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ACCELERATED COMMUNICATION

Dopamine D_2 Receptor Binding Subunits of $M_r \cong 140,000$ and 94,000 in Brain: Deglycosylation Yields a Common Unit of $M_r \cong$ 44,000

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SUMMARY

The ligand-binding subunit of the porcine striatal dopamine D_2 receptor was identified by photoaffinity labeling with [^{125}I]*N*-azidophenethylspiperone ([^{125}I]NAPS). Upon photolysis, [^{125}I]NAPS covalently incorporated into a broad band of apparent $M_r \cong 140,000$ with an appropriate pharmacological profile for D_2 receptors as assessed by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Smaller subunits of apparent $M_r \cong 94,000$ and 34,000 were specifically labeled by [^{125}I]NAPS with an appropriate D_2 receptor profile and were similar to the major ligand-binding subunits of photoaffinity-labeled canine striatal D_2 receptors. Photoaffinity labeling in the absence or presence of multiple protease inhibitors did not alter the migration pattern of the $M_r \cong 140,000/94,000$ subunits upon denaturing electrophoresis in either the absence or presence of thiol-reducing/alkylating reagents. In order to investigate the possible basis for the existence of these high molecular weight forms of the D_2 receptor, we assessed the carbohydrate nature of photolabeled D_2 ligand-binding subunits by the use of lectin affinity chromatography and specific exo- and endoglycosidase treatments. Both photoaffinity-labeled D_2 receptor proteins from porcine striatum ($M_r \cong 140,000$ and 94,000) were glycoproteins as indexed by their absorption and specific elution from wheat germ agglutinin lectin resins. The exoglycosidase neuraminidase altered the electrophoretic mobility of both the $M_r \cong 140,000$ and 94,000 labeled subunits to a single band of apparent $M_r \cong$ 51,000. Prior removal of sialic acid residues did not alter the reversible binding characteristics of [^{3}H]spiperone to D_2 receptors. Complete removal of receptor-associated *N*-linked carbohydrate by the endoglycosidase glycopeptidase F (peptide-N^4[*N*-acetyl-β-glucosaminyl]asparagine amidase) produced a further increase in the mobility of the $M_r \cong 51,000$ subunit to apparent $M_r \cong 44,000$. The porcine $M_r \cong 34,000$ photolabeled peptide is an *N*-linked glycoprotein as assessed by lectin affinity chromatography and susceptibility to digestion by glycopeptidase F to a peptide of apparent $M_r \cong 23,000$. These data suggest that the high molecular weight form of apparent $M_r \cong 140,000$ of the D_2 binding subunit observed in porcine striatal membranes results from glycosylation processing different from that observed in canine brain and that upon complete *N*-linked deglycosylation both porcine and canine striatal D_2 receptors share at least two common binding subunits of apparent $M_r \cong 44,000$ and 23,000. The exact molecular relation between these two subunits, as well as their role in the maintenance and expression of neuronal D_2 receptor-mediated events, is still unclear.

The recent synthesis and availability of specific radiolabeled photoaffinity probes for the D_2 dopamine receptor has allowed the identification of the ligand-binding subunit of this membrane protein (reviewed in Ref. 1). Photoaffinity labeling with [^{125}I]NAPS (2–4), or [^{3}H]azidomethylspiperone (5) followed by SDS-PAGE and autoradiography reveals a polypeptide of $M_r \cong 94,000$ as the major ligand binding subunit of D_2 dopamine receptors. Photoincorporation of these probes into the $M_r \cong 94,000$ subunit is antagonized in a stereoselective manner by dopaminergic agents with a pharmacological profile indicative of the D_2 receptor. In all species studied to date, including rat, hamster, dog, cow, guinea pig, and rabbit, [^{125}I]NAPS identifies a protein of apparent $M_r \cong 94,000$ as the ligand binding subunit of neuronal (striatal) D_2 receptors. The $M_r \cong 94,000$ [^{125}I]NAPS-labeled protein appears, at least in canine brain, to be heavily glycosylated because removal of *N*-linked carbohydrates by the endoglycosidase PNGase-F drastically alters the electrophoretic mobility of the $M_r \cong 94,000$ protein to apparent $M_r \cong 44,000$ (4).

Of the other tissues containing D_2 dopamine receptors, only

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ABBREVIATIONS: [^{125}I]NAPS, [^{125}I]*N*-azidophenethylsiperone; GlcNAc, *N*-acetylglucosamine; PNGase-F, glycopeptide-*N*-glycosidase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

in the neurointermediate lobe of rat pituitary has [^{125}I]NAPS been shown to label a protein of higher molecular weight (M_r ≅ 120,000) (3). In this report we document that [^{125}I]NAPS labels a high molecular weight form (M_r ≅ 140,000) of D_2 receptors from porcine striatal membranes. Moreover, we demonstrate with the use of specific exo- and endoglycosidases that the high molecular weight form of the receptor is due to carbohydrate moieties associated with the ligand binding subunit of the D_2 receptor and that upon complete deglycosylation the M_r of [^{125}I]NAPS-labeled receptors is the same in different species.

