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Development of Polyclonal Anti-D2 Dopamine Receptor Antibodies Using Sequence-Specific Peptides

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SUMMARY

Multiple subtypes of dopamine receptors with similar properties have been described. Ligands that have been shown to interact with a single subtype of receptor do not yet exist. The use of immunologic methods provides an alternative approach to distinguish receptors and receptor isoforms. Synthetic peptides corresponding to portions of the third intracellular loops of the two isoforms of the rat D2 dopamine receptor were used to elicit polyclonal antipeptide antibodies. Peptide D2-244 is unique to the $D2_L$ isoform, whereas peptide D2-284 is present in both the $D2_L$ and the $D2_S$ isoforms. Rabbits were immunized monthly with peptide coupled to keyhole limpet hemocyanin. The immunogenicity of the peptides was established using a solid-phase radioimmunoassay. Both immunogens elicited antipeptide antibodies within 10 weeks of the primary immunization, with titers of at least $1/10^4$. An immunoprecipitation assay using receptors in digitonin-solubilized extracts of rat or canine caudate labeled with the high affinity D2 antagonist ^{125}I-NCQ 298 showed that antipeptide antisera could recognize solubilized D2 receptors. At a dilution of 1/1000, antisera to peptide D2-284 quantitatively immunoprecipitated ^{125}I-NCQ 298 binding sites from both rat and canine striatal tissue, whereas antisera against peptide D2-244 immunoprecipitated 40% of the D2 receptors solubilized from rat caudate. The selectivity of the antisera was determined using 293 cells transfected with cDNA encoding the $D2_L$ or the $D2_S$ isoform of receptor. Antisera to D2-284, at a dilution of 1/1000, were able to quantitatively immunoprecipitate receptor from both 293-$D2_L$ and 293-$D2_S$ cells. Antisera to D2-244 were specific for the $D2_L$ isoform, immunoprecipitating ^{125}I-NCQ 298 binding sites from 293-$D2_L$ cells but not from 293-$D2_S$ cells. Anti-D2-284 specifically recognized multiple bands of 100 kDa, 68 kDa, and 50 kDa in immunoblots of denatured preparations of rat caudate. Immunohistochemical studies with anti-D2-284 demonstrated the presence of the D2 receptor in several regions of rat brain. Immunostaining was most dense in the striatum, with a lateral to medial gradient and patches of lighter staining. Immunoreactivity was negligible with preimmune serum or peptide-blocked immune serum. Immunoreactive processes were seen in the nucleus accumbens and ventral pallidum, as well as in the hypothalamus. The high affinity binding of agonist to D2 dopamine receptors was disrupted by anti-D2-284 but not anti-D2-244 antisera, implicating the internal region of the third intracellular loop represented by peptide D2-284 as a potential determinant of receptor-guanine nucleotide-binding protein coupling.

Two subtypes of dopamine receptors, D1 and D2, were identified on the basis of pharmacologic and biochemical criteria (1). At least five genes encoding subtypes of dopamine receptors have been isolated and categorized as D1-like or D2-like according to their nucleotide sequences and the pharmacologic profile of the expressed proteins. The D1-like receptors include the D1 (2-5) and the D5 (6) receptors, whereas the D2-like receptors include the two isoforms of the D2 receptor, $D2_S$ (7) and $D2_L$ (8-11), the D3 receptor (12), and the D4 receptor (13). The recently cloned D1b receptor (14) is believed to be the rat homologue of the human D5 receptor.

Pharmacologic characterization of the expressed receptors has revealed potentially important differences in their affinities for dopamine and atypical neuroleptic agents such as clozapine (6, 12-14). At the present time, radioligands shown to distinguish between the D1-like receptors or among the $D2_L$, $D2_S$, D3, and D4 receptors are not available. The fact that multiple species of mRNA are coexpressed in the same tissues (6, 8-14) and perhaps in the same cells (8) further emphasizes the problems inherent in the use of radioligands to define the properties of individual subtypes of receptors. The expression and characterization of multiple gene products have highlighted the need to develop antibodies as selective probes. In the absence of either nucleotide or amino acid sequence informa-

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ABBREVIATIONS: i3 loop, third intracellular loop; G protein, guanine nucleotide-binding protein; KLH, keyhole limpet hemocyanin; sulfo-MBS, sulfo-*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; FBS, fetal bovine serum; RIA, radioimmunoassay; PBS, phosphate-buffered saline; ^{125}I-NCQ 298, (*S*)-3-[^{125}I]iodo-2-hydroxy-5,6-dimethoxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]benzamide; ^{125}I-IBZM, (*S*)-3-[^{125}I]iodo-2-hydroxy-6-methoxy-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]benzamide; NPA, *N*-propylnorapomorphine; PNS, phosphate-buffered normal saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

tion, efforts to generate antibodies against dopamine receptors were largely unsuccessful. Several groups were able to raise antispiroperidol antibodies; however, cross-reacting anti-idiotypic antibodies were not obtained (15–17). The cloning of dopamine receptor subtypes presented the possibility of using sequence-specific peptides to elicit antireceptor antibodies.

The need for a selective immunologic probe is particularly compelling for the $D2_S$ and $D2_L$ isoforms of the D2 dopamine receptor. The two isoforms of the D2 receptor are encoded by splice variants of a single gene and differ only by the presence of an additional 29 amino acids in the i3 loop of the $D2_L$ receptor (8–11). Amino acid residues within the i3 loop of members of this family may play a role in the coupling of these receptors to G proteins (18–20). Modifications of the i3 loop do not, however, have marked effects on receptor-ligand interactions. Specific amino acid residues that have been shown to be involved in ligand binding are located primarily within the transmembrane regions (for review see Ref. 18). There is no evidence to suggest that the presence of the additional 29 amino acids in the i3 loop of the $D2_L$ isoform alters the affinity of D2 dopamine receptors for agonists or antagonists or the ability of D2 receptors to couple to G proteins. Isoform-selective anti-D2 receptor antibodies may allow examination of the role of the 29-amino acid, alternatively spliced insert, as well as localization of the isoforms.

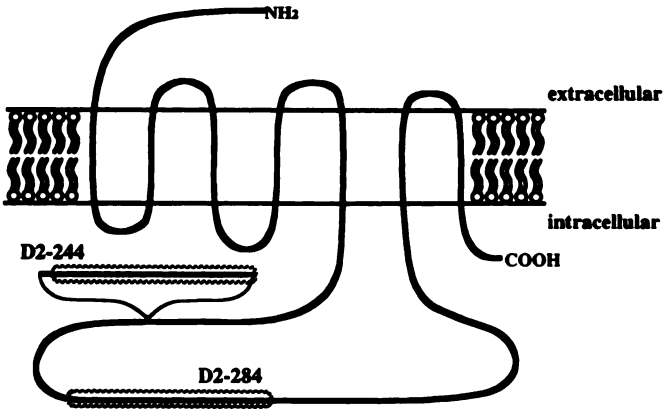
Recently, synthetic peptides have been used in several laboratories to develop antibodies against the D2 dopamine receptor. Antisera raised to six peptides (two from the amino terminus and four from the i3 loop of the D2 dopamine receptor) have been shown to immunoprecipitate photoaffinity-labeled receptors from Chinese hamster ovary cells transfected with D2 receptor cDNA and to label striatal neurons in immunocytochemical studies (21). Antibodies raised against a peptide representing the second extracellular loop inhibited the binding of an azido derivative of spiroperidol to rat striatal membranes (22). Antisera obtained after immunization with a peptide corresponding to the carboxyl terminus immunoprecipitated a 90-kDa photoaffinity-labeled site and, after proteolytic digestion, a 14-kDa labeled fragment (23). Antibodies against sequences in the i3 loop of the receptor that were reported to differentiate $D2_L$ and $D2_S$ receptors were characterized in immunocytochemical studies with transfected cells, although not with striatal tissue (24). Antipeptide antibodies to the second intracellular loop recognized several bands on Western blots of rat pituitary cells (25). In studies with partially purified preparations of receptors from bovine striatum, antisera raised against an i3 loop peptide recognized a 95-kDa band on Western blots and immunoprecipitated [^{3}H]spiperone binding sites (26).

