Expression of Dopamine D₃ Receptor Dimers and Tetramers in Brain and in Transfected Cells*

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The expression and characteristics of the dopamine D₃ receptor protein were studied in brain and in stably transfected GH3 cells. Monoclonal antibodies were used for immunoprecipitation and immunoblot experiments. Immunoprecipitates obtained from primate and rodent brain tissues contain a low molecular weight D₃ protein and one or two larger protein species whose molecular mass are integral multiples of the low molecular weight protein and thus appear to have resulted from dimerization and tetramerization of a D₃ monomer. Whereas D₃ receptor multimers were found to be abundantly expressed in brain, the major D₃ immunoreactivity expressed in stable D₃-expressing rat GH3 cells was found to be a monomer. However, multimeric D₃ receptor species with electrophoretic mobilities similar to those expressed in brain were also seen in D₃-expressing GH3 cells when a truncated D₃-like protein (named D₃af) was co-expressed in these cells. Furthermore, results from immunoprecipitation experiments with D₃- and D₃af-specific antibodies show that the higher-order D₃ proteins extracted from brain and D₃/D₃af double transfectants also contain D₃af immunoreactivity, and immunocytochemical studies show that the expression of D₃ and D₃af immunoreactivities overlaps substantially in monkey and rat cortical neurons. Altogether, these data show oligomeric D₃ receptor protein expression in vivo and they suggest that at least some of these oligomers are heteroligomeric protein complexes containing D₃ and the truncated D₃af protein.

D₃ receptors belong to the D₂-class of dopamine receptors known to couple to inhibitory subsets of heterotrimeric G proteins. The members of the superfamily of G-protein-coupled receptors have a predicted membrane topology of seven hydrophobic transmembrane domains connected by alternating extracellular and intracellular loops. Although it is generally thought that functional G-protein-coupled receptors contain a single receptor molecule, several recent observations suggest that these receptors also exist as oligomers. For example, muscarinic M2 receptor proteins purified from porcine atria contained, in addition to the monomer, multiples of the monomeric receptor with electrophoretic mobilities suggesting trimeric and tetrameric homologomers (1). Furthermore, the cooperative ligand binding profile of purified M2 receptors described by Wreggett and Wells (1) appears to fit best a model that assumes a tetrameric configuration of this receptor. Recently, Hebert et al. (2) showed that β₂-adrenergic receptors form homodimers in transfected cells. These homodimers were resistant to SDS and reducing agents. Most interestingly, this dimerization was found to be essential for β₂-receptor-mediated stimulation of adenyl cyclase, and agonist stimulation stabilized the dimeric receptor configuration, whereas inverse agonists favored the monomeric state (2).

Previous immunoblot and immunoprecipitation analyses of a variety of different G-protein-coupled receptors (expressed in insect or mammalian cells) also suggested that these receptors are capable of forming homologomers. For example, dopamine D₃ receptor immunoprecipitates contained two D₃-immunoreactive protein species, the larger (~93 kDa) being twice the size of the smaller (~44 kDa) (3). Similarly, dopamine D₂ receptors were found to migrate at ~50, 100, and 200 kDa (4) on SDS-PAGE. In addition, oligomers of the m5-HT₁b receptor (5), the mGluR1 receptor (6), the substance P receptor (7), and the platelet activating factor receptor (9) were resolved under reducing conditions on SDS-PAGE. Although these results in cell lines were often interpreted as nonspecific aggregation of incompletely folded intermediates, in each case the higher molecular weight species appeared to comprise multiples of a monomer.

Earlier studies on chimeric receptors already suggested the possibility that functional G-protein-coupled receptors can be comprised of multiple receptor molecules. For example, Maggio et al. (10) made chimeric α₂-adrenergic and M3 muscarinic receptors by replacing transmembrane domains VI and VII of one receptor with the corresponding domain of the other receptor. Although neither chimera was functional when expressed alone, functional receptors were formed when the two chimeras were co-expressed. These results demonstrate that intermolecular interactions can occur between G-protein-coupled receptors and that the resultant oligomeric receptor complexes are functional.

