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Fluorescent and Biotin Probes for Dopamine Receptors: D_1 and D_2 Receptor Affinity and Selectivity

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SUMMARY

Fluorophor and biotin derivatives of dopamine agonist and antagonist drugs were synthesized and evaluated for binding affinity and selectivity at D_1 and D_2 dopamine receptors in membranes prepared from monkey (*Macaca fascicularis*) caudate putamen. Binding was measured using [^{3}H]SCH 23390 to label D_1 receptors and [^{3}H]spiperone to label D_2 receptors. The selective D_1 antagonist SKF 83566, whether coupled to 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD), to fluorescein, or to biotin retained high affinity for D_1 dopamine receptors (K_i, 5.3 16 and 3.5 nM, respectively) and high D_1/D_2 receptor selectivity (130-, 300, and 600-fold, respectively). The selective D_2 antagonist derivative *N*-(*p*-aminophenethyl)spiperone, (NAPS) coupled either to biotin or to NBD via the *N*-aminoethylphenyl group, likewise retained high D_2 receptor affinity (K_i, 0.58 and 0.66 nM, respectively) and high D_2/D_1 selectivity (190- and 150-fold, respectively). The affinity of the NBD-coupled derivative of (*S*)-2-(*N*-phenylethyl-*N*-propyl)-amino-5-hydroxytetralin hydrochloride [(*S*)-PPHT], a selective D_2 agonist, was actually higher than that of the parent compound (K_i, 0.30 versus 2.1 nM), whereas the affinity of fluorescein-coupled (*S*)-PPHT was lower (K_i, 4.8 nM). Sensitivity to GTP, a characteristic of agonist binding at dopamine receptors, was demonstrated for NBD-coupled (*S*)-PPHT, because D_2 receptor affinity was somewhat reduced in the presence of GTP. PPHT-fluorescein fluorescence labeling rimmed cells in mo' 'ey and rat anterior pituitary and outlined cells in the striatum. Fluorescent and biotin probes based on selective high affinity ligands for dopamine receptors may expedite studies of receptor localization and mobility at the cellular level.

Brain dopamine systems have been implicated in psychiatric, neurological, and neuroendocrine disorders, and drugs that specifically target dopamine receptors are widely prescribed as therapeutic agents. At least two subtypes of dopamine receptors, D_1 and D_2, can be distinguished in brain on the basis of pharmacological specificity, anatomical distribution, and biochemical sequelae (1–6). The significance of each receptor subtype is not fully understood, because D_1 and D_2 receptors appear to mediate either synergistic or opposing actions depending on the behavioral or biochemical response measured (7–9). Receptor subtypes may be associated by proximity or by function in certain brain regions, but information on cellular and subcellular distribution of the receptors is limited. Mechanisms involved in dopamine receptor up- and down-regulation in response to drug treatment or brain lesions also remain obscure but might be clarified by improved detection of receptors *in situ*. Conventional autoradiographic techniques, although capable of providing important information on the regional distribution of brain D_1 and D_2 dopamine receptors (5, 10, 11), currently do not permit sufficient resolution for detection of receptor subtypes on single cells and on subcellular structures.

