0026-895X/91/040429-07$03.00/0

MOLECULAR PHARMACOLOGY, 39:429-435



*ACCELERATED COMMUNICATION*

# Photoaffinity Labeling of the Dopamine Reuptake Carrier Protein with 3-Azido[$^3$H]GBR-12935

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Received September 27, 1990; Accepted January 7, 1991

**SUMMARY**

A high affinity tritiated azido-diphenylpiperazine derivative, 3-azido[$^3$H]GBR-12935, was synthesized as a potential photoaffinity probe of the dopamine transporter. Initially, the reversible binding of 3-azido[$^3$H]GBR-12935 to crude synaptosomal membranes from the rat striatum was characterized. Specific binding was sodium dependent and inhibited by a variety of drugs that are known to potently inhibit dopamine uptake. Other neurotransmitter uptake inhibitors, as well as *cis*-flupenthixol, a potent inhibitor of [$^3$H]GBR-12935 binding to piperazine binding sites, failed to inhibit specific binding at concentrations of ≤10 μM. A good correlation was observed between the relative potencies of these drugs in inhibiting dopamine uptake into synaptosomes and in inhibiting specific 3-azido[$^3$H]GBR-12935 binding to rat striatal membranes ($r = 0.95$, $p < 0.01$). These data suggest that 3-azido[$^3$H]GBR-12935, like other diphenylpiperazines such as [$^3$H]GBR-12935 and [$^3$H]GBR-12909, binds primarily to the dopamine transporter under defined assay conditions. After UV photolysis of crude synaptosomal membranes preincubated with 3-azido[$^3$H]GBR-12935 (1–2 nM), a single radiolabeled polypeptide with an apparent molecular mass of 80 kDa was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Photoincorporation of 3-azido[$^3$H]GBR-12935 into this polypeptide was inhibited selectively by compounds that inhibit the uptake of dopamine (but not other biogenic amines) and was completely dependent on the presence of $Na^+$. No photolabeled proteins were observed when cerebellar membranes were substituted for striatal membranes. Essentially complete adsorption of the radiolabeled 80-kDa polypeptide to wheat germ agglutinin and elution with *N*-acetyl-D-glucosamine strongly suggest that the dopamine transporter polypeptide photolabeled by 3-azido[$^3$H]GBR-12935 is glycosylated.

The reuptake of biogenic amine and amino acid neurotransmitters into nerve terminals is generally regarded as the major inactivation mechanism for reducing the synaptic concentration of neurotransmitter following release (1). This carrier-mediated reuptake is sodium and energy dependent and can be selectively and potently inhibited by a variety of drugs. Moreover, the major psychopharmacological actions of many of these drugs can be directly attributed to a blockade of reuptake, resulting in an enhancement of the synaptic concentration of neurotransmitter (1). Several of the more potent reuptake inhibitors have also been used as radioligands for labeling specific neurotransmitter transporters, including those for serotonin (2), norepinephrine (3), and dopamine (3–9) (for review see Ref. 10). Recently, Ritz *et al.* (11) have shown that cocaine and related drugs compete with radiolabeled dopamine uptake inhibitors for binding sites on the dopamine transporter, with affinities that are highly correlated with their reinforcing properties. The latter has led to the hypothesis that the dopamine transporter itself may represent a pharmacologically relevant cocaine "receptor."

The physiological and pharmacological significance of these neurotransmitter reuptake mechanism(s) has prompted recent efforts at solubilizing, purifying, and characterizing the transporters for GABA (12), serotonin (13–15), and dopamine (16, 17). Grigoriadis *et al.* (16), as well as Sallee *et al.* (17), recently reported the photoaffinity labeling of a dopamine transporter protein from rat and dog striatal membranes by two high affinity radioiodinated diphenylpiperazine derivatives. Photoaffinity labeling of one protein (approximately 60 kDa) was inhibited by dopamine uptake inhibitors, with potencies that

**ABBREVIATIONS:** GABA, γ-aminobutyric acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate; 3-azido[$^3$H]GBR-12935, 1-[2-[bis(phenyl)methoxy]ethyl]-4-[3-(3′-azidophenyl)-[2,3-$^3$H]propyl]piperazine; GBR-12935, 1-[2-[bis(phenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine; GBR-12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenyl-2-propenyl]piperazine; [$^{125}$I]DEEP, 1-[2-[bis(phenyl)methoxy]ethyl]-4-[2-(4′-azido-3′-[$^{125}$I]iodophenyl)ethyl]piperazine.

parallel their relative potencies in inhibiting dopamine uptake. In both studies, however, multiple, presumably unrelated, photolabeled proteins were also observed, therefore limiting the utility of these radioligands for covalent labeling and subsequent purification of the dopamine transporter polypeptide. Recently, we synthesized a tritiated azido diphenylpiperazine derivative, 3-azido[$^3$H]GBR-12935 (18), which binds specifically and with high affinity to the dopamine transporter. Using 3-azido[$^3$H]GBR-12935, we now report the successful photoaffinity labeling of a dopamine transporter glycoprotein from rat striatal membranes, with an apparent molecular mass of approximately 80 kDa.

