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Photoaffinity labeling of the mammalian dopamine transporter

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A high affinity (1–2 nM) radioiodinated, photoaffinity probe for the dopamine transporter, 1-(2-[bis-(4-fluorophenyl)-methoxylethyl)-4-(2-[4-azido-3-[^{125}I]iodophenyl]ethyl)piperazine ([^{125}I]FAPP) has been synthesized. Upon photolysis, [^{125}I]FAPP incorporates into a striatal polypeptide of apparent M_r 62000 as visualized by autoradiography following sodium dodecyl sulfate-PAGE. Photoincorporation of [^{125}I]FAPP into the M_r 62000 polypeptide was stereoselectively inhibited by various dopamine uptake agents with a potency order typical of the dopamine transporter. The glycoprotein nature of the apparent M_r 62000 polypeptide was assessed following specific exo- and endoglycosidase treatment. The dopamine transporter appears to be associated with complex-type oligosaccharides as indexed by its susceptibility to neuraminidase but not α-mannosidase digestion. Complete *N*-linked deglycosylation of the neuronal dopamine transporter with the endoglycosidase, glycopeptidase-F, increased the electrophoretic mobility of the 62 kDa polypeptide to apparent M_r 48000. [^{125}I]FAPP should prove to be a useful probe for the molecular characterization of the dopamine uptake site in various tissues and under certain pathophysiological states.

Dopamine uptake; Transporter; Photoaffinity labeling; Deglycosylation

1. INTRODUCTION

[^{3}H]GBR-12935 (1-[2-diphenylmethoxy]ethyl)-4-(3-phenylpropyl)piperazine [1,2] and [^{3}H]GBR-12783 (1-[2-diphenylmethoxy]ethyl)-4-(3-phenyl-2-propenyl)piperazine) [3] have been widely used as selective and high affinity radiolabeled probes for the dopamine transporter in both rat and human brain [4–8]. These radioligands represent derivatives of a series of aryl-dialkylpiperazines with potent and selective dopamine uptake inhibiting properties [9,10]. The binding of [^{3}H]GBR-12935 to rat and human striatal membranes has been convincingly demonstrated, on both pharmacological and biochemical grounds to be related to the dopamine uptake site (see above refs).

In order to examine some of the molecular events associated with the dopamine uptake process, it would be helpful to be able to covalently label the dopamine transporter by means of a radioactive photoaffinity probe. Although both dopamine D_1 and D_2 receptors can now be so identified (see [11,12]) the elucidation of the structure of the dopamine transporter has been limited by the lack of a suitable radioiodinated photoaffinity label. In this paper, we document the synthesis and characterization of a high affinity, radioiodinated arylazide derivative of the dopamine uptake blocker GBR-12909, [^{125}I]FAPP. This ligand, upon photolysis, incorporates with high efficiency into a polypeptide of apparent M_r 62000 represen-

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Abbreviations: [^{125}I]FAPP, 1-(2-[bis-(4-fluorophenyl)methoxy]ethyl)-4-(2-[4-azido-3-iodophenyl]ethyl)piperazine; GBR-12909, 1-(2-[bis-(4-fluorophenyl)methoxy]ethyl)-4-(3-phenyl-2-propenyl)piperazine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PNGase-F, peptide *N*-glycosidase-F

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ting the neuronal ligand binding subunit of the dopamine transporter in both canine and human striatal membranes. Moreover, as a first step towards understanding the molecular structure of the neuronal dopamine transporter, we assessed the glycoprotein nature of [^{125}I]FAPP-labeled membranes following digestion with specific exo- and endoglycosidases.