Whether G-protein-coupled receptors, in cells in which they are natively expressed, exist in monomeric or oligomeric configurations is still unresolved. However, the demonstration of functional differences between dimers and monomers reported by Wreggett and Wells (1) and Hebert et al. (2) points to the importance of understanding the molecular composition of such receptors in vivo. The present study shows an oligomeric ex-

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pression of the dopamine D₃ receptor protein in brain which, at least in part, resulted from heteroligomerization of the D₃ protein and the truncated D₃-like protein D₃nt (see Refs. 11 and 12).

**EXPERIMENTAL PROCEDURES**

**Protein Extraction, Immunoprecipitations, Treatment of Proteins with N-Glycosidase, and Immunoblotting—**Our protocols for the extraction of proteins from transfected cells, SDS-PAGE, and Western blotting were previously described (12, 13). In addition, membranes were prepared from human brain by homogenizing 0.5 g of postmortem tissue in an ice-cold buffer containing 25 mM NaCl, 2 mM EDTA, and 50 mM Tris (pH 7.6). The homogenate was first centrifuged at 4 °C for 10 min at 500 × g to pellet nuclei, and the resultant supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C to obtain a pellet containing membranes. This pellet was resuspended in the same buffer, incubated at 37 °C for 30 min, and centrifuged again at 100,000 × g for 30 min at 4 °C.

For immunoprecipitation experiments, proteins from human, monkey, and rat brain tissue homogenates or membrane preparations and from transfected rat GH3 cells were solubilized in a buffer containing 0.1% Triton (pH 8.0), 0.15 M NaCl, 0.001% EDTA, 0.5% Nonidet P-40, 1% Triton X-100 supplemented with the protease inhibitors aprotinin (2 μg/ml) and phenylmethylsulfonyl fluoride (1 μM). Solubilized proteins were precleared with either protein G or protein A-agarose (40 μl; Boehringer Mannheim) and subsequently incubated with primary antibody (a D₃-specific monoclonal antibody IgG or a D₃nt-specific polyclonal antibody; see below) at 4 °C for 15 h. After adding 40 μl of protein G- or protein A-agarose slurry, the incubation was continued for at least 2 h. The immunoprecipitate was washed 4 times in a buffer containing 50 mM Tris pH 7, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100, followed by 2 additional washes in the same buffer without Triton X-100. The protein A- or G-antibody-antigen complexes were boiled for 5 min in 1 × Laemmli buffer (containing 5% β-mercaptoethanol) and analyzed by Western blotting (probed with the polyclonal D₃nt-specific antibody and the D₃-specific monoclonal antibody (IgM; see below)). Bound antigen was visualized using the appropriate peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-mouse IgG+M; Kirkegaard & Perry Laboratories, Gaithersburg, MD) in conjunction with enhanced chemiluminescence (Pierce, Rockford, IL).

Immunoprecipitated proteins subjected to treatment with N-glycosidase were separated from the agarose beads and antibody by boiling for 5 min in 5 mM Tris, 10 mM EDTA, containing 200 mM NaCl, 1% Nonidet P-40, and 1% Triton X-100. The samples were centrifuged for 10 min to collect the supernatant. Equal volumes of a buffer containing 100 mM sodium phosphate (pH 7.2), 20 mM EDTA, and 1% Nonidet P-40 was added to the supernatant. Samples were boiled again for 2 min and allowed to cool down to room temperature. N-Glycosidase F (0.6 to 1 units; Boehringer Mannheim) was then added and the samples were incubated at 37 °C for 16 h.