## Materials and Methods

**Synthesis of high specific activity 3-azido[$^3$H]GBR-12935.** The synthetic route to 3-azido[$^3$H]GBR-12935 ([$^3$H]-**I**) is outlined in Fig. 1. It commenced with 1-[2-[bis(phenyl)methoxy]ethyl]piperazine (**II**), as described by van der Zee *et al.* (19). Coupling of **II** with 3-nitrocinnamoyl chloride yielded 1-[2-[bis(phenyl)methoxy]ethyl]-4-[3-(3′-nitrophenyl)-1-oxo-2-propenyl]piperazine (**III**). The nitro group of **III** was selectively reduced with excess carbon monoxide in the presence of rhodium-triphenylphosphine complex, to give 1-[2-[bis(phenyl)methoxy]ethyl]-4-[3-(3′-aminophenyl)-1-oxo-2-propenyl] piperazine (**IV**). Lithium aluminum hydride reduction of **IV** afforded 1-[2-[bis(phenyl)methoxy]ethyl]-4-[3-(3′-aminophenyl)-2-propenyl] piperazine (**V**). Compound **V** served as a precursor for the synthesis of high specific activity [$^3$H]-**I**. Thus, catalytic tritiation of **V** with carrier-free tritium gas in the presence of 10% Pd/C afforded high specific activity (41.8 Ci/mmol) 1-[2-bis(phenyl)methoxy]ethyl]-4-[3-(3′-aminophenyl)-[2,3-$^3$H]propyl]piperazine (**VI**). The target compound, [$^3$H] -**I**, was synthesized in excellent yield by treatment of **VI** with excess aqueous nitrous acid, followed by quenching of the resultant diazonium cation with sodium azide. A complete description of the synthesis of [$^3$H]-**I** is described in detail elsewhere (18). This compound was stored in the dark at −25°, as a 2.13 mCi/ml (51 μM) solution in ethanol.

**Preparation of the crude synaptosomal fraction.** Male Sprague-Dawley rats (100–200 g), housed under diurnal lighting conditions (12/12 hr) with free access to food and water, were used in all experiments. Following decapitation, the corpus striatum was dissected out on ice, yielding approximately 100 mg of tissue (wet weight) from each rat. The crude synaptosomal fraction was obtained essentially as described (20). Briefly, tissue pooled from several rats was homogenized in 10 volumes of ice-cold 0.32 M sucrose containing 100 μM PMSF, with a Teflon-glass homogenizer, and was centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 23,000 × *g* for 20 min, and the resulting pellet (P2) was resuspended as described below.

**Reversible binding of 3-azido[$^3$H]GBR-12935 to P2 membranes and solubilized membrane proteins.** The binding of 3-azido[$^3$H]GBR-12935 to crude synaptosomal (P2) membranes was quantified as follows. The P2 pellet (from 100 mg of fresh tissue) was suspended in 20 ml of incubation buffer (50 mM Tris·HCl, pH 7.4, 120 mM NaCl, 0.01% bovine serum albumin). The incubation mixture contained 0.4 ml of membrane suspension, 0.1 ml of 3-azido[$^3$H]GBR-12935 (final concentration, 1–5 nM), 1.7 ml of incubation buffer, and 0.1 ml of the same incubation buffer with or without mazindol. The latter (final concentration, 10 μM) was added to determine nonspecific binding. After incubation at 25° in the dark for 45 min, the reaction was terminated by rapid filtration of the suspensions through Whatman GF/B glass fiber filters. The filters were rapidly washed with three 4-ml portions of ice-cold incubation buffer, and the radioactivity on the filters was determined following the addition of scintillation fluid. The counting efficiency was 50%. Data were analyzed by the nonlinear least-squares fitting program LIGAND of Munson and Rodbard (21).