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ABBREVIATIONS: 5-HT_2, 5-hydroxytryptamine$_2$; PPHT, 2-(*N*-phenylethyl-*N*-propyl)amino-5-hydroxytetralin hydrochloride; PPHT-NH_2 dihydrochloride, 2-(*N*-*p*-aminophenethyl-*N*-propyl)amino-5-hydroxytetralin; PPHT-FLU, 2-(*N*-propyl-*N*-fluoresceinylthioureidophenethyl)amino-5-hydroxytetralin; PPHT-NBD hydrochloride or dihydrochloride, 2-(*N*-*p*-(*N*-7-nitrobenzo-2-oxa-1,3-diazol-4-yl)aminophenethyl-*N*-propyl)amino-5-hydroxytetralin; SKF 38393, 7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF 38393-FLU hydrochloride, 7,8-dihydroxy-1-4′-fluoresceinylthioureidophenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF 83566, 7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF 83566-NH_2 dihydrobromide, 7-bromo-8-hydroxy-3-methyl-(4′-aminophenyl)-3-methyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF 83566-NBD dihydrochloride, 7-bromo-8-hydroxy-3-methyl-1-(4′-7-nitrobenzo-2-oxa-1,3-diazol-4-yl-aminophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; SKF 83566-FLU, 7-bromo-8-hydroxy-3-methyl-(4′fluoresceinylthioureidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; spiperone(NAPS)-biotin dihydrochloride, D-biotinylaminophenethylspiperone; SKF 83566; Spiperone (NAPS)-NBD dihydrochloride, 8-(4′-p-fluorophenyl-4′-oxo)n-butyl-3-(N-7-nitrobenzo-oxo-diazol-4-yl)aminophenethyl-1-phenyl-1,3,8-triazaspiro-[4,5] decan-4-one; SCH 23390, 7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine.

In recent years, fluorophor- and biotin-labeled probes have been successfully developed for localizing peptide and other receptors at the cellular and subcellular levels in peripheral tissues (12–15). Fluorophor-coupled compounds also have proven valuable for monitoring the mobility of hormone receptors within cell membranes following exposure to agonists (16). In pursuing a similar strategy for detecting brain dopamine receptors, we synthesized a series of fluorophor and biotin derivatives of dopamine agonist and antagonist drugs (17). The present study characterizes the binding of these novel compounds at D_1 and D_2 dopamine receptors in nonhuman primate brain and describes preliminary data on their binding to tissue sections. A conceptually similar study involving fluorophor-coupled dopamine antagonists has recently been reported (18, 19).

Materials and Methods

Source and preparation of tissue. Binding studies were performed on brain tissue of adult male and female cynomolgus monkeys (*Macaca fascicularis*). All tissues were stored at −85° in the primate brain bank of the New England Regional Primate Research Center, as described previously (4). No animals were sacrificed for these studies. The caudate putamen or frontal cortex regions were dissected from partially thawed coronal sections and homogenized in 10 volumes of Tris·HCl buffer (50 mM, using a Potter-Elvehjem homogenizer (glass-Teflon) at 2000 rpm. The suspension was centrifuged twice at 38,000 × *g* for 30 min at 4°, resuspended in 40 volumes of buffer, divided into aliquots, and stored frozen at −85° until use. Before assay, the suspension was diluted to a concentration corresponding to twice its concentration in the assay tubes and was dispersed with a Brinkmann Polytron, at setting 5, for 5 sec for D_2 receptor assays or 2 sec for D_1 receptor assays.

D_1 and D_2 dopamine receptor assays. D_1 and D_2 dopamine receptors were assayed by methods described previously (4), using [*N-methyl*-^{3}H]SCH 23390 (60.4–80.4 Ci/mmol; Dupont-New England Nuclear, Boston, MA) to label D_1 receptors and [*benzene ring*-^{3}H]spiperone (21.4–22.8 Ci/mmol; New England Nuclear) to label D_2 dopamine receptors. Stock solutions of the drugs were made in water, in ethanol (10–96 ml/100 ml) containing 2 M HCl (10–40 μl), or in ethanol containing dimethyl sulfoxide (5–50 ml/100 ml), depending on solubility. Each stock solution was diluted serially in the assay buffer to yield 12–20 concentrations. In control experiments, neither ethanol-HCl nor ethanol-dimethyl sulfoxide significantly affected the binding of [^{3}H]SCH 23390 or [^{3}H]spiperone at solvent concentrations up to 0.25 ml/100 ml of buffer, the range needed to dissolve conjugated compounds in concentrations up to 1 μM.