The monkey polyclonal antibody raised against the unique 60-amin acid residue-long carboxyl-terminal of the human D₃nt protein was previously characterized (12). Two monoclonal D₃ antibodies (IgG and IgM) were raised against a fusion protein incorporating amino acid residues 252–284 of the putative third cytoplasmic loop of the human D₃ receptor (14) fused in-frame to the carrier protein glutathione S-transferase. The fusion protein was expressed in bacteria and purified by affinity chromatography using glutathione according to the protocol provided by the manufacturer (Pharmacia Biotech, Piscataway, NJ). BALB/c mice were immunized with 50 μg of purified fusion protein. The animal with the highest titer for binding to the fusion protein was boosted with 24 μg of fusion protein administered intravenously. Single-cell suspensions of spleen cells were mixed with SP2/0 myeloma cells in serum-free media and fused using polyethylene glycol (4000). Cells were collected in Iscove's medium (10% fetal calf serum, G-418, sodium pyruvate, L-glutamine, hypoxanthine, and thymidine). After selecting for fused cells with asazenerine, supernatants from wells containing fluorescein isothiocyanate (FITC)- and rhodamine-conjugated anti-rabbit IgG secondary antibodies (Boehringer Mannheim; 1:100) were added to the biotinylated anti-mouse IgG secondary antibody. Prior to visualization, the sections were covered with Vectorshield mounting medium. A Zeiss Axioskop microscope equipped with appropriate filters to visualize fluorescein isothiocyanate and rhodamine was used for brightfield and epifluorescence microscopy. For confocal sections, images were collected using a Zeiss LSM 410 confocal microscope (Zeiss LSM 410) using Zeiss Neofluar ×40 objectives (Zeiss, Oberkochen, Germany). Rhodamine was visualized using an ArKr 488/568 laser with a 568-nm excitation filter and a 590-nm long-pass emission filter; fluorescein isothiocyanate was visualized with a 488-nm excitation filter and a 515–540-nm bandpass emission filter.
RESULTS

Polyclonal and monoclonal antibodies that were raised against peptide sequences specific for the human D₃ receptor were used to characterize the properties and distribution of D₃ receptor species expressed in mammalian brain and in stably transfected rat GH3 cells.

Characterization of the Antibodies—The characterization of one of the D₃ receptor antibodies used in this study has been reported previously (13). This antipeptide antibody, which was raised against the amino terminus of the human D₃ receptor (Cambio, Cambridge, UK), detected D₃ immunoreactivity in immunoblot experiments using proteins extracted from rat GH3 cells that stably express the human dopamine D₃ receptor under the transcriptional control of a tetracycline-responsive promoter. The expression of D₃ receptor mRNA and protein in these stably transfected cells is suppressed by including tetracycline in the culture medium and reaches steady-state levels 24 h after induction (13). Two novel monoclonal antibodies (IgM/D₃ and IgG/D₃), which were raised against peptide sequences constituting amino acid residues 252–284 that are part of the putative third cytoplasmic loop of the human D₃ protein, were then tested for their ability to detect the same approximately 50-kDa D₃-immunoreactivity recognized by the polyclonal antipeptide antibody raised against the amino terminus of the D₃ protein. As shown in Fig. 1A, all three antibodies recognize the same protein of approximately 50 kDa, which appears on immunoblots of proteins extracted 5–9 h after induction of D₃ expression in stably transfected GH3 cells. This D₃ immunoreactivity reaches steady-state levels 24 h after the induction of expression and is not detected in non-transfected GH3 cells. The expression of this D₃ immunoreactivity is abolished 3 days after the inhibition of D₃ mRNA expression by the addition of tetracycline (2 μg/ml) to the culture medium (Fig. 1A).

In addition, we synthesized a T7 RNA polymerase transcript of the human cDNA encoding the D₃ receptor (cloned into the plasmid vector pRe/CMV, Invitrogen) and translated this mRNA in vitro in rabbit reticulocyte lysates. The resulting proteins were analyzed by immunoblotting using monoclonal antibodies IgM/D₃ and IgG/D₃ as well as the polyclonal anti-D₃ antiserum. As shown in Fig. 1B, all three antibodies recognized an in vitro translation product that also migrated at approximately 50 kDa. When D₃ mRNA was omitted from the in vitro translation reaction, no D₃ immunoreactivity was detected on immunoblots, as shown in Fig. 1B for the polyclonal anti-D₃ antiserum. It is noted in both experiments on transfected GH3 cells and on in vitro translation products that all three antibodies also recognize smaller protein species of about 37 and 30 kDa. These proteins are likely to be degradation products of the D₃ protein.