**Photoaffinity labeling of striatal membranes.** To determine the nature of the protein(s) labeled with 3-azido[$^3$H]GBR-12935 and the degree of specificity of the labeling, the P2 fraction was suspended by vortexing in 10 mM sodium phosphate (pH 7.4) containing 0.2 M NaCl (4 ml/100 mg of fresh tissue). Where a sodium-free medium was desired, 10 mM potassium phosphate (pH 7.4) containing 0.2 M choline chloride was substituted. The suspension was incubated at 25° for 1 hr in the dark with 3-azido[$^3$H]GBR-12935 (final concentration, 1–2 nM), in the presence or absence of additional drugs (final concentration, 0.1–10 μM). Samples of 1 ml were placed in 3-ml quartz cuvettes (1-cm light path) placed approximately 1 cm from the surface of a vertically oriented UV transilluminator (model TM-36; UVP, Inc., San Gabriel, CA) having a peak wavelength of 302 nm and a peak intensity of 7.0 mW/cm$^2$. Photolysis was carried out for 45 sec. Essentially the same procedure was used to prepare larger amounts of photolabeled membranes for subsequent protein extraction. In this case, the P2 pellet from 10 pairs of striata was suspended at 25°, by gentle homogenization, in 12 ml of 10 mM sodium phosphate (pH 7.4) containing 0.2 M NaCl. After the addition of 3-azido[$^3$H]GBR-12935 (final concentration, ~20 nM), the suspension was incubated at 25° in the dark for 45 min, divided among four cuvettes, and photolyzed as described above. The membranes were pelleted by centrifugation at 23,000 × *g* for 10 min at 5°, suspended in 12 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.4) by homogenization, and repelleted.

**Solubilization of membrane proteins.** The osmotically shocked, 3-azido[$^3$H]GBR-12935-labeled P2 pellet from 10 striatal pairs was suspended by homogenization in 19 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.4) containing 10 mM sodium phosphate (pH 7.4), 0.5% CHAPS, 0.2 M NaCl, and 100 μM PMSF. The suspension was stirred for 20 min at 5° and then centrifuged at 150,000 × *g* at this temperature for 30 min, to yield the solubilized membrane proteins (approximately 1 mg/ml).

**Affinity chromatography.** Up to 10 ml of the solubilized protein fraction were applied (1 ml every 2.5 min) at 25° to a 2-ml column of wheat germ lectin-Sepharose 6MB (Pharmacia LKB, Piscataway, NJ). The column was subsequently washed with 6 ml of 10 mM sodium phosphate (pH 7.4) containing 0.5% CHAPS and 0.2 M NaCl, to yield

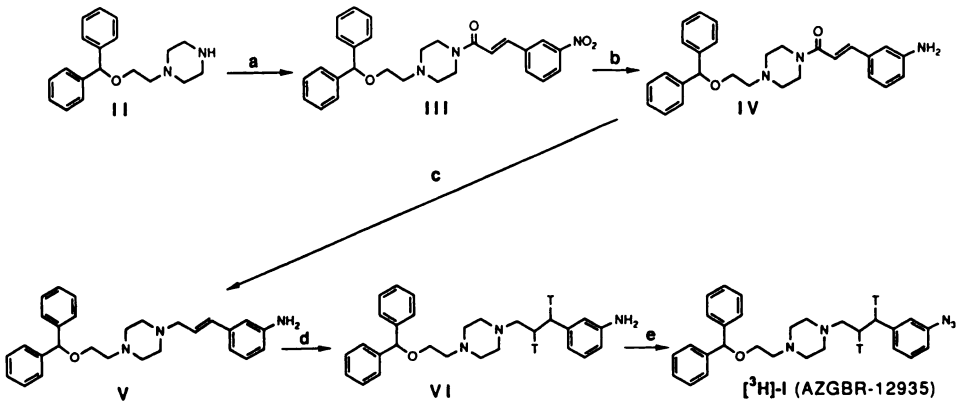


**Fig. 1.** Synthesis of 3-azido[$^3$H]GBR-12935. *a*, 3-Nitrocinnamoyl chloride; *b*, $Ru_3(CO)_{10}$/benzyltriethylammonium chloride/benzene/aqueous NaOH/CO; *c*, $LiAlH_4$/tetrahydrofuran; *d*, $^3H_2$/10% Pd-C; *e*, HONO/$NaN_3$.

the unadsorbed fraction. Desorption of bound glycoproteins was carried out by the addition to the column of 2 ml of the aforementioned solution containing *N*-acetyl-D-glucosamine (100 mg/ml). After 30 min, the proteins were eluted from the column with 6 ml of this solution, yielding the adsorbed fraction. Both fractions were concentrated in Centricon 10 ultrafilters (Amicon, Danvers, MA) and stored frozen before electrophoresis.