2. MATERIALS AND METHODS

[^{3}H]GBR-12935 (30 Ci/mmol) was obtained from New England Nuclear. Digitonin was obtained from Wako Chemical Co. and Sephadex G-50 was from Pharmacia. GBR-12909 was purchased from Research Biochemical Inc. and GBR-12937 was kindly provided by Dr P. Andersen of Novo Industries A/S. (+)- and (−)-diclofensine were obtained from Roche, and mazindol, nomifensine and methylphenidate were generously donated by the following pharmaceutical firms: Sandoz Research Inst., Hoechst Pharmaceutical, Ciba-Geigy Corp., and Sterling-Winthop. Dopamine, pemoline, cocaine, noradrenaline, neuraminidase (type x from *Clostridium perfringens*), α-mannosidase (Jack-bean), PMSF, benzamidine, leupeptin, pepstatin A and soybean trypsin inhibitor were from Sigma Chemicals. Glycopeptidase-F (peptide-N^4-[*N*-acetyl-β-glucosaminyl]asparagine-amidase) from *Flavobacterium meningosepticum* was obtained from Boehringer-Mannheim. Electrophoresis reagents were from Calbiochem Inc. and prestained molecular weight standards were from Amersham. All other drugs and reagents were obtained from sources previously described [12]. Canine brains were purchased from Pel Freez Biol. (Arkansas).

2.1. *Synthesis of [^{125}I]FAPP*

The complete synthesis and chemical characterization of IFAPP will be described in a forthcoming publication (Schwartz, E. et al., in preparation). The structure and general synthetic route of [^{125}I]FAPP are depicted in fig.1. Briefly, the synthesis of the non-radioactive photoaffinity label 7 began with the condensation of 4,4′-difluorobenzhydrol, **1** (19.6 mmol), and 2-chloroethanol (**1.5** e.g.) in benzene (20 ml, reflux, 30 min) under acid catalysis (0.1 ml conc. H_2SO_4) to provide the ether **2** in ca. 90% yield (R_f = 0.39, 10% EtOAc/hexane). The chloride **2** (10 mmol) was treated with an excess of piperazine and anhydrous K_2CO_3 (ca. 4.5 eq. of each) to afford the mono-alkylated piperazine **3** (ca. 90% yield; R_f = 0.10, 85% EtOAc/10% hexane/5% Et_3N). Subsequent alkylation of **3** (1.33 mmol) with 4-nitrophenethyl bromide (1.2 eq.) in acetonitrile (8 ml, reflux, 5 h) in the presence of an excess of anhydrous K_2CO_3 provided piperazine **4** in 55% yield after flash chromatography (R_f = 0.45, 85% EtOAc/10% hexane/5% Et_3N).

Reduction of the nitro group of **4** was accomplished using 5% palladium on activated carbon in ethanol under 1 atm. of H_2. Filtration of the reaction mixture through Celite followed by solvent removal gave the aniline **5** in 95–100% yield (R_f = 0.30, 95% EtOAc/5% Et_3N). Slow addition of iodine monochloride (1.3 eq. in 2 ml HOAc) to a solution of **5** (0.44 mmol) in 2 ml

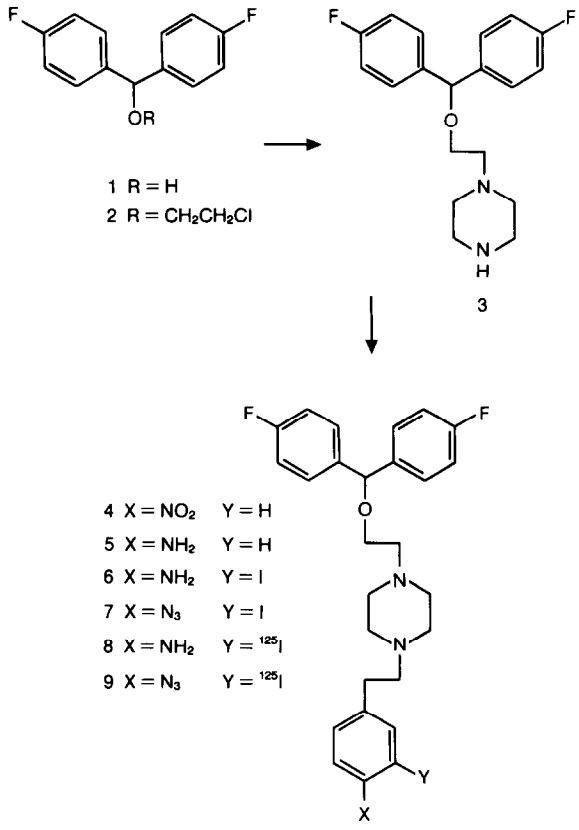

Fig.1. Structure and synthetic route of [^{125}I]FAPP.