In this manuscript, results obtained with synthetic peptides corresponding to portions of the i3 loops of the D2 dopamine receptor isoforms, coupled to the carrier protein KLH and used for the production of polyclonal antisera, are described. Peptide D2–244 corresponds to most of the 29-amino acid insert that is unique to the $D2_L$ isoform and elicited isoform-selective antisera able to immunoprecipitate binding sites for the high affinity D2 antagonist ^{125}I-NCQ 298 from solubilized extracts of rat caudate. Antisera to D2–284 were able to recognize both isoforms of the receptor and to quantitatively immunoprecipitate D2 dopamine receptors from solubilized extracts of canine and rat caudate and transfected cells expressing either isoform of the D2 receptor. Antisera to D2–284 were also characterized for use in immunoblots and immunohistochemistry. Examination of the association of the D2 receptor isoforms with G proteins using these antisera confirmed the importance of the i3 loop in receptor-G protein interactions and suggested that the 29-amino acid insert is not involved in this coupling reaction.

Materials and Methods

Generation of antisera. Peptides were synthesized and purified by high performance liquid chromatography by the Protein Chemistry Division, University of Pennsylvania School of Medicine. Peptide D2–284 is common to both isoforms of the D2 receptor and corresponds to amino acid residues 284–311 in the $D2_L$ isoform and amino acid residues 255–282 in the $D2_S$ isoform; peptide D2–244, composed of amino acid residues 244–270, is unique to the $D2_L$ isoform (Fig. 1). The peptides represent portions of the i3 loop of the two D2 receptor isoforms. The sequence of each peptide was modified to contain a norleucine residue, to make it possible to determine the efficiency of the conjugation reaction (27). Norleucine was substituted for the internal cysteine in peptide D2–244 and for the second leucine in peptide D2–284 (Fig. 1). The peptides were conjugated, through the amino-terminal cysteine in peptide D2–244 or through an extra carboxyl-terminal cysteine in peptide D2–284, to the carrier protein KLH (Calbiochem, La Jolla, CA) by using the water-soluble linker sulfo-MBS (Pierce, Rockford, IL), according to a previously published protocol (28). The D2–244 peptide was insoluble in water and was dissolved in 100% ethanol. The D2–244-KLH complex precipitated upon conjugation. Free peptides were not separated from the peptide-KLH complexes.

Four female New Zealand White rabbits were immunized monthly with each peptide conjugate. Conjugates were dried, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10.2 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.2) (500 μl/rabbit), combined with an equal volume of either Freund's complete (first injection) or Freund's incomplete (booster injections) adjuvant (Sigma Chemical Co., St. Louis, MO), and emulsified. Rabbits were injected with approximately 1 mg of protein in a final volume of 1 ml, using a multiple-site injection protocol (29). Rabbits were bled monthly beginning 10–14 days after the second



Peptide D2-244 NH_2- CTHPEDMKL*CTVIMKSNGSFPVNRRRM - COOH **(unique $D2_L$)**

Peptide D2-284 NH_2- LSSTSPPERTRYSPIPPSHHQL*TLPDPS(C) - COOH **(shared $D2_L$ / $D2_S$)**

Fig. 1. Schematic diagram of the dopamine D2 receptor. Following the generally accepted model of G protein-coupled receptors, the seven highly homologous hydrophobic regions are shown as membrane-spanning domains, whereas the more hydrophilic regions are depicted as intracellular or extracellular loops. The 29-amino acid insert within the i3 loop distinguishes the $D2_L$ from the $D2_S$ receptor variant. The boundaries and sequences of the two synthetic peptides (D2–244 and D2–284) are shown. *, Amino acids replaced with norleucine.

injection. Rabbits were also bled before initial immunization. Sera were stored at −20°.

Solid-phase RIA. Antisera were screened against peptides D2–244 and D2–284 by using a modification of the solid-phase RIA described by Luedtke *et al.* (29). Flexible 96-well microtiter plates (Falcon 3911; Becton-Dickinson, Oxnard, CA) were coated overnight at 4° with peptide (1.25 μg/well), washed five times with PBS, and incubated overnight with 10% FBS (Hyclone, Logan, UT) at 25°. Plates were washed five times with PBS; antisera were added at a dilution of 1/10,000 and were allowed to incubate for 2 hr at 25° and then for 2 hr at 4°. The plates were washed five times and secondary antibody, ^{125}I-goat anti-rabbit IgG (100,000 cpm) in PBS containing 10% FBS, was added and incubated as described above. After the final series of washes with PBS, the wells were counted individually. Goat anti-rabbit IgG (Southern Technology Associates, Birmingham, AL) was iodinated using a chloramine T-based procedure (30).

Preparation of solubilized striatal tissue. Canine caudate was dissected from hemisected brains (Pel-Freez Biologicals, Rogers, AR). Homogenates of canine caudate and digitonin-solubilized extracts of rat (Zivic Miller Laboratories, Zelienople, PA) and canine caudate were prepared, and solubilized extracts were chromatographed over a heparin-agarose column as described previously (20, 31). In brief, the heparin-agarose (Bio-Rad Laboratories, Richmond, CA) column was equilibrated with buffer [50 mM Tris, pH 7.4, 10 mM EDTA, 0.1% digitonin (Gallard-Schlesinger Industries, Inc., Carle Place, NY)], digitonin-solubilized tissue was applied, and the column was washed with equilibration buffer. Receptors were eluted with the same buffer containing 300 mM NaCl. Column fractions were assayed for the binding of ^{125}I-NCQ 298 or ^{125}I-IBZM (provided by Dr. Hank F. Kung, University of Pennsylvania School of Medicine, Philadelphia, PA, or New England Nuclear, Boston, MA). Protein was determined by the method of Bradford (32).

Ligand binding assays. The binding of ^{125}I-NCQ 298 or ^{125}I-IBZM to solubilized tissue extracts was measured using a charcoal adsorption assay (29, 33). Solubilized extracts eluted from a heparin-agarose column were incubated overnight at 4° with ^{125}I-NCQ 298 (0.5 nM), in a final volume of 100 μl. Activated charcoal (MCB Manufacturing Chemists, Inc., Cincinnati, OH) (50 μl of a 2%, w/v, suspension containing 5% FBS, 50 mM Tris, pH 7.4, 10 mM EDTA, and 0.1% digitonin) was added to the samples, which were then incubated on ice for 10 min. Charcoal was separated from the reaction mixture by centrifugation, and radioactivity in 100-μl aliquots of the supernatant was measured. Competition assays were performed with crude solubilized tissue diluted to a final digitonin concentration of 0.03%, using a modification of the procedure described by Chazot and Strange (34). These assays included 100 μl of NPA or vehicle (in 0.1 mM HCl), 100 μl of ^{125}I-NCQ 298 (0.15 nM in 100 mM Tris, pH 7.4, 450 mM NaCl, 0.1% bovine serum albumin), and 100 μl of solubilized rat caudate (in 50 mM Tris, pH 7.4, 0.09% digitonin). In experiments examining the effect of GTP, 300 μM GTP and 2 mM $MgCl_2$ were included. In experiments examining the effect of antisera, crude solubilized tissue (1% digitonin) was preincubated with antisera (anti-D2–284, 1/100; anti-D2–244, 1/30) for 16–18 hr at 4° before being diluted and incubated with ligand and inhibitor. Assays were incubated at 4° for 14–16 hr and were terminated by the addition of 5 ml of cold wash buffer (10 mM Tris, pH 7.4, containing 150 mM NaCl) followed by filtration, under low vacuum, though glass microfiber filters (GF-B; Whatman Labsales, Hillsboro, OR). Filters were washed with 10 ml of wash buffer and dried under vacuum. Radioactivity remaining on the filters was measured in a gamma counter. In all assays, nonspecific binding was determined in the presence of 2 μM (+)-butaclamol (Research Biochemicals, Inc., Natick, MA). Saturation binding data were transformed by the method of Scatchard (35). K_d and B_{max} values were estimated by linear regression analysis. Protein was determined by the method of Lowry *et al.* (36). Competition curves were analyzed by nonlinear regression using an iterative curve-fitting program written with the Solver program of Microsoft Excel 4.0 (Microsoft Corp., Redmond, WA). IC_{50} values were converted to K_i values as described by Cheng and Prusoff (37).