**Electrophoresis.** SDS-PAGE was carried out essentially as described by Laemmli (22). Samples of photolyzed membranes were diluted 10-fold with standard 1× sample buffer containing 2-mercaptoethanol, whereas concentrated samples of solubilized proteins were diluted with an equal volume of the 2× samples buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.0025% Bromophenol Blue). Samples were not heated before electrophoresis, in order to minimize the possibility of cleavage of labile ligand-protein bonds. They were electrophoresed in 80- × 100- × 1-mm slab gels having an 8–16% acrylamide gradient (Novex, Encinitas, CA), at 20 mA/gel (without cooling), until the tracking dye reached the bottom of the gel (approximately 2.5 hr). Alternatively, electrophoresis was carried out in 180- × 140- × 1.5-mm slab gels having an acrylamide gradient of 3–27% (Integrated Separation Systems, Hyde Park, MA), at 30 mA/gel (0°), until the tracking dye reached the bottom of the gel (5–6 hr). Apparent molecular masses were determined with 14.3–200-kDa standards (nonradioactive Rainbow markers; Amersham, Arlington Heights, IL). After electrophoresis, the gel was soaked in methanol/acetic acid/water (50:10:40), impregnated with a fluor (Entensify universal autoradiography enhancer; NEN/Dupont, Boston, MA), and dried onto Whatman 3MM chromatography paper under high vacuum. The gel was fluorographed at −70° for several days with Kodak X-OMAT AR5 film.

**Protein determination.** Protein concentrations were determined by the method of Bradford (23), with the Coomassie reagent from Pierce (Rockford, IL). Bovine serum albumin was used as the standard.

## Results

**Reversible binding of 3-azido[$^3$H]GBR-12935 to striatal membranes.** Our initial experiments were designed to characterize the reversible binding of 3-azido[$^3$H]GBR-12935 to crude synaptosomal (P2) membranes from the rat striatum. Specific binding, defined as the difference between total and nonspecific binding, was approximately 65% when a ligand concentration of 5 nM was used. Nonspecific binding was defined as the binding observed in the presence of mazindol (10 μM) or in the absence of $Na^+$ (see below), with both conditions yielding identical results. The binding to glass fiber filters was substantial. In fact, 65% of the nonspecific binding observed with tissue was also present without tissue. Nonspecific binding to crude synaptosomal membranes was, therefore, minimal (≤22%). The inhibition of specific 3-azido[$^3$H]GBR-12935 binding to striatal membranes by the potent dopamine reuptake inhibitor GBR-12909 was steep and of high affinity (Fig. 2).

To determine whether the binding site(s) for 3-azido[$^3$H]GBR-12935, like that of [$^3$H]GBR-12935, is associated with the dopamine transporter protein, we studied a series of drugs for their potencies in inhibiting specific binding of 3-azido[$^3$H]GBR-12935. Very good correlations between the logarithms of the relative and absolute potencies of these drugs in inhibiting specific 3-azido[$^3$H]GBR-12935 binding and dopamine uptake ($r = 0.96$, $p < 0.01$) were observed. Moreover, specific 3-azido[$^3$H]GBR-12935 binding was reversed stereospecifically by cocaine [(−)-cocaine > (+)-cocaine)] (Table 1) and was not inhibited by *cis*-flupenthixol (at concentrations of ≥10 μM). The latter is a purported inhibitor of GBR-12935 binding to the so called piperazine binding site, which is unrelated to the dopamine transporter protein (24). Furthermore, no specific binding was observed in membranes prepared from the cerebral cortex or cerebellum (data not shown), brain regions with low to undetectable levels of dopamine uptake sites (20). The reversible binding of 3-azido[$^3$H]GBR-12935 to striatal membranes was also sodium dependent, inasmuch as no specific binding was observed in incubations where choline chloride was substituted for NaCl (data not shown).

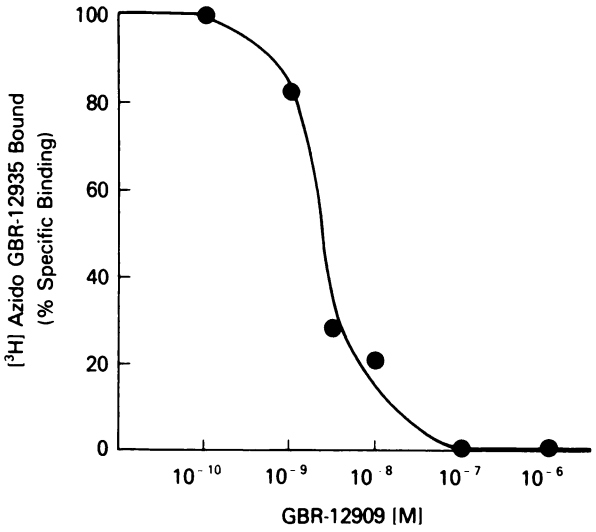


**Fig. 2.** Competition between GBR-12909 and 3-azido[$^3$H]GBR-12935 (5 nM) for reversible binding to crude synaptosomal (P2) membranes in rat striatum. Each *point* represents the average of triplicate determinations. A second independent experiment yielded similar results.