Binding of dopamine antagonists was evaluated in Tris·HCl buffer (50 mM, pH 7.4, at 23°) containing NaCl (120 mM), KCl (5 mM), $MgCl_2$ (4 mM), $CaCl_2$ (1.5 mM), and EDTA (1 mM). Binding of dopamine agonists was evaluated under identical conditions except that the antioxidant ascorbic acid (0.56 or 1 mM) was added and NaCl was omitted (20). Additional studies were conducted with D_2 agonists to determine the effects of the guanine nucleotide GTP. In these experiments, binding was measured in the presence of GTP (100 μM; Sigma Chemical Co.) and NaCl (120 mM) and was compared with binding in their absence.

To determine the affinity of drugs at D_1 or D_2 dopamine receptors, glass tubes (12 × 75 mm) received the following, in order: the test compound (0.25 ml), either [^{3}H]SCH 23390 (0.25 ml, 0.2–0.3 nM final concentration) or [^{3}H]spiperone (0.25 ml, 0.1 nM final concentration), and tissue (0.5 ml, 1 mg of original wet tissue weight/ml final concentration), to a final volume of 1 ml. At least 12 concentrations of drug were used in each assay and each concentration was studied in triplicate in two to six different brains.

Incubation was initiated by addition of membrane and was terminated after 2 hr at 22° by rapid filtration over Whatman GF/B glass fiber filters (M-24R cell harvester; Brandel, Gaithersburg, MD). The filters were washed three times (5 ml each) with Tris·HCl buffer (4°) and incubated overnight in polyethylene vials (7 ml) containing 4 ml of scintillation fluor (Beckman Ready-Solve, EP grade, or Ready-Value). Radioactivity was monitored for 2–5 min in a liquid scintillation counter (Beckman 1801) and cpm were converted to dpm by determination of the counting efficiency (45–53%) of each vial by external standardization. Variability between triplicate samples generally ranged from 0.5 to 4% but increased as radioactivity in the sample fell below 400 dpm. Total binding was measured in the presence of ineffective concentrations of the test compound (1 pM to 10 nM, depending on affinity), nonspecific binding was determined with (*S*)-butaclamol (1 μM), and specific binding was defined as the difference between the two values. For D_1 receptors, total binding was approximately 1800 dpm and nonspecific binding was 180 dpm; corresponding values for D_2 receptors were 1000 dpm and 200 dpm.

Data were analyzed by the EBDA and LIGAND programs (Elsevier-Biosoft, England). The EBDA program provided final parameter estimates for IC_{50} and n_H values by linear curve fitting. K_i values were computed by the LIGAND program by nonlinear curve fitting.

5-HT_2 serotonin receptor assays. 5-HT_2 serotonin receptors were assayed in frontal cortex using [*ethylene*-^{3}H]ketanserin (61.5 Ci/mmol; Dupont-New England Nuclear, Boston, MA) and procedures similar to the ones outlined above. All assays were performed in Tris·HCl buffer (50 mM, pH 7.4 at 23°) containing NaCl (120 mM), KCl (5 mM), $MgCl_2$ (4 mM), $CaCl_2$ (1.5 mM), and EDTA (1 mM), to obtain potency values using the same conditions as described for dopamine receptor binding. (In the absence of buffer salts, [^{3}H]ketanserin binding increased significantly and IC_{50} values were different then those reported herein.) To determine the affinity of the novel probes, glass tubes (12 × 75 mm) received the following in order: test compound (0.25 ml, 13–21 concentrations), [^{3}H]ketanserin (0.25 ml, 0.5 nM final concentration), and