Experimental Procedures

Materials. [^{125}I]NAPS (2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and [^{3}H]spiperone (85 Ci/mmol) was from Amersham (Arlington Heights, IL). Neuraminidase (purified Type X from *Clostridium perfringens*), Nonidet P-40, PMSF, benzamidine, leupeptin, soybean trypsin inhibitor, GlcNAc, and prestained electrophoresis molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO). Glycopeptidase F (peptide-*N*^{4}[*N*-acetyl-β-glucosaminyl]asparagine amidase) from *Flavobacterium meningosepticum* was from Boehringer Mannheim (Indianapolis, IN). *O*-Glycanase (endo-α-*N*-acetylgalactosaminidase) from *Diplococcus pneumonia* was from Genzyme (Inter Medico, Toronto, Canada). Digitonin was purchased from Wako Chemical Co. (Dallas, TX) and WGA-Sepharose was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Porcine and canine brains were obtained from Pel-Freez Biologicals (Rogers, AR). Electrophoresis reagents were from Bio-Rad (Richmond, CA). X-ray film (XAR-5) and developing solutions (D-19) were from Eastman Kodak (Rochester, NY). Dopaminergic agents were either purchased from Research Biochemicals (Natick, MA) or were generously donated by various firms as previously described (4, 5).

Membrane preparations. Striata dissected from partially thawed porcine or canine brains were prepared as previously described (4). Briefly, tissues were Teflon-glass homogenized (using 10 up and down strokes of the motor-driven rotating piston) in 20 volumes of ice-cold 25 mM Tris·HCl buffer containing 250 mM sucrose and the following protease inhibitors: 20 mM EDTA, 15 μg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, and 1 mM PMSF (pH 7.4 at 4°). Homogenates were centrifuged at 600 × *g* for 10 min and the supernatant was collected and recentrifuged at 48,000 × *g* for 20 min. The resulting pellet was resuspended in 25 mM Tris·HCl buffer containing 100 mM NaCl and protease inhibitors to yield a final protein concentration of ~2–3 mg/ml. For some experiments, membranes were prepared in 25 mM Tris·HCl buffer alone (pH 7.4 at 4°).

Photoaffinity labeling. Membranes (1 ml) were routinely incubated (in the dark) with 50 pM [^{125}I]NAPS at a D_2 concentration of ~50 pM in a total volume of 10 ml for 90 min at 22° in the presence or absence of dopaminergic agents as indicated. After incubation, samples were centrifuged at 48,000 × *g* for 10 min. Membrane pellets were resuspended in 10 ml of 25 mM Tris·HCl buffer containing 100 mM NaCl, protease inhibitors, and 0.5% bovine serum albumin and recentrifuged. Membranes were resuspended in 1 ml of buffer (without bovine serum albumin) and irradiated for 35 sec as previously described (5). [^{125}I]NAPS-labeled membranes were sedimented at 48,000 × *g* for 10 min and used as described below.

D_2 receptor solubilization. Photoaffinity-labeled membranes were solubilized (~10 mg/ml) in buffer containing protease inhibitors and 1% digitonin at 4° for 30 min with constant stirring. Samples were centrifuged at 48,000 × *g* for 1 hr and the supernatant was passed through a 220-nm Millex filter.

WGA-Sepharose chromatography. Digitonin-solubilized labeled receptors (1.8 ml) were applied to a 2-ml open column of WGA-Sepharose, preequilibrated with 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl, protease inhibitors, and 0.1% digitonin at 4°, as previously described (4). Columns were washed with 20 ml of the same buffer and absorbed receptors were specifically eluted with buffer (as above) containing 300 mM GlcNAc. Solubilized, nonabsorbed, and eluted fractions were lyophilized and subjected to SDS-PAGE, as described below.

Exoglycosidase treatments. For neuraminidase treatment, [^{125}I]NAPS-labeled membranes were washed twice in 30 ml of 100 mM sodium acetate buffer, pH 5.0, at 22°. Membranes were resuspended in the same buffer at 0.5–1.0 mg/ml, to which neuraminidase was added at a concentration of 2 units/ml. Aliquots were incubated for up to 1 hr at 37°. After incubation, membranes were washed and solubilized in SDS buffer, as described below.

Endoglycosidase treatments. [^{125}I]NAPS-photolabeled and neuraminidase-treated receptors (as described above) were washed twice in 30 ml of 200 mM sodium phosphate buffer containing 2.5 mM EDTA, pH 8.0, at 22°. Pellets were resuspended in a minimal volume (~300 μl) of the same buffer to which was added 0.5% SDS and 100 mM β-mercaptoethanol and incubated for 1 hr at 22°. Aliquots (30 μl; corresponding to 0.5–1.0 mg) were incubated with Nonidet P-40 (1.25% final concentration) and PNGase F (at a concentration of 50 units/ml) for 18–24 hr at 37° in a total volume of 60 μl as previously described (4). After incubation, samples were sedimented at 12,000 × *g* for 10 min and membranes were solubilized in SDS-PAGE sample buffer, as described below.