An additional polyclonal antibody that specifically recognizes a unique carboxyl-terminal peptide sequence contained only within the truncated D₃-like protein named D₃mut (11), but not within the D₃ protein, was used in some experiments. The characterization of this antibody has been reported previously (12).

D₃ Receptor Immunoreactivity Expressed in Brain—The monoclonal antibodies described above were used to characterize D₃ immunoreactivity expressed in brain tissue. Because of the low levels of the D₃ protein, immunoprecipitation experiments were performed using the monoclonal antibody (IgG/D₃). The composition of the immunoprecipitate was analyzed on immunoblots using the monoclonal IgM/D₃ antibody.

We first analyzed the expression of D₃ immunoreactivity expressed in human motor cortex. Fig. 2A shows the immunoprecipitates obtained with the IgG/D₃ antibody. A protein of ~50 kDa is clearly detected. However, an additional major D₃ immunoreactivity of more than 180 kDa (~200 kDa), and a smear of minor proteins migrating just below the largest protein, are also detected. The D₃ immunoprecipitate of solubilized total human brain proteins (Fig. 2A, lane 2) was then compared with the immunoprecipitate obtained from a membrane prep-
D3 protein species are integral membrane proteins. In lieu of the two major protein species indicating that both dopamine receptors are thought to be extensively glycosylated, treatment of the D3 immunoprecipitate with N-glycosidase F prior to blotting did not abolish the detection of the ~180-kDa D3 protein (Fig. 2A, lane 3). It did, however, abolish the smear of proteins seen directly below this protein species which revealed two sharp protein bands of slightly lower molecular mass. Furthermore, a doublet of proteins of ~50 kDa is detected after treatment of N-glycosidase F, indicating that removal of N-linked sugars in a substantial portion of the D3 proteins results in only a small shift of their electrophoretic mobility.

In further experiments, we tested the ability of the IgG/D3 antibody to immunoprecipitate the D3 protein expressed in monkey and rat brain. As shown in Fig. 2B, the antibody also immunoprecipitated the D3 protein expressed in these two species, and in both species the protein composition of the immunoprecipitate is very similar. In addition to a ~50-kDa protein, two larger proteins are contained in the immunoprecipitate that migrate at approximately 85 and 180 kDa. D3-immunoreactive signals were not detected when solubilized proteins from spleen or muscle tissue (which do not express D3 receptors) were incubated with the monoclonal IgG/D3 antibody (Fig. 2C).

The size of the 45-kDa D3 immunoreactivity obtained from monkey and rat brain tissue corresponds to the calculated molecular mass of the D3 core protein. Similar to the composition of the D3 immunoprecipitate obtained from human brain tissue, the two larger protein species appear to be multiples of the 45-kDa protein. The results, therefore, suggest that the D3 protein may be expressed as a monomer, a dimer, or a tetramer in rodent and primate brain. Interestingly, in all three species examined the tetrameric configuration of the D3 protein is the most abundant protein species present in the immunoprecipitate. In fact, only the tetrameric, but not the dimeric, D3 protein configuration was detected in human brain (Fig. 2A). In all three species, these higher-order structures of the D3 receptor were shown to be resistant to reducing agents (thus suggesting that monomers are not linked to each other via disulfide bonds) and were maintained after exposure to SDS.

**Fig. 2. D3-immunoreactivity expressed in human, monkey, and rat brain.** A, D3 immunoprecipitate of human motor cortical tissue. Lane 1, D3 immunoprecipitate obtained from 10 mg/ml protein of motor cortical membrane pellets. Lane 2, D3 immunoprecipitate obtained from 6.5 mg/ml protein of total motor cortical tissue homogenate. Lane 3, the D3 immunoprecipitate of motor cortical tissue protein was treated with 1 unit of glycosidase F prior to gel electrophoresis. B, D3 immunoprecipitate obtained from monkey and rat brain tissues. Lane 1, D3 immunoprecipitate of proteins extracted from monkey basal ganglia. Lane 2, D3 immunoprecipitate obtained from proteins extracted from rat prefrontal cortex. Proteins were immunoprecipitated with the monoclonal IgG antibody. C, D3 immunoprecipitates of different rat tissues. Lane 1, prefrontal cortex; lane 2, rat muscle; lane 3, rat spleen. All proteins were immunoprecipitated with the monoclonal IgG/D3 antibody. The immunoblots of these precipitates were probed with the monoclonal IgM antibody. Bound antigens are visualized as described in the legend to Fig. 1.
In total, these results suggest that in GH3 cells the formation of the 180-kDa D₃ immunoreactivity is specifically promoted by expression of the D₃nf protein. The results also suggest that it is the molar ratio of the two proteins, and not their absolute level of expression, that determines the appearance of the higher-order D₃ species; and the results do not therefore support the conclusion that the 180-kDa D₃ immunoreactivity results from nonspecific protein aggregation due to overexpression of the transfected proteins ("molecular crowding").