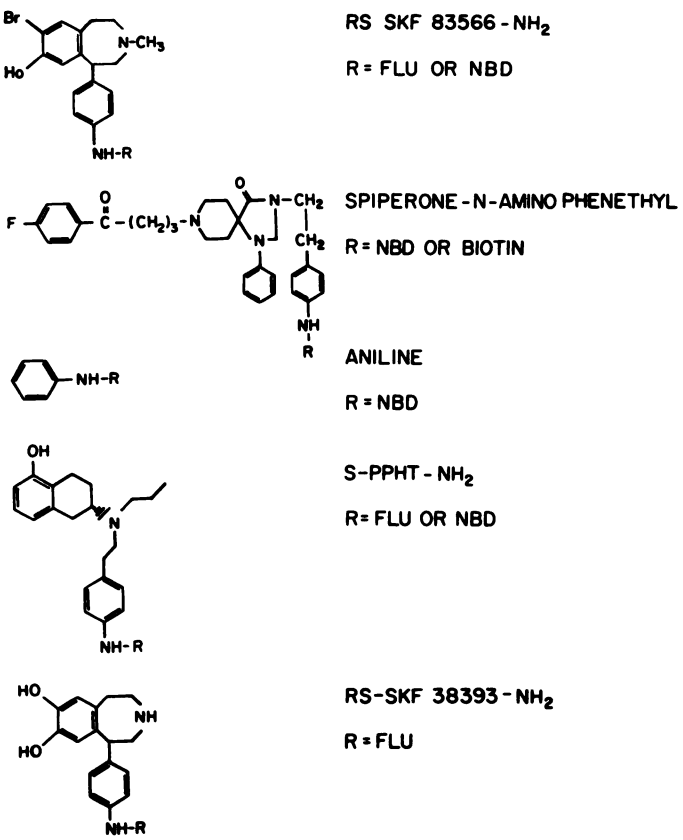

Fig. 1. Structures of novel probes.

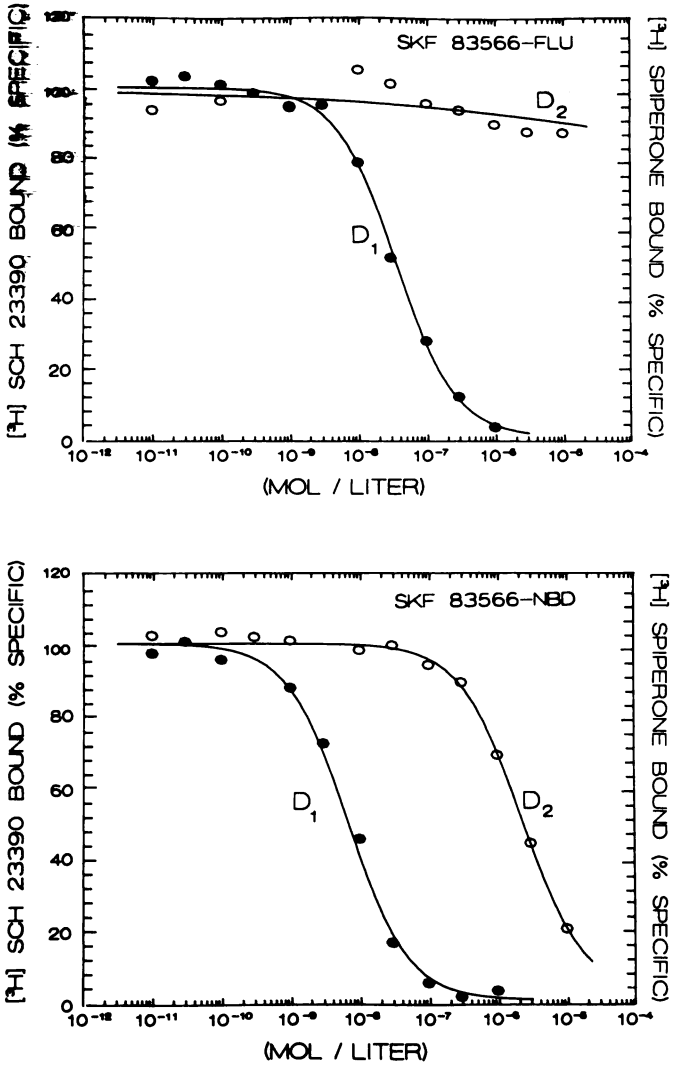

Fig. 2. Inhibition of specific binding of [^{3}H]SCH 23390 to D_1 receptors or of [^{3}H]spiperone to D_2 receptors by fluorescein and NBD conjugates of SKF 83566. Monkey caudate putamen membranes were incubated with [^{3}H]SCH 23390 (0.2 nM) or [^{3}H]spiperone (0.1 nM) and increasing concentrations of SKF 83566-FLU (*top*) or SKF 83566-NBD (*bottom*), as described in Materials and Methods. Each point is the mean of triplicate determinations in a representative experiment.