TABLE 1

**Inhibition of specific 3-azido[$^3$H]GBR-12935 binding to striatal membranes**

The displacement of 3-azido[$^3$H]GBR-12935 by various agents was carried out as described in the text. $IC_{50}$ values are the concentrations of unlabeled drug that inhibited the specific binding of 5 nM 3-azido[$^3$H]GBR-12935 by 50%. Each value represents the mean of values obtained in two or more experiments conducted in triplicate. $IC_{50}$ values for dopamine uptake are taken from the literature (20, 33–35). A correlation coefficient of $r = 0.96$, $p < 0.01$, was observed between the potency of these drugs in inhibiting specific 3-azido[$^3$H]GBR-12935 binding and dopamine uptake.

| Compound | 3-Azido[$^3$H]GBR-12935 displacement | [$^3$H]Dopamine uptake |
|---|---|---|
| | $IC_{50}$ (nM) | |
| Nomifensine | 1.8 | 49 |
| Mazindol | 2.3 | 37 |
| GBR-12909 | 2.6 | 3.5 |
| (−)-Cocaine | 14,750 | 2,500 |
| Clomipramine | 28,100 | 4,600 |
| Amitriptyline | 35,000 | 2,700 |
| Desipramine | 48,300 | 7,200 |
| *cis*-Flupenthixol | 51,200 | 8,000 |
| (+)-Cocaine | >100,000 | 160,000 |

**Photoaffinity labeling of the dopamine transporter protein with 3-azido[$^3$H]GBR-12935.** Following UV irradiation of rat striatal (P2) membranes that had been preincubated with 3-azido[$^3$H]GBR-12935, membranes were solubilized and the proteins were separated by SDS-PAGE. Fluorograms of these gels revealed a single radioactive polypeptide of mass ~80 kDa (Fig. 3). Typically, counting of the portion of the autoradiographic gel corresponding to the 80-kDa protein yielded approximately 200 dpm. This suggests that approximately 0.1% of the reversible 3-azido GBR-12935 binding de-