Immunoprecipitation assays. Immunoprecipitation was measured using a modification of a previously described protocol (38). Digitonin-solubilized receptors, eluted from a heparin-agarose column, were incubated with ^{125}I-NCQ 298 or ^{125}I-IBZM and antisera in the presence of 10 mg/ml bovine serum albumin and 0.1% digitonin, in a final volume of 200 μl, for 50–60 hr at 4°. Free ligand was removed by the addition of 50 μl of activated charcoal (as in ligand binding assays), followed by centrifugation. At 4° the binding of either ^{125}I-NCQ 298 or ^{125}I-IBZM to solubilized D2 receptors was stable; dissociation of the ligand from the receptor was negligible even after 9 hr (20, 31). A 200-μl aliquot of supernatant was incubated on a multipurpose rotator (model 151; Scientific Industries, Inc., Bohemia, NY) for 2 hr at 4° with 20 μl of Pansorbin (Calbiochem). Pansorbin was pretreated as described previously (39), except that the final resuspension buffer included 0.1% digitonin. Samples were diluted with 1 ml of TE buffer (50 mM Tris, pH 7.4, 10 mM EDTA, containing 0.1% digitonin), and the Pansorbin-antibody-receptor-radioligand complex was precipitated by centrifugation (16,000 × *g*, 2 min, 4°). Nonspecific immunoprecipitation was determined using preimmune antiserum or immune antiserum that had been preadsorbed with peptide. Percent precipitation was defined as the difference between precipitation observed with immune and preimmune antisera divided by the specific binding of radioligand determined in the absence of antiserum. Nonspecific binding was defined in the presence of 2 μM (+)-butaclamol.

Transfection of 293 cells. Human embryonic kidney 293 cells (40), grown in 5% CO_2 at 37°, were plated at a density of 5×10^6 cells/10-cm plate in 10 ml of medium [Eagle's minimal essential medium (GIBCO) supplemented with 10% FBS and penicillin/streptomycin] and allowed to attach overnight. The cDNAs encoding the human $D2_L$ and human $D2_S$ receptors, gifts of Dr. Peter H. Seeburg (University of Heidelberg, Heidelberg, Germany) and Dr. Dolan B. Pritchett (University of Pennsylvania School of Medicine, Philadelphia, PA), were ligated into the pRc/CMV expression vector (Invitrogen, San Diego, CA). pRc/CMV contains a neomycin resistance gene for selection of stable transformants. After overnight transfection (41) in 3% CO_2 at 35°, cells were fed with fresh medium, allowed to grow for 2 days, adjusted to a density of 2×10^6 cells/10-cm plate, and incubated for an additional 24 hr. Cells were continually selected for 3–4 weeks in medium containing 0.5 mg/ml levels of the antibiotic G418. Single colonies were then isolated, expanded, and harvested for radioligand binding assays to measure expression of receptors.

Immunoblots. Membranes from rat brain were prepared according to the reduction and alkylation procedure of Sternweis and Robishaw (42). Protein samples (100 μg/lane) were separated on 10% SDS-PAGE and immunoblots were performed using a modification of previously published procedures (43, 44). Proteins were transferred from polyacrylamide gels to nitrocellulose membranes (0.45 μm; Schleicher & Schuell) for 5 hr at 60 V using a Tris-glycine transfer buffer containing 20% methanol. The nitrocellulose membranes were washed with a high-salt Tris buffer (20 mM Tris·HCl, pH 7.5, 500 mM NaCl) and blocked for 1 hr at 25° with LD-Blotto (5% nonfat dry milk, 50 mM Tris·HCl, pH 8.0, 80 mM NaCl, 2 mM $CaCl_2$, 0.2% Nonidet P-40, 0.02% NaN_3) containing 10% normal goat serum. Membranes were incubated with anti-D2–284 antisera (1/1000 dilution in LD-Blotto) for 2 hr at 25°, washed with HD-Blotto (LD-Blotto supplemented to 2% Nonidet P-40 and 0.2% SDS), and incubated with secondary antiserum, ^{125}I-goat anti-rabbit IgG, F(ab')$_2$ fragment (NEN) (1×10^6 dpm/ml in HD-Blotto), for 2 hr at 25°. Membranes were washed with HD-Blotto, rinsed with 50 mM Tris·HCl, pH 8.0, 80 mM NaCl, 2 mM $CaCl_2$, and exposed to film at −80°. For peptide-blocking experiments, antisera (1/1000 dilution) were preincubated overnight at 4° with the peptide used for immunization (0.25 mg/ml).

Immunohistochemistry. Sprague Dawley rats were anesthetized with Equithesin (prepared according to the instructions of Jensen-Salsbury Labs, Kansas City, MO) and perfused transcardially with 0.9% saline (100–200 ml) followed by 600 ml of 4% paraformaldehyde

in 0.1 M phosphate buffer, pH 7.2. After perfusion, brains were removed, placed in a solution of phosphate-buffered sucrose (30% sucrose in 50 mM phosphate buffer) for cryoprotection, and incubated for 2–4 days at 4°. Brains were then frozen on crushed dry ice and stored at −70° until sectioned. Frozen brains were sectioned on an AO Histostat cryostat microtome. Sections (16–24 μm thick) were thaw-mounted on gelatin-coated slides and stored at 4° until used for immunohistochemistry. The distribution of immunoreaction product was confirmed by comparing cresyl violet-stained sections with the atlas of Paxinos and Watson (45).

Sections were washed twice (10 min each) with PNS (0.9% NaCl, 10 mM phosphate buffer, pH 7.2) and incubated for 1 hr at room temperature in 3% normal goat serum, 0.1% Triton X-100, in PNS. Before use, a diluted aliquot of anti-D2–284 antiserum was incubated with acetone-extracted liver powder (Sigma) for 2 hr at room temperature. After separation from the liver powder, the primary antiserum was brought to a final dilution of 1/500 in 1% normal goat serum, 0.1% Triton X-100, in PNS (T-GS-PNS), and was applied to the tissue sections, which were subsequently kept in a humidified chamber overnight at room temperature. Controls for specificity of staining included incubating sections without primary antiserum, with preimmune serum under the same conditions as with immune serum, and with immune antiserum that had been preadsorbed with the peptide immunogen (peptide D2–284). Preimmune serum was used on sections adjacent to each section incubated with immune serum. Selected sections were incubated either with blocked antibody or without primary antiserum. After incubation with control or primary antiserum, tissue was washed twice with T-GS-PNS and incubated with labeled secondary antibody. Rhodamine-conjugated goat anti-rabbit IgG (Fisher BioTech, Pittsburgh, PA) was diluted 1/100 in T-GS-PNS and applied to tissue sections for 1 hr at room temperature. Sections were then washed twice with PNS (20 min each) and rinsed with distilled water to remove buffer salts; coverslips were applied using Gel/Mount (Biomeda Corp., Foster City, CA). After immunohistochemical staining, tissue sections were viewed and photographed using a Leitz Aristoplan fluorescence microscope.

Results

Development and characterization of antisera. The synthetic peptides used as immunogens to prepare polyclonal antisera were chosen because of their predicted antigenicity and their location in the i3 loops of the D2 dopamine receptors (Fig. 1). Peptide D2–284, common to both isoforms of the D2 receptor, was modified to include a carboxyl-terminal cysteine to permit coupling to KLH by using the bifunctional agent sulfo-MBS. Peptide D2–244 corresponds to 27 of the 29 amino acids of the insert that is unique to the $D2_L$ receptor isoform and has an amino-terminal cysteine residue. Antisera were initially screened with a solid-phase RIA against uncoupled peptides. Preimmune sera were inactive, whereas antisera obtained from the second bleed contained antibodies that were able to recognize the peptide antigens at a dilution as high as 1/10,000 (Fig. 2). These titers remained high and constant through at least the fourth bleed, as measured in an RIA. Antisera to D2–284 were able to specifically recognize peptide D2–284, whereas antisera to D2–244 were able to recognize only peptide D2–244 (Fig. 2). Neither antiserum exhibited significant cross-reactivity to the peptide not used for immunization.