HOAc afforded the iodoaniline **6** in 64% yield after neutralization of the reaction mixture (aq. $NaHCO_3$), extraction with Et_2O, and flash chromatography (R_f = 0.29, 95% EtOAc/5% Et_3N). Diazotization of the amine **6** (0.16 mmol, 1.5 eq. $NaNO_2$ in 7 ml 3 M aq. HOAc and 2 ml MeOH, 0°C) followed by direct reaction with NaN_3 (1.5 eq.) provided the iodoazide **7** in 80% yield after flash chromatography (R_f = 0.53, 85% EtOAc/10% hexane/5% Et_3N).

As is to be described elsewhere (Schwartz et al., in preparation), [^{125}I]FAPP (2200 Ci/mmol) was custom-prepared and purified by New England Nuclear.

2.2. *Membrane preparation*

Striata dissected from partially thawed canine brains were prepared as previously described [12]. For some experiments, membranes were prepared in 25 mM Tris-HCl buffer alone (pH 7.4 at 4°C) without the addition of protease inhibitors.

2.3. *Photoaffinity labeling*

Membranes (~200 μg) were routinely incubated (in the dark) with 1 nM [^{125}I]FAPP at a dopamine uptake site concentration of ~800 pM in a total volume of 1 ml for 90 min at 4°C in the presence or absence of dopaminergic agents as indicated. After incubation, samples were centrifuged (Eppendorf no.5415) at 14000 rpm for 2 min. Membrane pellets were resuspended in

1 ml of 25 mM Tris-HCl buffer containing 100 mM NaCl and protease inhibitors (where indicated), and irradiated for 35 s as previously described [12]. [^{125}I]FAPP-labeled membranes were sedimented at 14000 rpm for 2 min and used as described below.

2.4. *Exoglycosidase treatments*

For neuraminidase treatment, [^{125}I]FAPP-labeled membranes were washed twice in 1 ml of 100 mM sodium acetate buffer, pH 5 at 22°C. Membranes were resuspended in the same buffer at 0.5–1.0 mg/ml, to which neuraminidase was added at a concentration of 2 U/ml and incubated for 15 min at 37°C. After incubation, membranes were washed and solubilized in SDS-buffer as described below. For α-mannosidase treatment, [^{125}I]FAPP-labeled membranes were washed twice with 1 ml of 50 mM sodium citrate buffer (pH 4.5 at 22°C) containing 100 μM PMSF and 3 μg/ml pepstatin A and resuspended in the same buffer to which was added up to 6 U/ml of α-mannosidase and incubated overnight at 22°C. Pelleted membranes were then processed for SDS-PAGE as described below.

2.5. *Endoglycosidase treatments*

[^{125}I]FAPP-photolabeled receptors (as described above) were washed twice in 1 ml of 200 mM sodium phosphate buffer containing 10 mM EDTA, pH 8.0, at 22°C. Pellets were resuspended in the same buffer to which was added 0.5% SDS and 100 mM β-mercaptoethanol and incubated for 1 h at 22°C in a total volume of 60 μl. Nonidet P-40 (1.25% final concentration) was then added to the samples followed by the addition of PNGase-F (at a concentration of 50 U/ml) and incubated for 6–22 h at 37°C in a total volume of 90 μl. After incubation, samples were pelleted and membranes solubilized in SDS-PAGE sample buffer, as described below.

2.6. *SDS-PAGE and autoradiography*

Electrophoresis and autoradiography were performed as previously described [12].