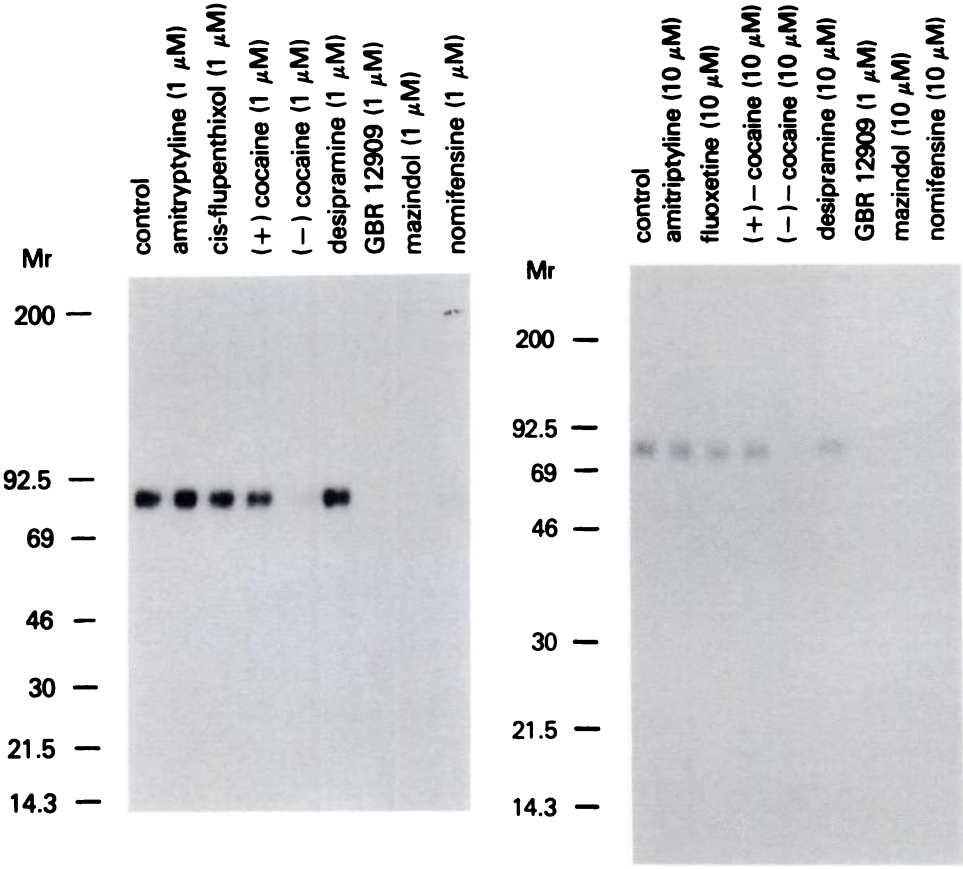


**Fig. 3.** Pharmacological specificity of 3-azido[$^3$H]GBR-12935 photoincorporation into the 80-kDa protein in crude synaptosomal (P2) membranes of rat striatum. Samples were subjected to UV irradiation in the absence (control) or presence of potential inhibitors of binding (*cis*-flupenthixol and various inhibitors of catecholamine and serotonin reuptake) at the concentrations indicated. The apparent molecular weights of standard proteins are shown × $10^{-3}$.

scribed above was retained covalently following separation of proteins by SDS-PAGE. The photoincorporation of 3-azido[$^3$H]GBR-12935 into this polypeptide was sodium dependent (Fig. 4) and enriched in crude synaptosomal (P2) membranes, compared with a "total" membrane preparation (where a band was faintly visible on the fluorograph). As shown in Fig. 3, photoincorporation was also inhibited by various dopamine reuptake inhibitors. Relatively low (1 μM) concentrations of [$^3$H]GBR-12909, (−)-cocaine, and nomifensine completely prevented the photoincorporation of 3-azido[$^3$H]GBR-12935 into this striatal membrane polypeptide. In contrast, (+)-cocaine, amitriptyline, desipramine, fluoxetine, and *cis*-flupenthixol (at concentrations of ≥10 μM) failed to block the specific photolabeling of the 80-kDa polypeptide by 3-azido[$^3$H]GBR-12935. No photoincorporation of 3-azido[$^3$H]GBR-12935 into any protein was observed when cerebellar membranes incubated under identical assay conditions were used (data not shown).

Previous reports have shown that the dopamine transporter labeled by various radiolabeled diphenylpiperazines is a glycoprotein, because it binds to immobilized lectin and is substantially reduced in size following incubation with glycosidases (16, 17). The essentially complete adsorption of the radiolabeled polypeptide onto wheat germ lectin-Sepharose and its subsequent elution with *N*-acetyl-D-glucosamine is shown in Fig. 5.

## Discussion

Diphenyl-substituted piperazine derivatives, such as GBR-12935 and GBR-12909, are among the most potent and selective dopamine reuptake inhibitors that have been described (19). We (4, 20) and others (9, 24–26) have previously shown that [$^3$H]GBR-12935 and [$^3$H]GBR-12909 can be used to selectively label the dopamine transporter of mammalian, including human (27–29), brain. The specific binding of [$^3$H]GBR-12935 to crude synaptosomal (P2) membranes is of high affinity, saturable, sodium dependent, and only observed in brain regions with relatively high densities of dopamine nerve terminals (e.g., the striatum and nucleus accumbens) (30). These findings, coupled with the structure-activity relationships reported by a number of laboratories (20, 24, 26), as well as the effects of specific neurotoxins on specific binding (20, 24), strongly suggest that [$^3$H]GBR-12935 selectively labels the dopamine transporter under defined assay conditions (see below).

The findings described above prompted the development of a suitable photoaffinity ligand (e.g., an azido derivative of GBR-12935) for covalent labeling of the dopamine transporter protein. In a previous report we described the synthesis of 3-azido[$^3$H]GBR-12935 (18). In the present study we have characterized both the reversible and the irreversible binding of 3-azido[$^3$H]GBR-12935 to rat striatal membranes. The data (Table 1, Fig. 2) strongly suggest that this azido derivative of GBR-12935 labels the same population of binding sites as GBR-12935 itself and that these sites are structurally associated with the dopamine transporter. It should be pointed out that, by using crude striatal membrane preparations, others have also reported the "specific" binding of azido-labeled GBR derivatives to a "piperazine acceptor" site that is unrelated to the dopamine transporter (24). Recently, Niznik *et al.* (31) provided evidence that the piperazine binding site for [$^3$H]GBR-12935 is an isozyme of a cytochrome P450-dependent monooxygenase (P450IID1). In the present studies, we have found that the