The data presented in this paper include antisera obtained from one rabbit per immunogen. Two of the three other rabbits immunized with peptide D2–284 produced antibodies with characteristics similar to those of the one presented. The fourth rabbit was slow to respond and had a lower titer of anti-D2 antibodies. Two of the other rabbits immunized with peptide D2–244 died early in the study and the third produced antibodies with characteristics similar to those of the one presented.

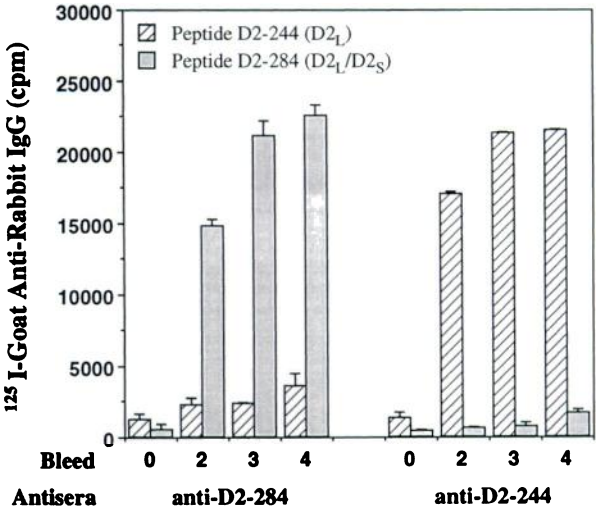


Fig. 2. Solid-phase RIA against peptide antigens. Flexible 96-well microtiter plates were coated with either peptide D2–284 or peptide D2–244 (1.25 μg/well) and blocked with FBS. Antisera (preimmune serum and bleeds 2–4) were added at a dilution of 1/10,000 and allowed to incubate for 2 hr at 25° and then for 2 hr at 4°. The plates were washed and secondary antiserum, ^{125}I-goat anti-rabbit IgG (100,000 cpm), was added and incubated as described above. After a final series of washes with PBS, the wells were individually counted. Data shown are mean ± range of two determinations.

An immunoprecipitation assay using digitonin-solubilized extracts of canine caudate eluted from a heparin-agarose column and the high affinity radioligand ^{125}I-NCQ 298 was used to monitor antiserum production and to determine whether antipeptide antibodies could recognize solubilized receptors. Previous studies have shown that canine D2 dopamine receptors solubilized with 1% digitonin could be enriched 2–3-fold by chromatographic separation using heparin-agarose (31). This procedure also permits reduction of the concentration of digitonin. ^{125}I-NCQ 298 has been reported to be a high affinity D2 antagonist in studies using rat striatal membranes (46) and in solubilized preparations of canine and rat striatal tissue (20). The affinity of D2 receptors for ^{125}I-NCQ 298 decreased by 3–4-fold upon solubilization, such that the K_d was 0.14 nM. Nonetheless, ^{125}I-NCQ 298 displays high affinity for both membrane-bound and soluble D2 dopamine receptors.

Although the earliest bleeds of anti-D2–284 antisera had high titers of antipeptide antibodies, these antisera showed limited ability to immunoprecipitate labeled receptors from solubilized extracts of canine caudate (Fig. 3A). By the sixth bleed the titer of the antisera to D2–284 had improved such that 75–85% of the labeled sites were precipitated at antisera dilutions of 1/1000 (Fig. 3A). This dramatic increase in the titer of anti-D2–284 was such that the antisera from the sixth bleed not only immunoprecipitated almost 90% of the ^{125}I-NCQ 298 binding sites at a dilution of 1/1000 but also achieved 50% precipitation at dilutions between 1/2500 and 1/5000 (Fig. 4). In contrast, the second through sixth bleeds of antisera to D2–244 demonstrated no significant immunoprecipitation of labeled receptors from solubilized extracts of canine caudate at dilutions of 1/1000 to 1/10,000 (Fig. 3B and Fig. 4, *inset*).

Antisera were tested for their ability to immunoprecipitate receptors from solubilized extracts of rat caudate. Antisera from rabbits immunized with peptide D2–284 were able to immunoprecipitate 80–90% of the ^{125}I-NCQ 298 binding sites from

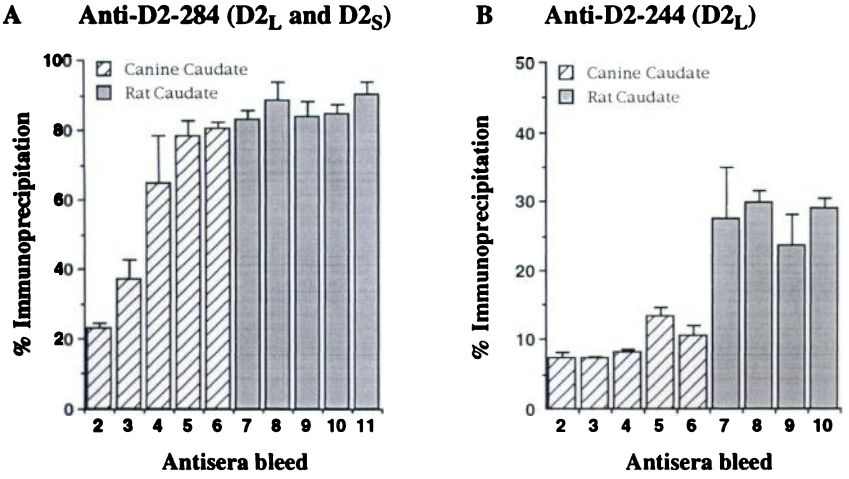


Fig. 3. Immunoprecipitation of ^{125}I-NCQ 298 binding sites from digitonin-solubilized canine and rat caudate. Caudate from either dog or rat was solubilized with 1% digitonin and chromatographed on a heparin-agarose column. Aliquots of eluate were incubated with a 1/1000 dilution of antisera to peptide D2–284 (A) or to peptide D2–244 (B) and ^{125}I-NCQ 298 for 60 hr. The antibody-receptor-^{125}I-NCQ 298 complex was precipitated with Pansorbin, and radioactivity in the pellet was determined. Percent precipitation was defined as the difference between precipitation with immune and preimmune sera divided by the specific binding of ^{125}I-NCQ 298 measured in the absence of antisera. Data shown are mean ± range (or standard error) from two (or three) independent determinations.

solubilized rat caudate at a dilution of 1/1000 (Fig. 3A). The titer of antireceptor antibodies was high; 50% of the sites were immunoprecipitated with anti-D2–284 at dilutions between 1/5000 and 1/10,000 (Fig. 5). Immunoprecipitation was completely abolished when antisera had been preadsorbed to peptide (data not shown). Antisera raised against peptide D2–244 were also able to immunoprecipitate labeled receptors from solubilized extracts of rat caudate (Fig. 3B). The titer of these antisera was low, such that only 30–40% immunoprecipitation was achieved at a dilution of 1/1000 (Figs. 3B and 5).

To establish the selectivity of anti-D2–244 antisera for the $D2_L$ isoform of the D2 dopamine receptor, homogeneous populations of the receptor isoforms were needed. The cDNAs encoding the two isoforms of the D2 dopamine receptor were subcloned into the mammalian expression vector pRc/CMV, and recombinant plasmids were used to transfect 293 cells. Two stable cell lines, 293-$D2_L$ and 293-$D2_S$, were established. The density of receptors ranged from 200 to 800 fmol/mg of protein, and the K_d of the expressed receptors for ^{125}I-NCQ 298 was approximately 0.04 nM when measured in membranes prepared from either cell line. Antisera to D2–284 immunoprecipitated 95–100% of the labeled receptors from digitonin-solubilized extracts of both 293-$D2_L$ and 293-$D2_S$ cells (Fig. 6). Antisera to D2–244 were able to immunoprecipitate approximately 40% of the ^{125}I-NCQ 298 binding sites from 293-$D2_L$ cells (Fig. 6A) and, as expected, were unable to immunoprecipitate binding sites for ^{125}I-NCQ 298 from 293-$D2_S$ cells (Fig. 6B).