3. RESULTS AND DISCUSSION

The product described here yields a novel analogue of the potent dopamine uptake blocker, GBR-12909. The compound, IFAPP, possesses an azido-phenyl substituent that renders it as a potent photoaffinity probe for the dopamine transporter. The capacity of IFAPP to interact with dopamine uptake sites was assessed in competition binding experiments with [^{3}H]GBR-12935. IFAPP displayed a high affinity for digitonin-solubilized [^{3}H]GBR-12935 binding sites with an estimated K_i

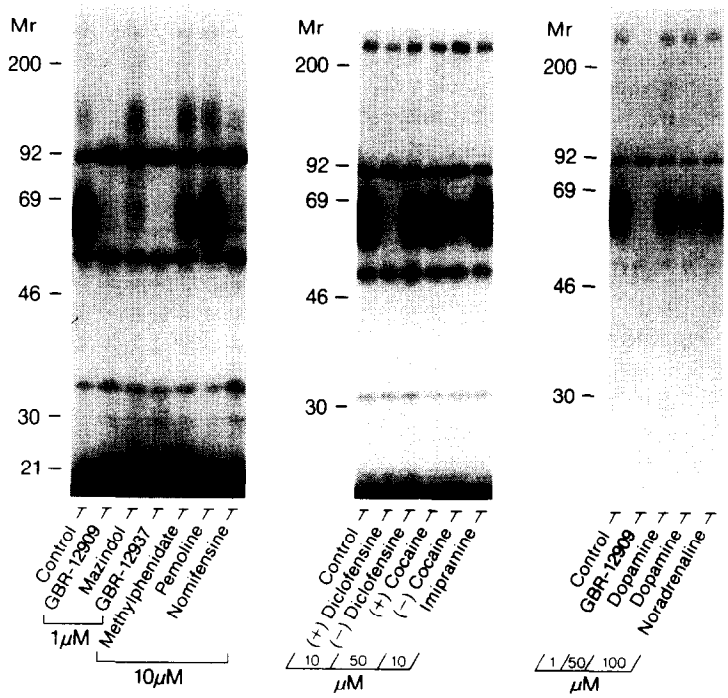

Fig.2. Pharmacological specificity of [^{125}I]FAPP-labeled membranes from canine striatum. Samples were photolyzed in the absence or presence of the indicated concentrations of dopaminergic uptake agents and subjected to SDS-PAGE and autoradiography as described in section 2. M_r of known molecular weight standards are shown $\times 10^{-3}$. These results are representative of 3 independent experiments.

value of ~3 nM. Thus, the introduction of both iodo and azido substituents did not interfere with the capacity of IFAPP to interact with the dopamine transporter compared to the parent compound, GBR-12909 (K_i ~ 3 nM).

The major objective of this work was to develop an arylazide derivative of GBR-12909 that can upon photolysis incorporate into the ligand binding subunit of the dopamine transporter. Fig.2 illustrates the results obtained when canine striatal membranes were incubated with [^{125}I]FAPP, subjected to photolysis, SDS-PAGE and autoradiography. A broad band was labeled at apparent M_r 62000. The specificity of labeling was shown by virtue of the fact that GBR-12909 blocked the photoincorporation of [^{125}I]FAPP into the M_r 62000 polypeptide. Three non-specifically labeled bands at apparent M_r 90000, 50000 and 32000 were also seen. The pharmacological specificity of [^{125}I]FAPP photoincorporation was further assessed by examining the capacity of various dopamine uptake blockers and substrates to block labeling of the M_r 62000 polypeptide. As seen in fig.2 the photo-dependent labeling of the 62 kDa polypeptide was stereoselectively antagonized by diclofensine and cocaine and by dopamine uptake inhibitors and substrates such as mazindol, methylphenidate, nomifensine and dopamine, but not by the serotonin uptake inhibitor, imipramine or noradrenaline. These data strongly suggest that the 62 kDa labeled polypeptide is the ligand binding subunit of the dopamine transporter. Similarly, a 62 kDa specifically labeled polypeptide was identified in membranes from human putamen (data not shown).