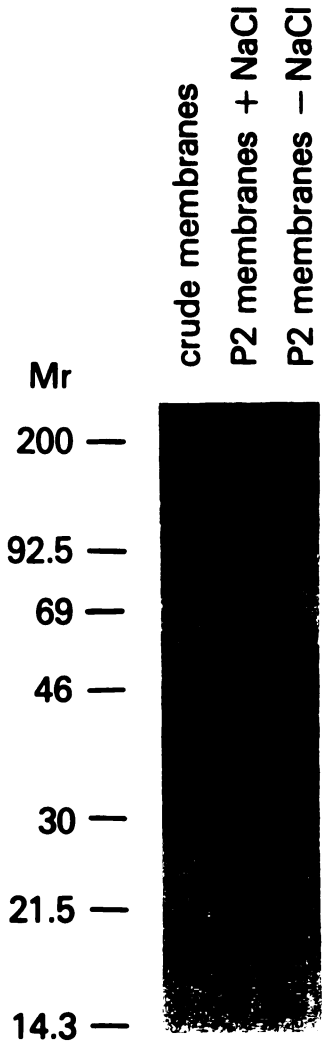


**Fig. 4.** Photoincorporation of 3-azido[$^3$H]GBR-12935 into the 80-kDa protein in the pellet (crude membranes) prepared by a single 20-min, 23,000 × *g* centrifugation of rat striatal homogenate, and the sodium dependence of photoincorporation into this protein in the crude synaptosomal (P2) membranes. The apparent molecular weights of standard proteins are shown × $10^{-3}$.

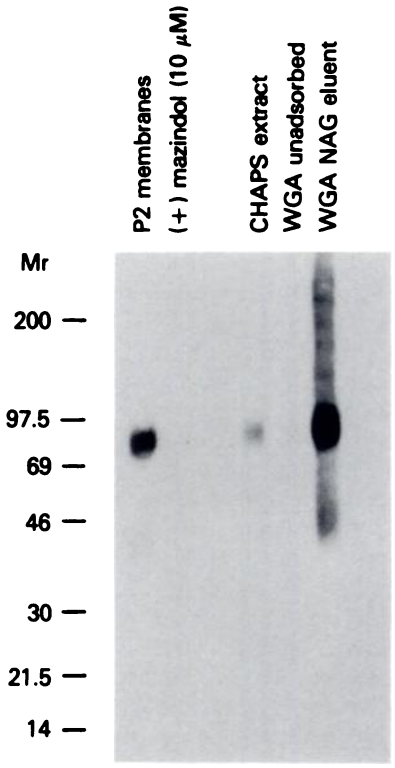


**Fig. 5.** Complete inhibition by mazindol (10 μM) of photoincorporation of 3-azido[$^3$H]GBR-12935 into the 80-kDa protein in the crude synaptosomal (P2) membranes of rat striatum, and the partial purification of this protein in the CHAPS extract by its adsorption to immobilized wheat germ agglutinin (*WGA*) and elution with *N*-acetyl-D-glucosamine (*NAG*). The apparent molecular weights of standard proteins are shown × $10^{-3}$.

specific reversible binding of 3-azido[$^3$H]GBR-12935 to a freshly prepared (nonfrozen) crude synaptosomal (P2) membrane preparation from rat striatum has an absolute dependence on $Na^+$ (data not shown) and is unaffected by low micromolar concentrations of *cis*-flupenthixol; this indicates that under our assay conditions there is little, if any, binding of 3-azido[$^3$H]GBR-12935 to the piperazine binding site. The good correlations between the potencies of various drugs in inhibiting the binding of 3-azido[$^3$H]GBR-12935 to striatal membranes and in blocking dopamine uptake into synaptosomes further suggest that 3-azido[$^3$H]GBR-12935 binds preferentially to the dopamine transporter. Some discrepancies were apparent, however, in that cocaine was a substantially weaker inhibitor of 3-azido[$^3$H]GBR-12935 binding and both mazindol and nomifensine were more potent than as inhibitors of dopamine reuptake. We reported a similar discrepancy in our earlier characterization of [$^3$H]GBR-12935 binding (cocaine was a substantially weaker inhibitor of binding than dopamine reuptake) (20). Likewise, in the pharmacological characterization of [$^{125}$I]DEEP (16), a structurally related analog of GBR-12909, some drugs are more or less potent as inhibitors of binding than of dopamine reuptake. Nonetheless, given that these two phenomena are measured under different experimental conditions (and that uptake studies are often confounded by a mixture of reuptake and release), we feel that these discrepancies do not negate the very good overall correlation between inhibition of 3-azido[$^3$H]GBR-12935 binding and of dopamine reuptake.