[^{3}H]Spiperone binding assay. Membranes were prepared for neuraminidase treatment (as described above) and washed twice in 100 mM sodium acetate buffer, pH 5.0. Membranes were treated with 2 units/ml of neuraminidase for 15 min at 37°. After treatment, membranes were washed twice in 30 ml of 50 mM Tris·HCl buffer containing 1 mM EDTA, 120 mM NaCl, 1.5 mM $CaCl_2$, and 4 mM $MgCl_2$ and resuspended in the same buffer at a concentration of ~0.8 mg/ml. Aliquots (0.5 ml) of control or neuraminidase-treated receptors were incubated with increasing concentrations of [^{3}H]spiperone (0.1–2.0 nM) for 90 min at 22° in a total volume of 1.5 ml and assayed for D_2 receptor activity by rapid filtration as previously described (4, 5). Nonspecific binding was defined in the presence of 1 μM (+)-butaclamol. Computer-assisted analyses (LIGAND) of all ligand binding data were as previously described (5).

SDS-PAGE and autoradiography. Electrophoresis was performed as previously described (4, 5). Briefly, photoaffinity-labeled membranes were washed once in Tris·HCl buffer and incubated with 50 mM Tris, 10% glycerol, 10% SDS, and 5% β-mercaptoethanol, pH 6.8, for 1–2 hr at 22°. For lyophilized samples containing digitonin, or for PNGase-F-treated membrane, samples were solubilized in the above buffer containing 20% SDS. Aliquots (100–300 μg) were loaded on slab gels containing either a 10% or 12% acrylamide separating gel and a 6% stacking gel and electrophoresed overnight. In an attempt to gain greater resolving power in the molecular weight range of interest, a longer separating gel (16–17 cm) was used in most experiments. After electrophoresis, gels were dried and exposed to Kodak film with one intensifying screen at −70° for various amounts of time. To document quantitative incorporation of [^{125}I]NAPS into D_2 receptor subunits, gel regions of interest were cut out and counted in a Packard gamma counter at ~80% efficiency. Subsequent autoradiography of the cut gels confirmed that the appropriate section was taken. Molecular weights were determined graphically by plotting the log molecular weight of known protein standards versus the R_F (relative migration) of these proteins. The apparent M_r of photolabeled receptors was estimated by determining the R_F (from the center of the band) and interpolating this value on the standard curve. The values given are the means of several experiments.

Results and Discussion

Photoaffinity labeling and pharmacological specificity of [^{125}I]NAPS photoincorporation in porcine striatal membranes. The ligand binding subunit of striatal D_2 dopamine receptors has been shown to reside on an apparent M_r ≅

94,000 protein in numerous species by photoaffinity labeling with [^{125}I]NAPS (see Introduction). Fig. 1A depicts the results obtained when porcine striatal membranes were incubated with [^{125}I]NAPS and photolyzed and samples were subjected to SDS-PAGE and autoradiography. A broad band was labeled at apparent $M_r \cong 140{,}000$. The specificity of photoaffinity labeling was shown by virtue of the fact that covalent photoincorporation of [^{125}I]NAPS into the $M_r \cong 140{,}000$ subunit was blocked by 100 nM (+)-butaclamol. Moreover, smaller specifically labeled subunits of apparent $M_r \cong 94{,}000$ and 34,000 were also seen. The stoichiometry of [^{125}I]NAPS photoincorporation into these three subunits was ~4:2:1. In addition, a nonspecifically labeled band of apparent $M_r \cong 53{,}000$ was sometimes seen.

The pharmacological specificity of [^{125}I]NAPS photoincorporation was assessed further by examining the ability of various dopaminergic agents to block covalent labeling of these subunits. As seen in Fig. 1A, the photolysis-dependent covalent labeling of the subunits at $M_r \cong 140{,}000$, 94,000, and 34,000 by [^{125}I]NAPS was stereoselectively blocked by (+)- and not (−)-butaclamol and by a selective D_2 receptor antagonist (eticlopride) and an agonist (*N*-propylnorapomorphine). In contrast, coincubation of [^{125}I]NAPS with a selective D_1 receptor antagonist, SCH-23390, the serotonergic S_2 receptor antagonist methysergide, or the adrenergic agonist noradrenaline did not prevent the covalent incorporation of [^{125}I]NAPS into these subunits. Taken together, the data strongly suggest that the apparent $M_r \cong 140{,}000$, 94,000, and 34,000 proteins are the ligand-binding subunits of the porcine striatal D_2 receptor.

For comparative purposes, Fig. 1B depicts the results obtained when [^{125}I]NAPS was incubated with striatal membranes from porcine and canine brain, photolyzed, and subjected to SDS-PAGE and autoradiography. In canine striatal membranes, a broad band centered at $M_r \cong 94{,}000$ was specifically labeled, as defined by 1 μM (+)-butaclamol. In addition, another specifically labeled subunit at apparent $M_r \cong 34{,}000$ was also seen, consistent with previous observations (3, 4) Because both photolabeled porcine and canine striatal membrane were run on the same polyacrylamide gel and subjected to the same experimental procedures, the data suggest that both tissues share the apparent $M_r \cong 94{,}000$ and 34,000 protein as common ligand-binding subunits but the porcine striatal membrane D_2 receptor contains, in addition, a high molecular weight subunit of apparent $M_r \cong 140{,}000$. (It should be noted that, due to the differences in D_2 receptor density between porcine and striatal tissues and stiochiometry of photolabeling, the autoradiograph depicting photolabeled porcine D_2 receptors is slightly underexposed, resulting in the apparent loss of specific labeling of the $M_r \cong 34{,}000$ subunit.) The purpose of the present investigation was, therefore, to investigate the possible molecular basis for the high molecular weight form of the porcine striatal D_2 receptor.