In order to assess whether the [^{125}I]FAPP-labeled polypeptide of M_r 62000 is a derivative of a larger molecular weight protein or contains disulfide linkages, we examined the electrophoretic mobility of [^{125}I]FAPP-labeled membranes photolabeled in either the absence or presence of multiple protease inhibitors (see [12]) or treated with thiol-reducing reagents prior to SDS-PAGE. As shown in fig.3, neither of these conditions altered the electrophoretic mobility of the dopamine transporter. These data suggest that the M_r 62000 labeled polypeptide is probably not a proteolytic derivative of a larger molecular weight species and is not linked by intramolecular disulfide bridges.

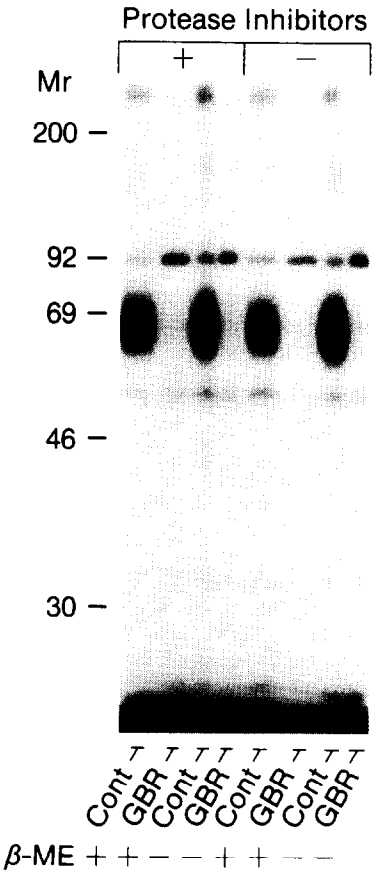

Fig.3. Effect of protease inhibitors and reducing agents on the electrophoretic mobility of [^{125}I]FAPP-labeled membranes. Samples were photolyzed in the absence or presence of multiple protease inhibitors (20 mM EDTA, 15 μg/ml benzamidine, 5 μg/ml leupeptin and soybean trypsin inhibitor, 1 mM PMSF) and solubilized in sample buffer in the absence or presence of 10% β-mercaptoethanol (β-ME). M_r of known molecular weight standards are shown × 10^{-3}.

As illustrated in fig.4, the M_r 62000 labeled polypeptide appears to be associated with glycan chains containing sialic acid/*n*-acetylglucosamine residues since neuraminidase but not α-mannosidase treatment altered the electrophoretic mobility of the dopamine transporter to apparent M_r 56000. Sequential treatment with both enzymes, irrespective of order, did not further increase the mobility of the 62 kDa polypeptide. These data are consistent with the observations that the dopamine uptake site is a complex type glycoprotein as indexed by the interaction of solubilized [^{3}H]GBR-12935 binding sites with