The ability of the antisera to recognize denatured proteins was examined using immunoblots. In immunoblots of SDS-solubilized membranes prepared from rat striatum, preimmune serum detected a single faint band at approximately 60 kDa (Fig. 7, *lane 1*). In addition to detecting this faint band, anti-D2–284 antisera (1/1000 dilution) recognized a prominent band at an approximate molecular mass of 100 kDa, a lighter band at 68 kDa, and in some blots an even lighter band at 50 kDa (Fig. 7, *lane 2*). The nonspecific 60-kDa protein was also seen in blots incubated with immune sera that had been preadsorbed with peptide (Fig. 7, *lane 3*). The three specific bands were not detected in blots incubated with preimmune serum (Fig. 7, *lane 1*) or with peptide-blocked immune serum (Fig. 7, *lane 3*).

Localization of D2 dopamine receptors in rat brain. The highest density of immunoreactivity detected using anti-D2–284 antisera was in the caudate-putamen. The distribution of staining was not homogeneous. There was a lateral to medial gradient evident at low magnification, as well as patches of reduced immunoreactivity (Fig. 8a). Negligible staining was seen when adjacent sections were incubated with preimmune

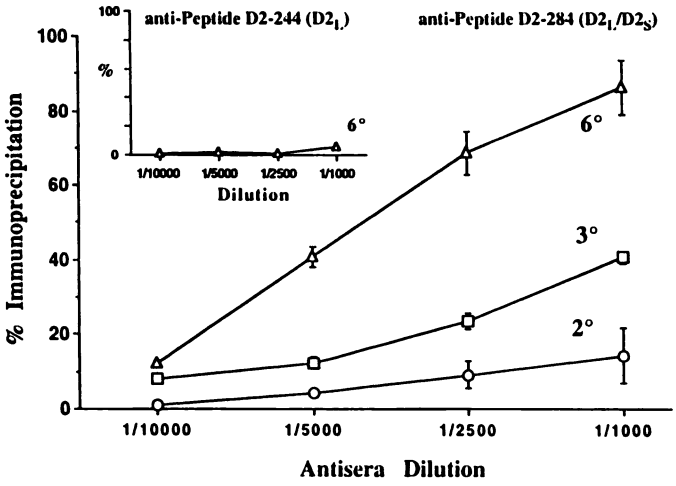


Fig. 4. Titration of antisera to peptides D2–284 and D2–244 using an immunoprecipitation assay with canine caudate. Immunoprecipitation assays were performed as described in the legend to Fig. 3, using selected bleeds for anti-D2–284 and the sixth bleed for anti-D2–244. Data shown are mean ± standard error from three independent determinations.

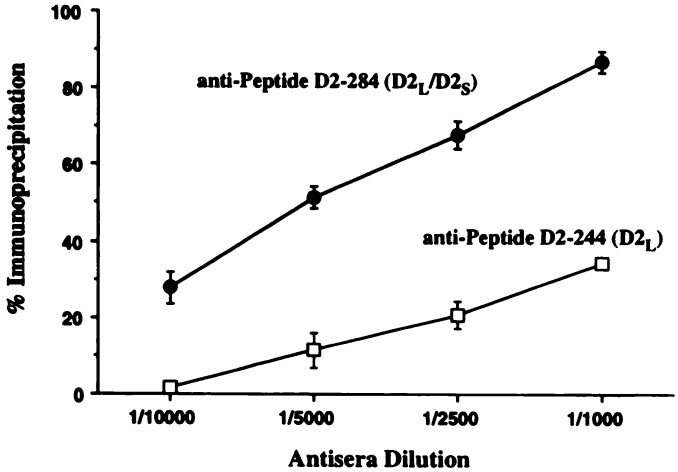


Fig. 5. Titration of antisera to peptides D2–284 and D2–244 using an immunoprecipitation assay with rat caudate. Immunoprecipitation assays were performed as described in the legend to Fig. 3, using the eighth bleed of anti-D2-284 and the seventh bleed of anti-D2-244. Data shown are mean ± standard error from three independent determinations.

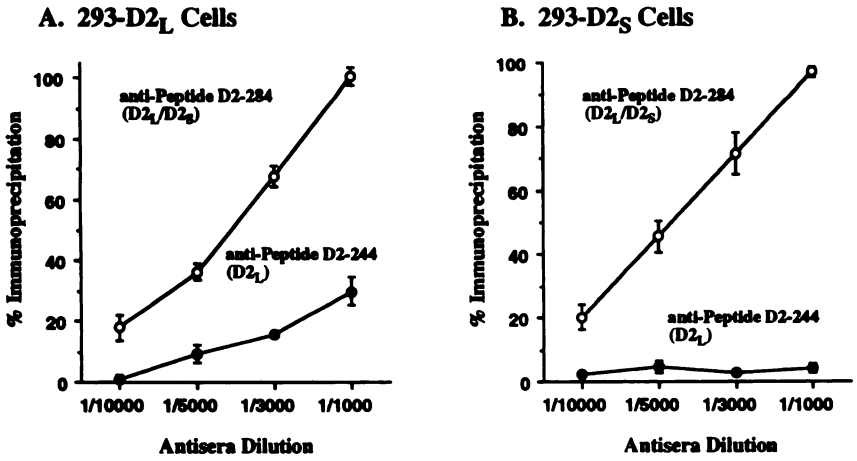


Fig. 6. Selective immunoprecipitation of $D2_L$ and $D2_S$ receptors. 293-$D2_L$ cells (A) and 293-$D2_S$ cells (B) were harvested and membranes were prepared and solubilized with 1% digitonin. Solubilized extracts were incubated with antisera (eighth bleed for anti-D2–284 or seventh bleed for anti-D2–244) and ^{125}I-NCQ 298 for 60 hr. The antibody-receptor-^{125}I-NCQ 298 complex was precipitated with Pansorbin. Data shown are mean ± standard error from three independent determinations.

serum (Fig. 8b). Neither cell bodies nor individual processes were discernable because of the high density of immunoreactivity throughout the area (Fig. 8c). Incubation of an adjacent section with immune sera that had been preadsorbed with peptide showed an almost complete elimination of staining (Fig. 8d). In the nucleus accumbens, the density of labeling was much lower than that seen in the striatum. Instead of a dense plexus preventing the resolution of individual processes, a network of very fine immunoreactive processes as well as punctate staining was seen. The same pattern was seen in the ventral pallidum, although less immunoreactivity was observed. A few labeled processes were seen in the lateral septum and globus pallidus. Labeled soma were not detected in any of these areas. Within the hypothalamus, immunoreactivity was seen in the paraventricular nucleus (Fig. 9). Negligible staining was seen with preimmune sera (Fig. 9a). The magnocellular part of the nucleus showed prominently labeled neurons as well as fibers and punctate immunoreactivity (Fig. 9, b-d). More medially, the parvocellular portion of the nucleus was filled with fine punctate immunoreactivity but labeled neurons were not seen (Fig. 9b). Specific immunoreactivity was present in other areas of the hypothalamus, including the supraoptic nucleus, where there was labeling of cell soma, and the zona incerta, which had a low density of punctate immunoreactivity with no labeled cell bodies. Specific immunoreactivity was not seen in other areas of the hypothalamus.

Examination of D2 receptor-G protein interactions. NPA inhibited the binding of ^{125}I-NCQ 298 to solubilized rat caudate (0.03% digitonin) with a K_i of 51 ± 6.4 nM. The addition of GTP caused a shift of the curves to the right (K_i = 107.9 ± 11.9 nM) (data not shown). Preincubation of crude solubilized tissue with preimmune antiserum did not significantly affect the high affinity agonist binding, which was shifted 2–3-fold by the addition of GTP (Fig. 10). Preincubation of the tissue with anti-D2–284 antisera resulted in loss of the high affinity agonist binding (Fig. 10A). Anti-D2–284 caused a shift of 2–3-fold in the affinity of NPA for the receptor (K_i = 137.0 ± 17.1 nM). GTP had no effect on the binding of agonist to tissue preincubated with anti-D2–284 antisera (Fig. 10A). In contrast, preincubation of crude solubilized rat caudate tissue with anti-D2–244 antisera did not affect the high affinity agonist binding (K_i = 64.0 ± 10.1 nM). As was seen with tissue preincubated with preimmune serum or buffer, the addition of GTP to tissue preincubated with the anti-D2–244 antisera resulted in a shift in the K_i of NPA for the receptor to 120.4 ± 15.8 nM (Fig. 10B).