In further experiments, proteins from the three double transfectants that express the 180-kDa D₃ immunoreactivity were immunoprecipitated with the monoclonal IgG/D₃ antibody and the protein composition was analyzed on immunoblots probed with the monoclonal IgM/D₃ antibody (Fig. 3C). As shown in Fig. 3C, in each of the 3 clones, strong immunoreactive signals of approximately 180, 90, and 50 kDa were detected with monoclonal antibody IgM/D₃. Thus, the pattern of D₃ immunoreactivity contained in the immunoprecipitate of proteins from these D₃/D₃nf double transfectants is very similar to that seen in immunoprecipitates of proteins extracted from brain tissues (Fig. 2). It is noted, however, that the dimeric D₃ immunoreactivity is only detected in immunoprecipitation, but not immunoblotting, experiments on proteins of the GH3 double transfectants and that it is also not detected in immunoprecipitates of proteins extracted from human brain (Figs. 2 and 3). Although the reason for this discrepancy remains to be resolved, it is likely that the pellets of the immunoprecipitates contained mostly the 180-kDa protein complex which, during the preparation for SDS-PAGE analysis, could then be dissociated into its various protein constituents and that the detection of the dimeric D₃ protein complex is therefore only possible when large amounts of the 180-kDa complex (i.e. those found in transfected cells, but not in brain tissue) are expressed.

Protein Composition of the Higher-order D₃ Immunoreactivity in Brain and in D₃/D₃nf-expressing GH3 Cells—One possible explanation for the appearance of the ~90- and 180-kDa protein species in co-transfected cells is that D₃ and D₃nf form heteroligomers. Both proteins have identical amino acid sequences that extend into the amino-terminal sequence of the putative third cytoplasmic domain, and thus include transmembrane spanning domains I through V. Although the D₃nf protein is unlikely to have the additional transmembrane spanning domains VI to VII found in the D₃ receptor protein, previous studies have shown molecular interactions between split G-protein-coupled receptor proteins (21) or chimeric G-protein-coupled receptors (10). The following experiments therefore tested whether the ~85/90- and 180-kDa D₃ immunoreactivities seen in immunoprecipitates of proteins extracted from D₃/D₃nf expressing GH3 cells are present, and most importantly, from brain tissue also contain D₃nf immunoreactivity.

The results shown in Fig. 4 were obtained with proteins extracted from rat prefrontal cortex (lane 1) and from GH3 D₃/D₃nf double transfectants (lane 2) that were immunoprecipitated with the monoclonal IgG/D₃ antibody. The immunoblot of these precipitates was probed with the D₃nf-specific polyclonal antibody. Both the 180- and the ~85/90-kDa D₃ immunoreactivities detected possessed binding activity, and the results (not shown) are identical to the results shown in Figs. 2 and 3C, e.g. all three D₃-immunoreactive protein species, including the ~50-kDa D₃ monomer, were detected. In addition, proteins extracted from the rat brain were immunoprecipitated with the D₃nf-specific polyclonal antiserum and the immunoblot of this precipitate was probed with the monoclonal IgM/D₃ antibody.
D3nf immunoreactivity was found in every cortical area that as the primary motor and prefrontal cortex (data not shown). Which varied on a laminar and regional basis, with the greatest activities overlap in monkey and rodent neurons. As shown in Fig. 4 (lane 3), both the higher-order D3 species, but not the 45-kDa D3nf immunoreactivity seen in single D3nf transfectants (see Fig. 3A), are also recognized by the D3-specific antibody. (In general, however, we found the D3nf antisemur less suitable for immunoprecipitation experiments because such an immunoprecipitate also contains additional bands that are weakly recognized by the monoclonal IgM/D3 antibody. Whether these bands result from a partial degradation or partial dissociation of the immunoprecipitated 180-kDa protein complex remains to be elucidated.)