After UV photolysis of membranes preincubated with 3-azido[$^3$H]GBR-12935, and the subsequent solubilization, separation, and visualization of the photolabeled proteins by SDS-PAGE and fluorography, we observed a single radiolabeled polypeptide of mass 80 kDa (Figs. 3 and 4). Photoincorporation of 3-azido[$^3$H]GBR-12935 into this protein was sodium dependent (Fig. 4) and inhibited by low concentrations of drugs that potently block dopamine uptake (Fig. 3). Like dopamine uptake, the inhibition of photoincorporation was also stereospecific, inasmuch as it was inhibited by the (−)- but not by the (+)-, optically active enantiomer of cocaine. Photoincorporation was also unaffected by a variety of serotonin and norepinephrine uptake inhibitors (Fig. 3). As is the case with the reversible binding of 3-azido[$^3$H]GBR-12935, *cis*-flupenthixol failed to inhibit photoincorporation (Fig. 3). Moreover, no radiolabeled proteins were detected in crude synaptosomal (P2) membranes prepared from the cerebellum (data not shown), a brain region devoid of dopamine nerve terminals but containing the piperazine binding sites for [$^3$H]GBR-12935 (24). Taken together, these data strongly suggest that the 80-kDa striatal protein photolabeled by 3-azido[$^3$H]GBR-12935 is structurally associated with the dopamine transporter.

Our data obtained with 3-azido[$^3$H]GBR-12935 contrast with those of Grigoriadis *et al.* (16) and Sallee *et al.* (17). These investigators, using two structurally related radiodinated azido derivatives of GBR-12935 and GBR-12909, respectively, reported photoincorporation into multiple proteins in total membranes prepared from frozen and thawed rat and canine striata. In both studies, photolabeling of one of these striatal proteins was inhibited by dopamine uptake inhibitors and did not occur in membranes prepared from the cerebellum. On the basis of its pharmacological and biochemical characteristics, these investigators proposed that this polypeptide is the dopamine transporter. In both studies, however, the apparent molecular mass of this striatal photolabeled polypeptide was 58–62 kDa, a value substantially lower than the 80 kDa we obtained for the protein labeled with 3-azido[$^3$H]GBR-12935. The reasons for these discrepancies are unclear but could involve differences resulting from halogenation of the radioligand, differences in the membrane preparation used (fresh versus frozen, P2 versus whole membranes), or differences in the amount of proteolysis occurring before SDS-PAGE. Conceivably, introduction of the bulky iodine atom could have caused the GBR compounds to be incorporated into a different subunit of the transporter, if, indeed, the transporter consists of more than a single polypeptide chain. Alternatively, differences in the type of gels or molecular weight markers used might contribute to the difference in reported molecular weights. Work is currently in progress in collaboration with other investigators to resolve these discrepancies. In agreement with Grigoriadis *et al.* (16) and Sallee *et al.* (17), we have observed adsorption of the 3-azido[$^3$H]GBR-12935-labeled polypeptide onto a wheat germ agglutinin-immobilized matrix and elution with *N*-acetyl-D-glucosamine. These data, therefore, also suggest that the dopamine transporter polypeptide observed in our experiments is glycosylated. Recently, Radian *et al.* (12), using a variety of chromatographic methods, have purified the GABA transporter from rat brain; these workers reported that the purified polypeptide is a glycoprotein with a mass of 80 kDa. Subsequent work by Guastella *et al.* (32), utilizing protein sequence data derived from this purified GABA transporter polypeptide, has resulted in the isolation of full-length cDNA clones encoding the GABA transporter. The nucleotide sequence of this cDNA predicts a 67-kDa protein, compatible with a larger glycosylated native protein of approximately 80 kDa. Given the similarities in size and biochemical properties (e.g., sodium dependence) between the purified and recombinantly expressed GABA transporter and the dopamine transporter labeled with 3-azido[$^3$H]GBR-12935, it is conceivable that both are members of a neurotransmitter transporter superfamily. Our findings that 3-azido[$^3$H]GBR-12935 can be used to covalently label a single polypeptide, from rat striatal membranes, that has the pharmacological properties of the dopamine transporter should facilitate the purification of the transporter. In fact, we are currently in the process of purifying a radiolabeled proteolytic fragment of the 80-kDa dopamine transporter protein from rat striatum using high performance liquid chromatography and SDS-PAGE. The latter may prove useful for obtaining a partial amino acid sequence and for eventual cloning of the dopamine transporter cDNA.

**Acknowledgments**

The authors thank the National Institute on Drug Abuse for generous support of this project. The authors are grateful to the National Institute of General Medical Sciences, Pharmacology Research in Training Program, for support of S.P.B. Ms. Kay Kuhns provided excellent secretarial and editorial assistance.

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# Erratum

Volume 39, No. 1 (1991), in the article, "Characterization of Polyclonal Antibodies to the *Ah* Receptor Prepared by Immunization with a Synthetic Peptide Hapten," by Alan Poland, Edward Glover, and Christopher A. Bradfield, p. 21: in the fourth paragraph of the first column, the amino acids starting in the sixth line down read

Val-Gly-Lys-Thr . . .

they should read

Val-Gln-Lys-Thr . . .