Discussion

Study of the distribution and properties of dopamine receptors has become increasingly complex since molecular studies led to the discovery of at least five genes encoding at least six receptors. Although pharmacologic analysis of receptors expressed in mammalian cell lines has revealed differences in their affinities for various ligands, the six subtypes follow the pharmacologic profile of either the D1 or the D2 dopamine receptor. Pharmacologic methods presently available cannot distinguish between the D1-like receptors or among the D2-like receptors. Using Northern blot analysis, *in situ* hybridiza-

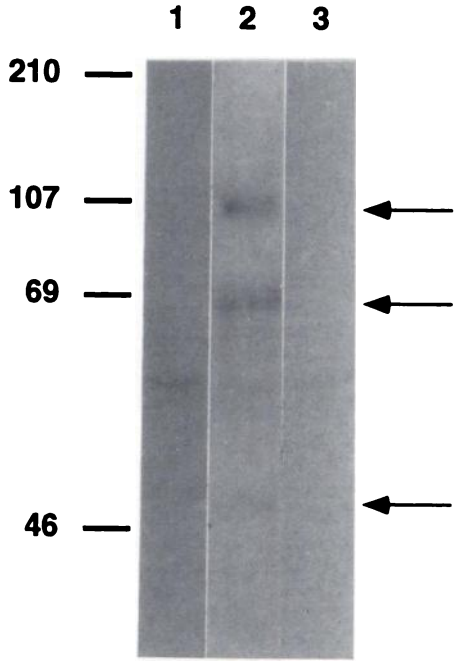


Fig. 7. Immunoblot of rat striatal membranes with anti-D2–284 antisera. Extracts of membranes prepared from rat striatum (100 μg/lane) were size fractionated on 10% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with LD-Blotto with 10% goat serum at 25° for 1 hr. Filters were incubated with preimmune serum (*lane 1*), anti-D2–284 antisera (*lane 2*), or anti-D2–284 antisera preadsorbed with D2–284 peptide (*lane 3*), washed, and then incubated with a secondary antiserum, ^{125}I-goat anti-rabbit IgG, F(ab′)$_2$ fragments. Filters were washed, dried, and exposed to film at −80°. Three specific bands (*arrows*) are visible at 100 kDa, 68 kDa, and 50 kDa. The results shown are representative of three independent experiments.

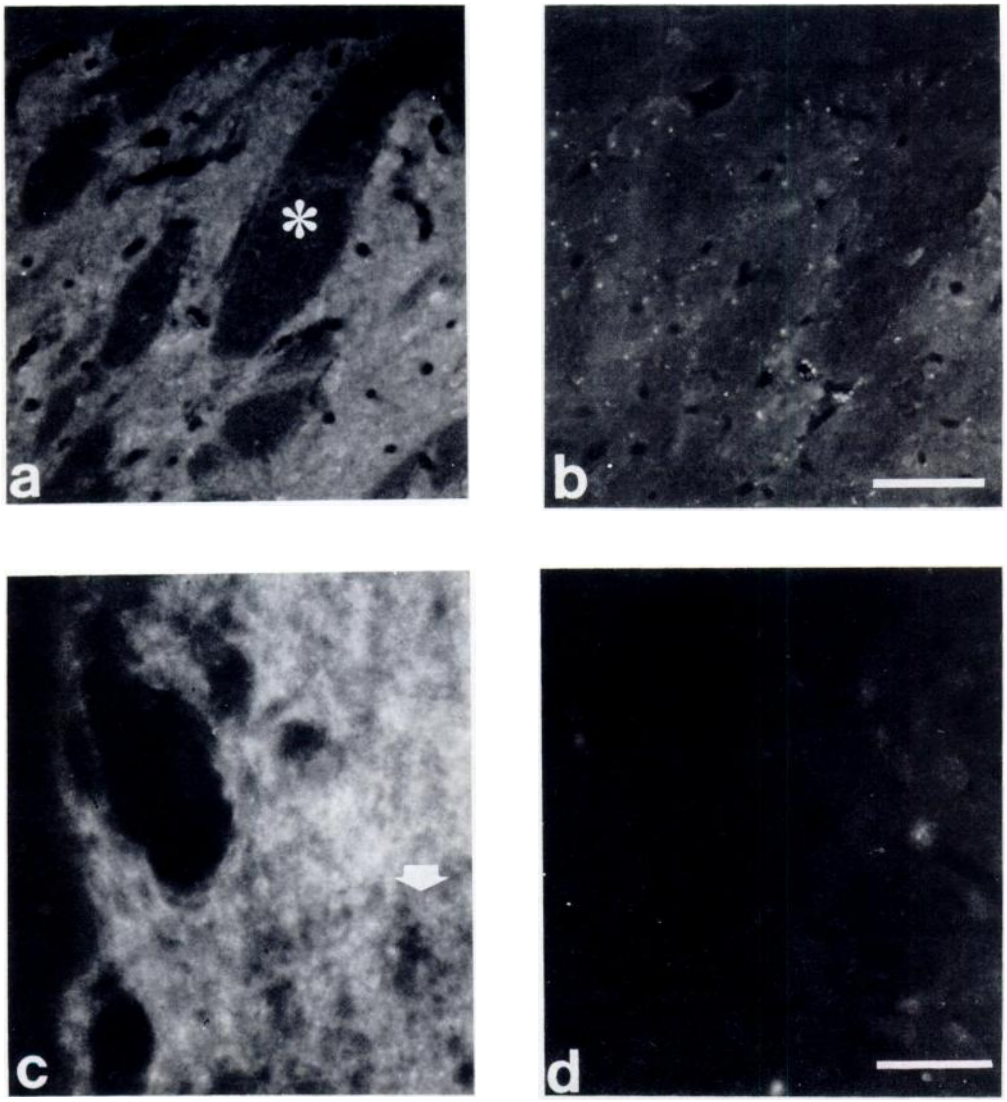


Fig. 8. Photomicrographs of coronal sections through the rat caudate. a and b, Micrographs of a section after incubation with anti-D2–284 (a) and of an adjacent section after incubation with preimmune antiserum (b) (*bar* = 50 μm). Immunohistochemistry was performed as described in Materials and Methods. There is no specific labeling in the fascicles of fibers perforating the striatum (*). Little immunoreaction product is present in the section incubated with preimmune antiserum. c and d, Higher magnification micrographs of coronal sections through the dorsolateral region of the caudate after incubation with anti-D2–284 (c) or with antisera blocked with the peptide D2–284 (d) (*bar* = 100 μm). At this magnification, dense staining of processes in the caudate is visible, as are lighter patches of immunoreaction product (*arrow*). The immunoreaction product visualized in the sections incubated with blocked serum (d) is restricted to very few cells and processes.

tion, or nuclease protection assays, it is possible to measure the mRNA encoding the subtypes of dopamine receptors. These approaches have made it possible to define the tissue distribution of the mRNA encoding the dopamine receptors and receptor isoforms (2–14). It is also important to demonstrate that the receptors are being expressed at the cell surface and to determine whether receptors or receptor isoforms are expressed in ratios comparable to their mRNA levels. The need to monitor protein expression is particularly evident in studies of the regulation of the receptor, where changes in protein may or may not correspond to changes in mRNA levels. Immunologic methods may be the only way to distinguish among receptors and receptor isoforms.

The immunization of rabbits with synthetic peptides has proven to be an appropriate method for the production of polyclonal antibodies to many G protein-coupled receptors, including β-adrenergic receptors (47) and muscarinic acetylcholine receptors (38, 48). The use of synthetic peptides coupled to a protein carrier such as KLH allows for the presentation of specific epitopes. This is important because the proteins of interest exhibit a high degree of sequence identity with other members of the G protein-coupled receptor family. The $D2_L$ and D3 receptors exhibit 52% homology overall and 75% homology within the transmembrane domains (12), whereas the homology between the $D2_L$ and D4 receptors is 41% overall (13). The two isoforms of the D2 receptor, encoded by splice variants of a single gene, differ by only 29 amino acids. The i3 loop, excluding the eight to 10 amino acids closest to the fifth and sixth transmembrane domains, is the region of the D2 dopamine receptor that displays the least homology with other members of the G protein-coupled receptor family and therefore should provide unique epitopes for the production of antireceptor antibodies. The peptides chosen share little sequence identity with the other dopamine receptors, and one of the peptides in particular, D2–244, is unique to the $D2_L$ isoform.