Effects of protease inhibitors. Numerous studies have shown that failure to prevent endogenous protease activity during photoaffinity labeling may result in the generation of lower molecular weight protein fragments displaying the same pharmacological profile as the native receptor (5–8). In order to assess whether both the $M_r \cong 94{,}000$ and 34,000 subunits were proteolytically derived receptor fragments from the ap-

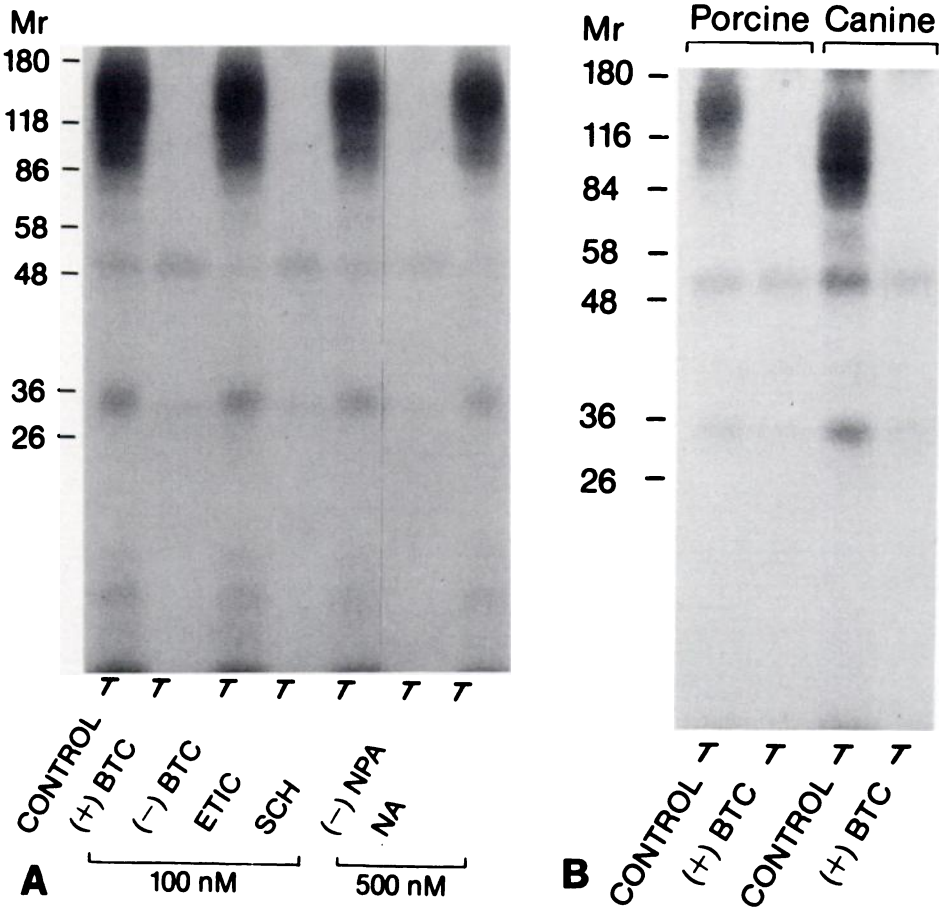

Fig. 1. Photoaffinity labeling and pharmacological specificity of [^{125}I]NAPS photoincorporation into porcine striatal membranes. Membranes were prepared and photoaffinity-labeled with [^{125}I]NAPS alone (*control*) or in the presence of indicated concentrations of competing ligands, as described under Experimental Procedures. Samples were then solubilized and electrophoresed on a 12% acrylamide gel. The M_r of known prestained standards are shown ×1000. The results shown are representative of four similar experiments. *BTC*, butaclamol; *ETIC*, eticlopride; *SCH*, SCH-23390 [(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine]; NPA, *N*-propylnorapomorphine; and *NA*, noradrenaline.

parent M_r ≅ 140,000 polypeptide, porcine striatal membranes were prepared and photoaffinity labeled in the absence or presence of multiple protease inhibitors. As illustrated in Fig. 2, preparing and photoaffinity labeling porcine striatal membranes in Tris buffer alone did not significantly affect the pattern of [^{125}I]NAPS incorporation into the subunits of apparent M_r ≅ 140,000, 94,000 and 34,000, as compared with samples prepared and photolyzed in the presence of multiple protease inhibitors. In the absence of protease inhibitors, a M_r ≅ 52,000 peptide appeared to be partially displaceable. This band appeared to correspond to the nonspecifically labeled peptide of M_r ≅ 52,000 observed in canine striatal membranes (Fig. 2). Thus, the inclusion of multiple class-specific protease inhibitors, which have been shown to be useful in preventing receptor degradation in other systems (6–8) and even for photoaffinity-labeled D_2 receptors of rat striatum (3), did not prevent the specific labeling of "lower" molecular weight subunits of apparent M_r ≅ 94,000 and 34,000 of porcine striata or the M_r ≅ 34,000 subunit of canine striata, consistent with previous observations (3, 4). These data suggest that either an inappropriate protease inhibitor cocktail was chosen or that the M_r ≅ 94,000 and 34,000 labeled subunits are not simply proteolytic degradation products of the M_r ≅ 140,000 protein.