Immunocytochemical Analysis of D3 and D3nf Protein Expression—The results shown above suggest a heteroligomerization of the D3 and D3nf proteins. A prerequisite for such an intermolecular interaction between D3 and D3nf in vivo must therefore be that both proteins co-localize in the same neuron. The following series of immunocytochemical experiments sought to address whether, and to what extent, D3 and D3nf immunoreactivities overlap in monkey and rodent neurons.

Light microscopic studies revealed that both D3- and D3nf-immunoreactive structures are abundantly found in rat and monkey neocortex. No significant differences were observed in the overall pattern of immunoreactivity for the two species. D3 immunoreactivity was present in all neocortical areas studied, and it was most pronounced in neuronal somata (Figs. 5, A and C; 6, A and C). Dendritic immunostaining was also evident, which varied on a laminar and regional basis, with the greatest density in areas of most dense dopaminergic innervation, such as the primary motor and prefrontal cortex (data not shown). D3nf immunoreactivity was found in every cortical area that contained D3 immunoreactivity. However, as shown in Figs. 5 and 6, its cellular distribution differed. As noted, D3 immunoreactivity was found most prominently in neuronal perikarya, whereas D3nf-like immunoreactivity (while present in perikarya) was most intense more distally in pyramidal neuron apical dendrites. In neocortex, areas in which intense D3 receptor somatic labeling was evident also contained thick D3nf-immunoreactive dendrites, as well as fine (presumably dendritic) processes throughout the neuropil. D3nf-immunoreactive dendrites were frequently found in bundles. These dendrites were especially prominent in layer V, and they were also seen coursing through layers IV and III (Figs. 5D and 6, B and D).

Because of the noted difference in the most pronounced cellular distribution of D3 and D3nf immunoreactivity (as illustrated in Figs. 5 and 6), we examined with confocal microscopy whether both proteins show regions of co-expression in the same neuron. Indeed, confocal analysis of double labeled material confirmed the presence of both proteins in the same pyramidal-like neurons. In agreement with the results obtained with light microscopic analysis of immunolabeled tissue, D3 immunoreactivity was concentrated in the somata, and D3nf immunoreactivity was concentrated in the dendritic profiles. However, as shown in Figs. 7, C and E, the expression of both proteins also overlaps. Whereas D3 immunoreactivity tapers off in intensity with distance from the soma, D3nf immunoreactivity gradually increases, with the result that the region of greatest co-localization of both proteins is the proximal portion of the apical dendrite.

DISCUSSION

The present study shows that dopamine D3 receptors expressed in brain exist as oligomers. Furthermore, results obtained with transfected GH3 cells suggested that the detection of D3 receptor oligomers is significantly enhanced when the truncated D3-like protein D3nf is co-expressed in these cells. Although the enhancing effect of D3nf expression on the detection of D3 oligomers may be unique to GH3 cells, results from these studies have pointed to the possibility of a D3/D3nf heteroligomerization. Indeed, D3 and D3nf immunoreactivities expressed in brain and in D3/D3nf-expressing GH3 cells co-precipitate in immunoprecipitation experiments, and both immunoreactivities co-migrate at approximately 85/90 and 180 kDa. The heteroligomerization of D3 and D3nf proteins in vivo is further supported by results of immunocytochemical studies demonstrating that D3 and D3nf proteins co-localize in distinct regions of cortical neurons.