The immunogenicity of the peptides and the specificity of

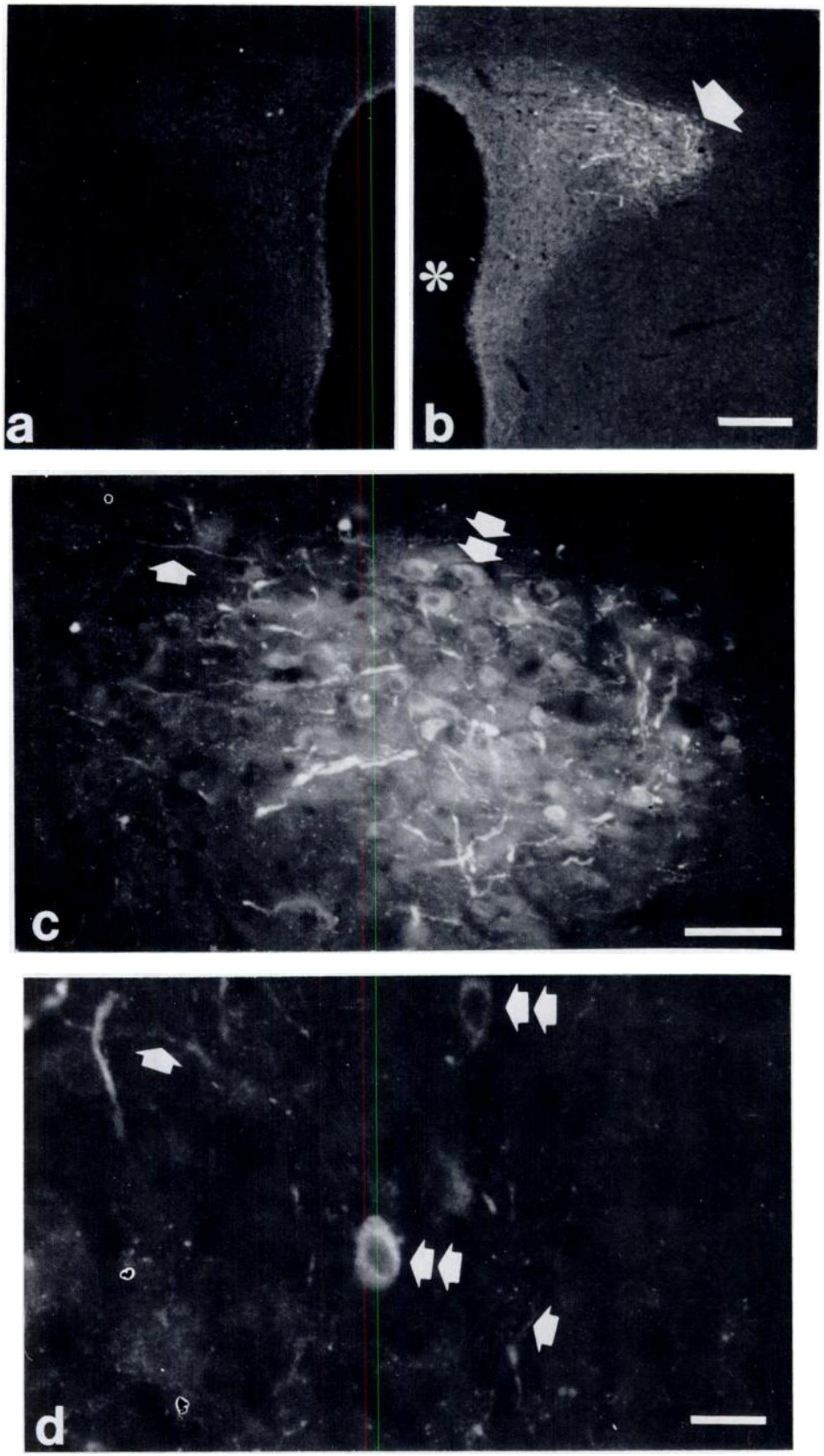


Fig. 9. Photomicrographs of coronal sections through the rat hypothalamus. a and b, Low power micrographs of sections incubated with preimmune (a) or anti-D2–284 (b) sera, as described in Materials and Methods (*bar* = 200 μm). There is intense immunofluorescence in the magnocellular part of the paraventricular nucleus (*arrow*) and finer, more diffuse staining in the parvocellular aspects nearing the third ventricle (*). The parvocellular region contains no labeled cell bodies. There are no structures labeled using preimmune serum (a). c and d, Higher magnification of the paraventricular nucleus shows intensely labeled cell bodies (*double arrows*) as well as heavily labeled processes (*arrows*) (c, *bar* = 100 μm; d, *bar* = 20 μm). The processes appear to be oriented at right angles to the third ventricle.

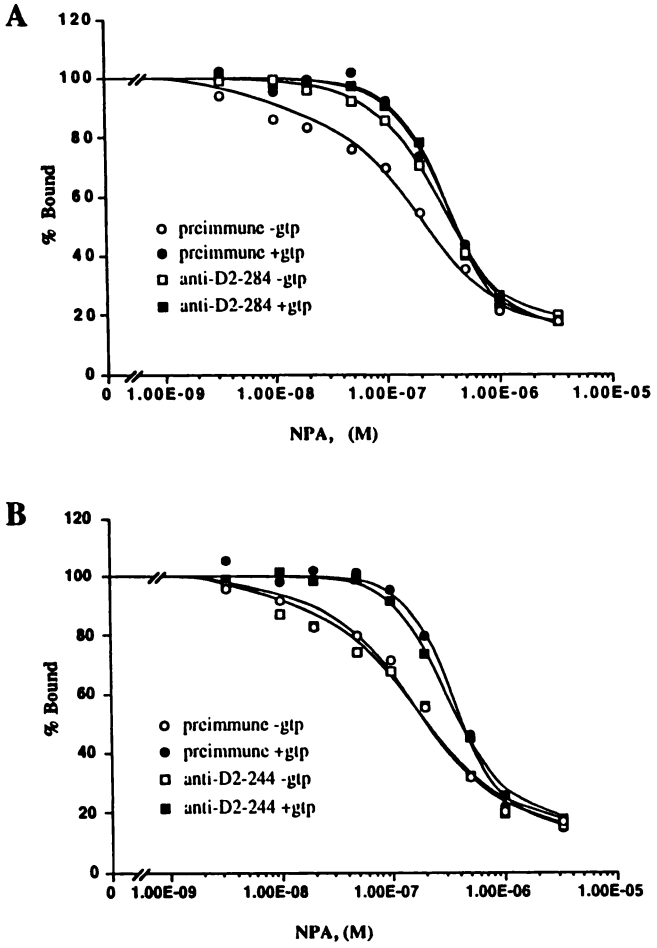


Fig. 10. Effect of anti-D2–284 (A) and anti-D2–244 (B) antisera and GTP on the binding of NPA to solubilized rat caudate. The effects of antisera (preimmune or immune) and/or GTP on the ability of NPA to inhibit the binding of ^{125}I-NCQ 298 to digitonin-solubilized rat caudate were measured using a vacuum filtration assay as described in Materials and Methods. Nonspecific binding was determined by the addition of 2 μM (+)-butaclamol. Results shown are representative of data obtained in three independent experiments.

the antipeptide response were demonstrated using a solid-phase RIA. Antisera were specific for the peptide used for immunization. Cross-reactivity with other peptides was negligible. Production of antibodies to native receptors was initially monitored by immunoprecipitation. This assay requires a stable preparation of detergent-solubilized D2 receptors and a selective, high affinity radioligand with high specific activity. Results of previous studies have demonstrated the usefulness of digitonin-solubilized extracts of canine and rat caudate for the study of D2 dopamine receptors using the high affinity dopamine antagonists ^{125}I-IBZM and ^{125}I-NCQ 298 (20, 31). Pansorbin, Protein A-coated cells used to precipitate the antibody-receptor-radioligand complex, binds free ^{125}I-NCQ 298, necessitating the removal of free ligand from the samples before the addition of Pansorbin. Negligible dissociation of ^{125}I-NCQ 298 occurs during a 2-hr incubation with Pansorbin at 4° (20). The maximal concentration of antiserum used was a 1/1000 dilution. An increase in antiserum concentration beyond a 1/1000 dilution required more Pansorbin, which increased nonspecific binding.