Previous work on both the insulin (9) and β-adrenergic receptors (10) have shown that the M_r of receptors on SDS-PAGE can vary considerably, depending on the reduction-oxidation state of the protein. In order to investigate the possible subunit structure of the apparent M_r ≅ 140,000 and 94,000 photolabeled subunits, the ability of dithiothreitol (or β-mercaptoethanol) and *N*-ethylmaleimide to alter the migration pattern of photolabeled receptors was investigated. *N*-Ethylmaleimide (at 10 mM), dithiothreitol (10 mM) or β-mercaptoethanol (10%) added during receptor solubilization in SDS sample buffer did not alter the migration patterns of porcine striatal photoaffinity-labeled bands of apparent M_r of 140,000 and 94,000 (data not shown), as compared with samples prepared in SDS buffer alone. These data indicate that neither the M_r ≅ 140,000 nor M_r ≅ 94,000 subunits contain intramolecular disulfide bridges.

Glycoprotein nature of photolabeled D_2 dopamine receptor from porcine brain. [^{125}I]NAPS photoaffinity-labeled proteins of M_r ≅ 94,000 and 34,000, representing the ligand-binding subunit of canine striatal D_2 dopamine receptors, have been shown to bind to and be specifically eluted from WGA-Sepharose columns with GlcNAc (4). In order to assess whether photoaffinity-labeled porcine striatal D_2 binding subunits of apparent M_r ≅ 140,000, 94,000, and 34,000 are glycoproteins, [^{125}I]NAPS-labeled membranes were solubilized and applied to WGA-Sepharose columns. As depicted in Fig. 3, all three photolabeled subunits of apparent M_r ≅ 140,000, 94,000, and 34,000 were extracted from porcine striatal membranes by the detergent digitonin. Moreover, under the conditions used, virtually all of the labeled receptor present bound to the resin, as

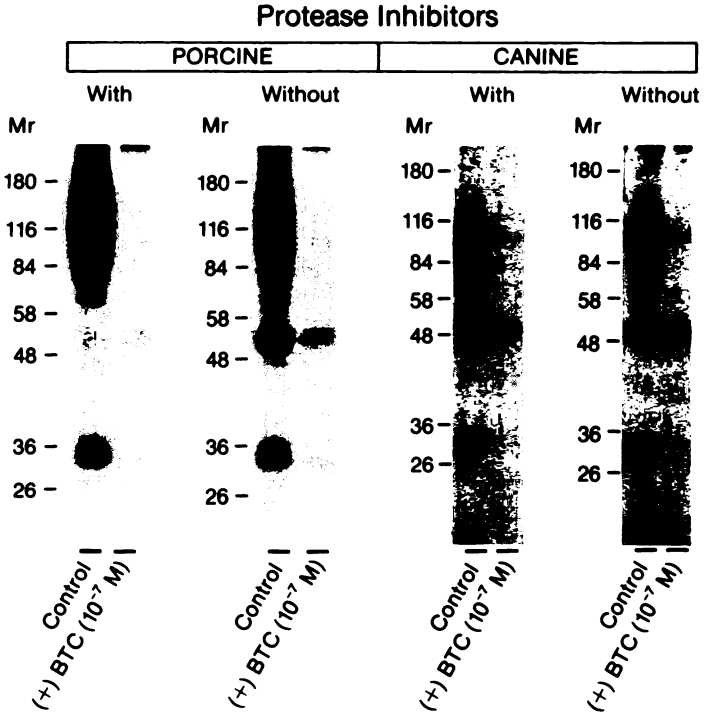

Fig. 2. Effect of protease inhibitors on the photoaffinity labeling pattern of [^{125}I]NAPS into D_2 receptors of porcine and canine striatal membranes. Membranes were prepared, photolyzed in the complete absence (Tris buffer alone) or presence of protease inhibitors, and subjected to SDS-PAGE, as described in Experimental Procedures, using a 12% acrylamide gel. The results shown are typical of three similar experiments. Included in the gel are lanes demonstrating nonspecific labeling, as defined by 100 nM (+)-butaclamol, (*BTC*). M_r of molecular weight standards are shown ×1000.