Two novel monoclonal antibodies (raised against a peptide sequence of the human D3 protein that constitutes a region of the unique third cytoplasmic domain of the receptor) were used in this study. The ability of these antibodies to detect the expression of recombinant human D3 receptor proteins (translated either in vitro or stably expressed in GH3 cells with active tetracycline-responsive promoters), as well as the similarity of the results obtained with the monoclonal antibodies and with an antipeptide D3 receptor antibody raised against a completely different (amino-terminal) epitope, suggest that these antibodies react specifically with the D3 receptor protein. Furthermore, these antibodies do not cross-react with the truncated D3-like protein D3nf (Figs. 3A and 4), and they fail to recognize the homologous D2 receptor protein (Fig. 3B). It is worth noting, however, that these antibodies immunoprecipitated not only the D3 protein expressed in human brain. As shown in Fig. 2B, they also immunoprecipitated the D3 protein expressed in rodent and monkey brain tissue, and they produce similar immunocytochemical staining of monkey and rat brain tissue sections (Figs. 5 and 6). Because of the considerable divergence of the peptide sequence of the third cytoplasmic domain of the receptor protein between primates and rodents, it is likely that the monoclonal antibodies described here recognize either a distinct structural motif of the D3 receptor protein (rather than a linear peptide sequence) that is common to both rodent and primates or that they recognize a very small number of amino acid residues. Neither scenario would be unusual for monoclonal antibodies.

The availability of D3-specific monoclonal antibodies and a polyclonal D3nf-specific antibody that recognizes peptide sequences that are not present in the D3 protein enabled us to use these antibodies sequentially in immunoprecipitation and Western blot experiments. Results from these studies showed that the smallest D3 protein has a molecular mass of ~50 kDa in human motor cortex, and ~45 kDa in rodent and primate brain. In addition to these low molecular mass proteins, however, two larger D3 protein species of ~85 and ~180 kDa were immunoprecipitated from rodent and primate brain, and one protein species of ~200 kDa was detected in immunoprecipi-
tates of human brain tissues. These results suggest for the first time the existence of D₃ receptor dimers and tetramers in brain tissue. Surprisingly, similarly abundant oligomeric D₃ receptor species were not observed in GH3 cells singly transfected with cDNA encoding the D₃ receptor. However, D₃ oligomers were found to be abundantly expressed in GH3 cells that co-express the D₃ and D₃nf proteins. Furthermore, only in double transfectants in which the relative expression levels of the D₃ and D₃nf proteins were similar, a substantial proportion of the D₃ immunoreactivity is expressed in the tetrameric form (see Fig. 3, A and B). Whereas these results already suggested the possibility of a heteroligomerization of the D₃ protein, the demonstration that D₃ and D₃nf proteins co-precipitate in immunoprecipitation experiments provided further evidence for a physical interaction between D₃ and D₃nf molecules both in brain and in transfected cells (Fig. 4).

A presently unresolved issue is the relative contribution of the D₃ and D₃nf proteins to the oligomeric states of the D₃ protein. It is noted that a small amount of the D₃ protein expressed in single GH3 transfectants also appears to form higher-order structures suggesting that, in addition to the heteroligomerization described here, a proportion of the D₃ protein can also form homoligomers. Furthermore, because of the different nature of the different antibodies used in this study, we cannot determine the relative contri-

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**FIG. 5. Low-magnification photomicrographs of dopamine D₃ receptor and D₃nf immunoreactivity in rat and monkey neocortex.** In both rat (A and B) and macaque monkey (C and D) neocortex, D₃ immunoreactivity (A and C) is most pronounced in neuronal somata, and to a lesser extent in dendrites. D₃nf immunoreactivity (B and D) is also present in somata, but is much more intense in dendritic profiles, which often appear in bundles (arrows). Scale bar = 100 μm.