The early bleeds of antisera to D2–284 were able to immunoprecipitate significant percentages of canine D2 dopamine receptors that had been partially purified by chromatography on a heparin-agarose column. The first six bleeds of antisera to D2–244 ($D2_L$ selective) were unable to immunoprecipitate ^{125}I-NCQ 298 binding sites from solubilized extracts of canine caudate. Binding sites were immunoprecipitated from solubilized extracts of rat caudate with antisera from later bleeds from rabbits immunized with either peptide. Antisera to D2–244 were found to have a relatively low titer, whereas anti-D2–284 antisera displayed a high titer, quantitatively immunoprecipitating the soluble rat D2 receptors eluted from the heparin-agarose column. The difference in precipitation across species might be due to a lack of sequence homology between the i3 loops of the canine and rat D2 dopamine receptors. It is also possible that the $D2_L$ receptor isoform is not expressed in canine caudate. Even if both isoforms are present in canine caudate, single or multiple amino acid substitutions within the insert of the canine $D2_L$ receptor could result in the absence of the specific epitopes recognized by the anti-D2–244 antisera.

A major band at an approximate molecular mass of 100 kDa and lesser bands with approximate molecular masses of 68 kDa and 50 kDa were labeled in an immunoblot of rat striatal membranes. The protein molecular mass of the $D2_L$ isoform is 50 kDa. It is possible that the larger bands represent the receptor in different states of glycosylation.

The distribution of immunoreaction product in tissue sections was in general agreement with those reported by McVittie *et al.* (21) and Brock *et al.* (49) for the areas examined. There was a dense staining within the striatum that was most prominent dorsolaterally and decreased ventromedially. This gradient of D2 receptors was comparable to the distribution of receptors measured by using quantitative autoradiography (50), as well as in the distribution of mRNA encoding the D2 receptor measured by using *in situ* hybridization histochemistry (51). Neuronal cell bodies were not detected in the striatum. This may be due to the dense network of immunoreactive processes found in this area, which would obscure cell soma. Within the striatum there were patches of less intense immunoreactivity that may correspond to the striosomal patches seen using *in situ* hybridization. By using *in situ* hybridization it has been demonstrated that the majority of neurons that express mRNA coding for the D2 receptor are localized outside the striosomal patches that are defined by [^{3}H]naloxone binding (51).

A notable discrepancy in the localization of D2 receptors using immunologic techniques, in comparison with the distribution of receptors seen using radioligand binding, is within the hypothalamus. Quantitative autoradiography studies have reported low levels of D2 receptors with diffuse binding throughout the hypothalamus (50, 52), whereas *in situ* hybridization studies have shown low to moderate amounts of mRNA encoding the D2 receptor in the areas of the hypothalamus, including the paraventricular nucleus (52). We have demonstrated prominent labeling of neuronal processes as well as cell soma in the paraventricular nucleus. This is in general agreement with the localization of mRNA encoding the D2 receptor and indicates that receptor protein may be present in areas where receptors cannot be quantitated or localized using radioligand binding.

The antibodies raised against the peptides from the i3 loops of the dopamine receptor isoforms were used to probe the site of interaction between receptor and G protein, because this domain has been implicated in the coupling of various other members of the G protein-coupled receptor family to their respective G proteins (18, 19). The importance of the i3 loop in

receptor-G_i interactions has been demonstrated in studies of chimeric receptors and deletion mutants involving receptors coupled to the inhibition of adenylyl cyclase (α_2-adrenergic and m2 and m4 muscarinic acetylcholine receptors). The amino-terminal portion of the i3 loop in the m2 muscarinic receptor has been shown to be a sufficient but not exclusive determinant of coupling (53, 54). In addition, a peptide from the carboxyl-terminal portion of the i3 loop of the α_2-adrenergic receptor has been shown to inhibit high affinity binding of agonists (55), whereas a peptide from the same region of the m4 muscarinic receptor has been shown to activate G_i and G_o at nanomolar concentrations (56). Receptor G protein interactions were recently studied using antireceptor antibodies raised against peptides within the i3 loops of the m1, m2, and m4 muscarinic receptors (57). Three of the antisera raised to peptides from the amino-terminal portion of the m4 i3 loop, the middle of the m4 i3 loop, and the middle of the m2 i3 loop were able to inhibit high affinity binding and agonist-stimulated guanosine-5′-*O*-(3-thio)triphosphate binding. Antibodies raised to a peptide from the middle of the m1 i3 loop had negligible effects on receptor-G protein interactions. Each of these antisera were capable of immunoprecipitating 60–70% of the respective muscarinic receptor subtype (58). The antibodies whose properties are described here were elicited in response to immunization with peptides in the internal portion of the i3 loop of the D2 dopamine receptor isoforms. Our results showing the loss of high affinity binding in the presence of antisera implicate the middle portion of the i3 loop in receptor-G protein interactions. Similar results have been obtained with antibodies raised against fusion proteins including the middle of the D2 i3 loop (20), as well as with another D2 i3 loop antipeptide antibody described recently (26). Although it is possible that the antibodies are sterically hindering access to the receptor, the results described above with anti-m1 muscarinic receptor antibodies (57, 58) and our work described here with the anti-D2–244 antisera suggest that it is possible for an antibody to bind to a receptor without inhibiting G protein coupling. As with the anti-m1 antibodies, the anti-D2 antisera that did not modulate agonist binding were capable of immunoprecipitating receptor. Anti-D2–244 and anti-D2–284 immunoprecipitated ^{125}I-NCQ 298 binding sites not only from solubilized rat caudate (0.1% digitonin) but also from preparations of solubilized rat caudate where the digitonin concentration had been reduced to 0.03% to allow G protein coupling. The long-isoform-selective antibody, anti-D2–244, has a significantly lower titer than the antiserum capable of recognizing both isoforms, anti-D2–284. At a dilution (1/1000) where anti-D2–284 can immunoprecipitate 100% of the D2 receptors from 293-$D2_L$ or 293-$D2_S$ cells, anti-D2–244 can immunoprecipitate only 40% of the receptors. Recognizing this difference, we used 3 times as much anti-D2–244 antiserum in the competition assays (1/300 final dilution), and still modulation of the high affinity agonist binding was not seen. Thus, our results are consistent with previously published reports suggesting that internal portions of the i3 loop can modulate or participate in receptor-G_i interactions. In addition, our results suggest that the 29-amino acid insert in the $D2_L$ isoform is not involved in G protein coupling.

In summary, polyclonal antibodies to D2 receptors were raised against synthetic peptides corresponding to regions of the i3 loop of the two isoforms of D2 dopamine receptor. Antisera generated against a peptide that is common to both isoforms were capable of quantitatively immunoprecipitating ^{125}I-NCQ 298 binding sites from solubilized extracts of canine caudate, rat caudate, and 293 cells expressing either the long or the short isoform of the D2 receptor. Antisera raised against a peptide within the 29-amino acid insert of the $D2_L$ dopamine receptor were specific for this isoform of the receptor and immunoprecipitated ^{125}I-NCQ 298 binding sites from solubilized extracts of rat caudate as well as 293-$D2_L$ cells, demonstrating that the receptor protein encoded by the $D2_L$ mRNA is expressed in rat caudate. Localization studies indicate that anti-D2-284 is suitable for the high resolution localization of D2 receptors and can detect receptor protein present at levels below the detection limit of radioligand autoradiography. The use of antireceptor antibodies raised against amino acids 284–311 of the i3 loop of the D2 receptor to prevent the high affinity binding of agonists implicates this portion of the i3 loop as a potential determinant for G protein coupling.

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