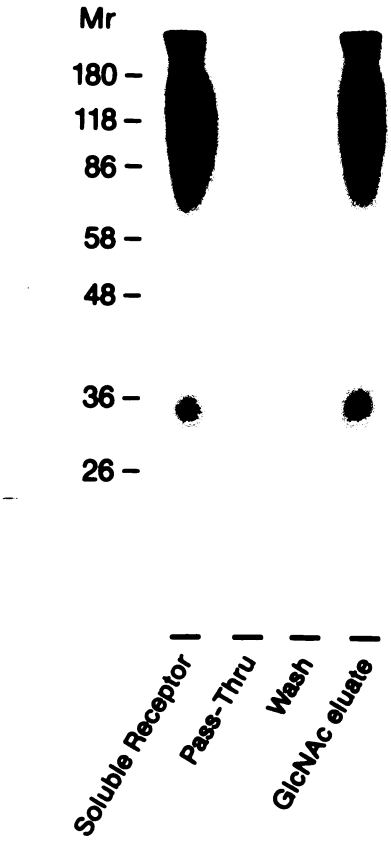

Fig. 3. WGA-Sepharose chromatography of [^{125}I]NAPS photoaffinity-labeled D_2 receptors. Porcine striatal membranes were photoaffinity labeled with [^{125}I]NAPS, solubilized in digitonin, and subjected to WGA-Sepharose chromatography as described in Experimental Procedures. Equal amounts of radioactivity of digitonin-solubilized (~1.0 mg) and GlcNAc-eluted (200 μg) samples were lyophilized and processed for SDS-PAGE on a 12% acrylamide gel, as described in Experimental Procedures. The results shown are representative of two other experiments. M_r of known prestained standards are shown ×1000.

indicated by the lack of radioactivity found in the pass-through and column wash fractions. The addition of 300 mM GlcNAc eluted the labeled receptor-binding subunits with >80% recovery. These data clearly suggest that [^{125}I]NAPS-labeled subunits of the porcine D_2 receptor are glycoproteins. The exact mode of porcine D_2 receptor interaction with WGA-Sepharose is unknown, although previous data indicate that D_2 receptor sialic acid residues interact only weakly with the resin (4) and so suggest that the primary site of binding to WGA is probably via poly(*N*-acetyllactosamine)-type glycans (11).

To further investigate the nature of the high molecular weight form of the D_2 receptor in this tissue, we analyzed the carbohydrate composition of these photolabeled proteins with the use of specific exo- and endo-glycosidases. Fig. 4A depicts the effects of neuraminidase digestion on both photoaffinity-labeled canine and porcine D_2 dopamine receptors. As previously reported (4), neuraminidase at a concentration of 2 units/ml altered the mobility of the apparent $M_r \cong 94{,}000$ subunit to apparent $M_r \cong 54{,}000$. Similarly, neuraminidase digestion of [^{125}I]NAPS-labeled porcine striatal membranes drastically altered the mobility of the $M_r \cong 140{,}000$ and 94,000 subunits to a distinct band at apparent $M_r \cong 52{,}000$. Concentration-effect experiments with neuraminidase revealed that this was the maximal effect seen (data not shown). Inasmuch as neuraminidase specifically cleaves terminal sialic residues, both the [^{125}I]NAPS-labeled subunits of $M_r \cong 140{,}000$ and 94,000 contain terminal sialic acid. The $M_r \cong 34{,}000$ subunit of porcine brain, although not clearly visible in this underexposed autoradiogram, did display a marginal sensitivity to neuraminidase digestion (change of $M_r \cong 1000$). This is consistent with similar observations made in canine brain (4). However, it should be noted that the M_r of these lower molecular weight peptides cannot be accurately determined on these acrylamide gels.

The effect of neuraminidase digestion on the electrophoretic mobility of photolabeled D_2 receptors of canine brain resulted in a diffuse band at apparent $M_r \cong 54{,}000$ (Fig. 4A). By increasing the concentration of acrylamide (from 10 to 12%) and the length of the separating gel (from 12 to 16 cm), this band could be resolved into a doublet of apparent $M_r \cong 54{,}000$ and 51,000. In contrast, neuraminidase-treated photolabeled striatal membranes from porcine brain migrate as a single band of apparent $M_r \cong 51{,}000$ under these conditions (Fig. 4B). Mixing solubilized porcine and canine photolabeled receptors before SDS-PAGE and autoradiography confirmed the existence of post-neuraminidase heterogeneity in canine striatal binding subunits. These data suggest that both porcine and canine labeled striatal D_2 binding subunits share at least one common subunit of $M_r \cong 51{,}000$ after neuraminidase treatment. The exact molecular nature of the apparent $M_r \cong 54{,}000$ subunit observed in canine striatal membrane is unknown but does not appear to be due to the presence of high mannose-type chains, because sequential neuraminidase and α-mannosidase treatment (12 units/ml) did not alter the migration pattern of the $M_r \cong 54{,}000$ subunit (data not shown). Possibly, the $M_r \cong 54{,}000$ subunit represents an undifferentiated D_2 receptor fragment whereas the $M_r \cong 51{,}000$ subunit is a fully processed form of the receptor. Moreover, it is still unclear whether the neuraminidase-induced change in the electrophoretic mobility of the photolabeled $M_r \cong 140{,}000$ and 94,000 binding subunits represents the quantitative cleavage of large amounts of receptor-associated sialic acids or simply relieves the interference caused by negatively charged residues on protein migration in SDS-PAGE.