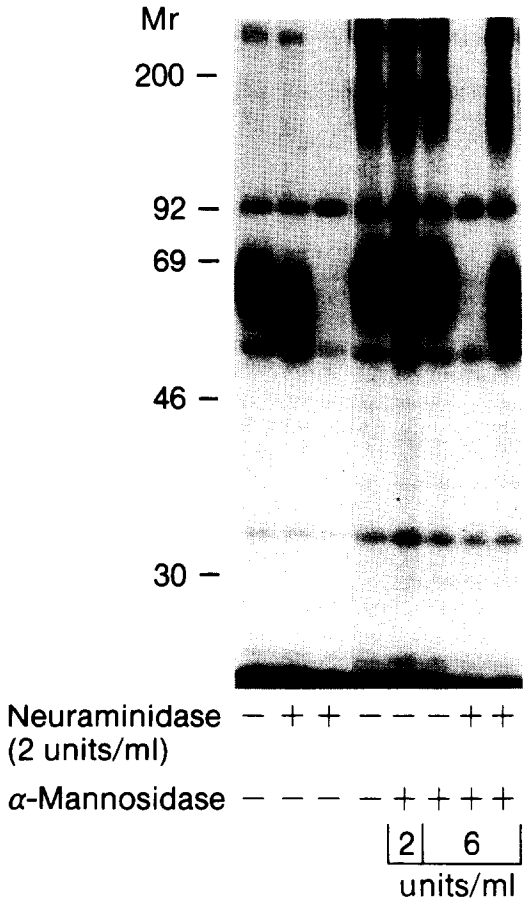

Fig.4. Exoglycosidase treatment of [^{125}I]FAPP-labeled dopamine uptake sites. Membranes were labeled with [^{125}I]FAPP, washed with the appropriate buffer and incubated with buffer alone or with the indicated concentrations of neuraminidase or α-mannoside as described in section 2. Included in this gel are lanes demonstrating non-specific labeling as defined by 1 μM GBR-12909, and sequential neuraminidine and α-mannosidase treatments. M_r of protein standards are shown $\times 10^{-3}$.

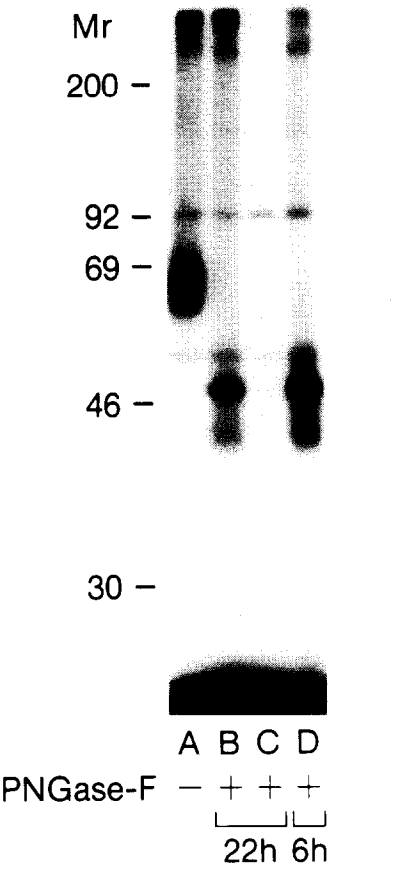

Fig.5. Effect of PNGase-F treatment on the electrophoretic mobility of the dopamine transporter. [^{125}I]FAPP-labeled membranes were prepared for enzyme treatment as described. Aliquots were incubated with buffer alone (A) or with 50 U/ml PNGase-F for 22 h (lane B) at 37°C. Lane C, same as B except photolabeling in the presence of 1 μM GBR-12909; lane D same as B except for a 6 h incubation.

various lectins [13,14] and the role of sialic acid residues in the dopamine uptake process [15].

The peptide backbone of the dopamine transporter was assessed following complete *N*-linked deglycosylation with PNGase-F [16]. As seen in fig.5, PNGase-F treatment of [^{125}I]FAPP-labeled membranes for either 6 or 22 h increased the electrophoretic mobility of the 62 kDa labeled polypeptide to apparent M_r 48000. In addition, a minor (~10%) polypeptide of apparent M_r 44000 was also seen. At present it is unknown whether this fragment represents a proteolytic degradation product of the M_r 48000 protein or is evidence for multiple *N*-linked glycosylation sites on the transporter. Moreover, it is difficult to ascertain whether the 48 kDa polypeptide represents just the ligand binding subunit alone or the entire dopamine transport protein. Final assessment will have to await the purification of dopamine uptake site and its functional reconstitution and/or the isolation of cDNA clones encoding its synthesis.

In any event, the introduction of this specific, high affinity, radioiodinated, photoaffinity probe for the dopamine transporter should prove to be of

value for the subsequent molecular characterization and purification of this important membrane protein.

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