**FIG. 6. High-magnification photomicrographs of D₃ and D₃nf immunoreactivity in rat and monkey motor cortex.** At this magnification, the difference in localization of these two proteins is evident, with somata intensely immunoreactive for D₃ and more distal portions of the apical dendrite preferentially containing D₃nf (arrows). Letters A-D correspond to those of Fig. 5. Note the presence, in D, of lightly stained large neuronal somata adjacent to intensely labeled dendrites. Scale bar = 25 μm.
The D₃ receptor oligomers described here are resistant to SDS and reducing agents. This finding is similar to results obtained for higher-order structures of other G-protein-coupled receptors (see above). Several studies have now shown that oligomers of G-protein-coupled receptors are characteristically of defined size and that they always comprise multiples of the monomer. Although random intermolecular interactions between hydrophobic molecules might be expected to yield similar large protein aggregates, such interactions would also be expected to yield aggregates of varying size and intermediate molecular mass, and if they resulted from random formations of disulfide bonds they would be disrupted by reducing agents. Furthermore, if all studies that identified oligomers of G-protein-coupled receptors suffer from the same artifactual oligomerization of such hydrophobic molecules in solution, one may expect that mixing protein extracts from two single transfectants would also result in apparent oligomerization. However, we have never observed higher-order D₃ species after mixing and boiling extracts of D₃ and D₃nf single transfectants in the presence of SDS (not shown). Finally, if the higher-order structures described in this study are in fact aggregates forming under denaturing conditions prior to electrophoresis (this procedure is identical for all samples) it would be difficult to explain the absence of such aggregates in protein samples obtained single GH3 transfectants. The weight of the evidence therefore suggests that the observed intermolecular interactions between D₃ and D₃nf molecules (and the likely interactions between D₃ molecules) are specific, a conclusion also supported by our finding that D₃nf but not the homologous D₃ receptor, induces D₃ receptor oligomerization.

It has recently been shown that the heterodimeric assembly of a full-length and a truncated amino-terminal peptide requires only that the truncated peptide still possesses an amino-terminal hydrophobic transmembrane-spanning domain (22). Furthermore, it has been shown that a dimer with a single carboxyl terminal can still be functional (23, 24). But what is the functional relevance of oligomerization of G-protein-coupled receptors and, more specifically, what role do D₃/Δ₃nf heteroligomers play in vivo? Because G-protein-coupled receptors are integral membrane proteins, a prerequisite for a functional role of receptor oligomers is their presence in such membranes. As shown in Fig. 2A, both D₃ monomers and tetramers could be immunoprecipitated from membranes prepared from human brain tissues, demonstrating that oligomers are indeed inserted into membranes. Furthermore, our immunocytochemical analysis shows that D₃ and D₃nf immunoreactivities colocalize within individual neurons with overlapping, but also different intracellular distributions. The existence of functional heteroligomers could perhaps explain several puzzling characteristics of these proteins and their distribution. For example, the functionally competent form of the D₃ receptor protein is predominantly localized in the soma, whereas the bulk of dopaminergic terminals synapse on dendritic shafts and spines (25–27). Conversely, the D₃nf protein has an anatomic distribution consistent with that of a dopamine receptor, but it is not expected itself to act as a functional receptor. Thus, one role of the D₃nf protein, which is located more peripherally in the dendritic tree (and in the vicinity of dopaminergic afferents), could be to target a proportion of the D₃ receptor pool to those areas in which D₃nf is expressed. Thus the localization of the D₃nf protein, not the D₃ receptor protein itself, would signal the presence of functional heteroligomers. Interestingly, the β₃-adrenergic receptor has been shown to dimerize, and the presence of agonist promotes dimer formation (2). If such a mechanism is operable in the dopamine D₃ receptor, the localization of both D₃nf and D₃ receptor protein might be influenced by the activity of dopaminergic afferents impinging on different parts of the dendritic arbor. Thus, future studies on transfected polarized cells that express D₃ in the presence and absence of D₃nf will have to test whether D₃nf influences the trafficking of the D₃ protein.

Finally, different molecular sizes of oligomers might have differing anatomic distributions and affinities for receptor ligands. For example, if only the most abundant form, the tetrameric D₃ receptor, were recognizable by the D₃-specific ligands currently in use, this could explain the discrepancies between immunocytochemical (28, 29) and ligand-binding studies of receptor distribution (30–33).

Although the functional consequences of D₃ receptor oligomerization remain to be elucidated, the results reported here suggest that D₃ receptor oligomers represent the predominant form of the receptor in brain. Results from transfected GH3 cells also suggest that co-expression of the D₃nf protein influences the assembly of the D₃ receptor, further suggesting that alterations of D₃nf expression may have important functional consequences for dopaminergic neurotransmission.
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