As listed in Table I, porcine striatal D_2 receptors, after neuraminidase treatment, bound the selective D_2 receptor antagonist [^{3}H]spiperone in a saturable manner. Moreover, the affinity of [^{3}H]spiperone for the neuraminidase-treated receptor was similar to that for control membranes. These data suggest that the receptor-associated sialic acid residues are not necessary for ligand binding to the $M_r \cong 51{,}000$ subunit, as has

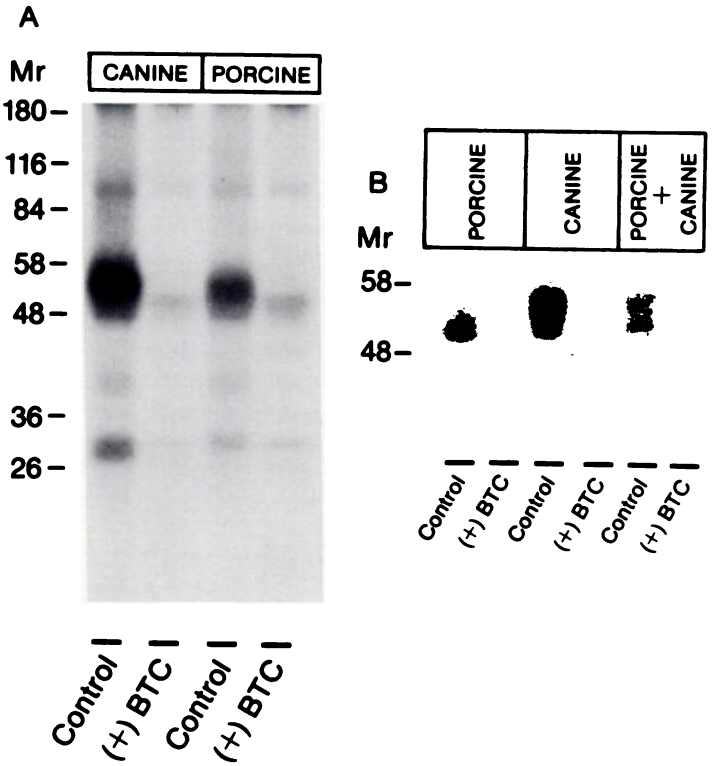

Fig. 4. Effect of neuraminidase treatment on the D_2 dopamine receptor of the porcine and canine striata. A, [^{125}I]NAPS-labeled membranes were prepared for enzyme treatment as described under Experimental Procedures. Neuraminidase was added to the indicated aliquots at 2 units/ml and incubated at 37° for 1 hr. Included in this gel is a lane demonstrating nonspecific labeling (neuraminidase-treated) as defined by (+)-butaclamol. After treatment the samples were prepared and subjected to SDS-PAGE, as described under Experimental Procedures using a 10% acrylamide gel. The relative molecular weights are shown ×1000. This experiment was repeated three times with similar results. B, Porcine or canine striatal [^{125}I]-labeled D_2 receptors were treated with neuraminidase as described above and prepared for SDS-PAGE using a 12% acrylamide separating gel of 16–17 cm. An equal mixture of neuraminidase-digested porcine and canine striatal D_2 receptors were also analyzed.

TABLE 1

[^{3}H]Spiperone binding to neuraminidase-treated D_2 receptors

Membranes were prepared and treated with 2 units/ml neuraminidase for 15 min at 37° as described in Experimental Procedures. After incubation, membranes were washed in Tris·HCl buffer and assayed for D_2 receptor activity, as described in Experimental Procedures.

B_{max} and K_D values for [^{3}H]spiperone binding were derived from computer-assisted analysis of the data (LIGAND) and are the means ± standard error obtained from three experiments. Simultaneous analysis of control and neuraminidase-treated curves revealed no statistical difference in either the K_D or B_{max}

Condition	K_D	B_{max}
	pM	
Control	40 ± 5	18.5 ± 1
Neuraminidase-treated	62 ± 16	18.9 ± 2

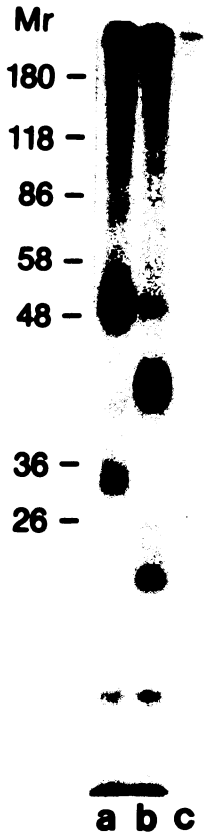

Fig. 5. Effect of PNGase-F treatment on the D_2 dopamine receptor binding subunit of porcine striatal membranes. Membranes were labeled with [^{125}I]NAPS, treated with 2 units/ml neuraminidase and incubated with sodium phosphate buffer containing 0.5% SDS and 100 mM β-mercaptoethanol for 60 min at 22° as described in Experimental Procedures (*lane a*). PNGase-F (50 units/ml) was added to the indicated aliquots (*lanes b* and *c*) and incubated for 18 hr at 37°. Control samples (*lane a*; neuraminidase-treated) were subjected to the same experimental procedures but without PNGase-F. Included in this gel is a lane (*lane c*) demonstrating nonspecific labeling. After treatment, samples were prepared and subjected to SDS-PAGE as described under Experimental Procedures using a 12% acrylamide gel. Molecular weight markers are shown ×1000.

been similarly demonstrated for the canine striatal D_2 receptor (4).

