as a result of this process and this may partly explain why their endosomal location has not been described previously. However, the presence of receptors in the cell body, rather than dendritic processes, may make it easier to identify dopamine-responsive neurons by immunohistochemistry. For example, radioligand binding studies detected $D_2$-like receptors in layers V and VI of the cortex, but binding in layers I–III was also found (Martres et al. 1985; Bouthenet et al. 1987). This might be explained by projection of apical dendrites from pyramidal neurons in layers V and VI into layers I–III, since ligand binding detects active receptors on dendrites whereas endosomes will be most evident in the neuronal cell body. These two studies also found $D_2$-like receptors in the hippocampus and this was confirmed by later studies with more specific ligands for DRD2 (Camps et al. 1989) and DRD3 (Levesque et al. 1992; Herroelen et al. 1994; Hall et al. 1996; Bancroft et al. 1998). The distribution of cytoplasmic vesicle labelling is therefore in broad agreement with earlier radioligand studies.

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