It is still possible, however, that altering the carbohydrate composition of the receptor and, therefore, subunit molecular mass after neuraminidase treatment may influence dopaminergic agonist interaction with D_2 receptors, as has been suggested for the γ-aminobutyric-benzodiazepine receptor complex (12, 13).

The next series of experiments were conducted to gain an approximation of the relative molecular mass of the protein backbone of porcine striatal labeled D_2 receptors. For these experiments neuraminidase-treated labeled membranes were partially denatured and incubated with peptide-N^4[*N*-acetyl-β-glucosaminyl]asparagine amidase (*N*-glycanase; PNGase-F), an enzyme that is capable of removing high mannose, hybrid, and complex-type oligosaccharide chains via hydrolysis of the peptide-*N*-acetylglucosamine-asparagine bond (14). As illustrated in Fig. 5, control labeled membranes (neuraminidase-treated), which were subjected to the same protocol (but without PNGase-F), migrated on SDS-PAGE with an apparent $M_r \cong 51{,}000$ and 34,000 (as described above). The addition of PNGase-F, at 50 units/ml, further increased the electrophoretic mobility of the $M_r \cong 51{,}000$ subunit to apparent $M_r \cong 44{,}000$. In addition, the $M_r \cong 34{,}000$ subunit was susceptible to PNGase-F digestion, as indicated by its increased mobility to an apparent $M_r \cong 23{,}000$. Included in this gel was a lane demonstrating nonspecific labeling. The lack of other new lower molecular weight bands on the gel suggested that little proteolysis was occuring during enzyme treatment. Moreover, the quantitative recovery (>96%) of labeled peptides after treatment suggested that receptor activity was not being degraded to any great extent. The apparent $M_r \cong 21{,}000$ subunit seen in Fig. 1A and Fig. 5 is a specifically labeled D_2 receptor binding subunit that does not appear to be glycosylated. The exact molecular nature of this low molecular weight subunit, which is always observed, is unknown.

The significant increase in receptor mobility on SDS-PAGE caused by PNGase-F treatment (>30% of the $M_r \cong 34{,}000$ labeled subunit and 15% of the $M_r \cong 51{,}000$ subunit) suggests that a large component of receptor-associated carbohydrate chains are *N*-linked at asparagine residues. In agreement with previous observations on canine striatal membranes (4), the deglycosylated form of porcine D_2 receptors resides in subunits of apparent $M_r \cong 44{,}000$ and 23,000, which appear common to both species. Whether there are any additional sites for *N*-linked glycosylation, as has been suggested for canine striatal D_2 receptors (4), is unknown. Moreover, preliminary evidence suggests that there are no carbohydrate chains *O*-linked to serine or threonine residues, inasmuch as sequential PNGase-F and *O*-glycanase treatment (at 1 unit/ml) did not further increase the electrophoretic mobility of the apparent $M_r \cong 44{,}000$ or 23,000 subunits. However, given the rather extreme site-specific cleavage requirements for *O*-glycanase (15), these results must be interpreted with caution.

The exact functional role, if any, of D_2 receptor carbohydrate is unknown. Previous work on β- (16–19) and α_1- (20) adrenergic, acetylcholine (21), and adenosine A_1 (22) receptors revealed little effect of deglycosylation or the inhibition of *N*-glycosylation in the acquisition of ligand receptor binding characteristics and transmembrane signalling processes. In contrast, receptors for epidermal growth factor (23) and low density lipoprotein (24, 25), and the γ-aminobutyric benzodiazepine receptor complex (12, 13) appear to require receptor carbohydrate for normal ligand binding and function. At least for neuronal D_2 receptors, simple removal of sialic acid is not necessary for saturable and high affinity antagonist binding (this study and Ref. 4). Further work with cultured cell lines may yield more information on the role of *N*-linked oligosaccharides in receptor function.

In summary, the data presented in the communication indicate that the porcine striatal D_2 receptor is a glycoprotein containing complex-type carbohydrate chains. The relative molecular mass of the *N*-deglycosylated [^{125}I]NAPS-labeled protein backbone residues in two subunits of apparent $M_r \cong 44{,}000$ and 23,000, similar if not identical to completely *N*-deglycosylated D_2 receptors of canine striatal membranes (4). Thus, despite the existence of a high molecular weight form of photoaffinity-labeled neuronal porcine D_2 receptor binding subunit, deglycosylated subunits from both species migrate with similar molecular masses on SDS-PAGE; this suggests that, although different glycosylation pathways or processing occurs in both species, the molecular nature of the D_2 receptor appears to be

similar. It is unclear, however, whether the apparent $M_r \cong$ 44,000/23,000 subunits represent the functional D_2 receptor moiety or merely the ligand-binding subunit alone and whether these two proteins are noncovalently or covalently coupled under physiological conditions. Ultimately, studies of the predicted peptide core structure based on cDNA sequences (once obtained) will resolve this issue.

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