tissue 0.5 ml, final concentration 1 mg of original wet tissue weight/ml). At this concentration, approximately 60% of the receptors were saturated. Each concentration of the test compound was studied in triplicate, and the experiment was repeated at least twice. Total binding was measured in the presence of ineffective concentrations of the test compound (1 pM to 10 nM, depending on affinity), nonspecific binding was determined with spiperone (1 μM) or cinanserin (1 to 10 μM), and specific binding was defined as the difference between the two values. Data were analyzed by the EBDA program to obtain IC_{50} and n_H values, and K_i values were computed from the dissociation constant (K_d) for [^{3}H]ketanserin (1.5 nM ± 0.21, mean ± SD).

Tissue sections. Frozen tissue sections (15–20 μm) from *M. fascicularis* pituitary (5) or from Sprague-Dawley rats (300 g, $n = 4$) were mounted on Chromalum-coated slides and stored overnight at −20° (rodent) or −85° (monkey). Sections could be stored for at least 2 weeks without apparent loss of binding activity of the fluorescent probe. For monkey tissue, each slide was mounted with two to three sections and four or more slides were used for each determination. The sections were brought to room temperature, preincubated for 30 min with Tris·HCl, pH 7.4 (50 mM), KCl (5 mM), $MgCl_2$ (4 mM), $CaCl_2$ (1.5 mM), EDTA (1 mM), ascorbic acid (1 mM), and then incubated with the probe in buffer for 2 hr at 0° (monkey) or at room temperature (rodent) in the dark. The slides were rinsed two times (monkey) or four times (rodent) for 30 sec and dipped in double-distilled H_2O before being dried under a cool airstream.

Primate sections were incubated with (*S*)-PPHT-FLU (20–500 nM) in Tris-salt buffer for 2 hr in the dark. Four controls were used to evaluate specificity of the fluorescent signal, by incubating slide-mounted tissue with 1) (*S*)-PPHT-FLU simultaneously with or before preincubation with an excess of (*RS*)-PPHT (50–200 μM) to mask the binding of the fluorophor and, thereby, ascertain the extent of nonspecific binding; 2) buffer alone to measure the extent of tissue autofluorescence; 3) (*RS*)-PPHT alone to measure the contribution, if any, of the masking agent (*RS*)-PPHT to the fluorescent signal; or 4) (*S*)-PPHT-FLU in combination with ethanol at ethanol concentrations required to solubilize (*RS*)-PPHT, to determine the contribution of ethanol to the fluorescent signal. Several dopamine receptor antagonists were rejected for masking purposes. Sulpiride, if incubated with tissue sections alone at concentrations sufficient to block fluorophor binding, appeared to emit fluorescence on some tissue sections. In combination with PPHT-FLU, sulpiride did not reduce overall tissue fluorescence. Another benzamide, eticlopride, generated fluorescence in tissue sections at high ligand concentrations. Butaclamol, YM 09151-2, and spiperone, if used at the high concentrations calculated to block binding of the probes (≥50 μM), required solvent (ethanol/HCl) levels in the buffer that interfere with ^{3}Hneuroleptic binding to either D_1 or D_2 dopamine receptors.

Dopamine receptor binding in lightly fixed tissue was evaluated initially by radioreceptor assay in monkey brain tissue homogenates and sections with fluorescent probes on rodent tissue sections. Light fixation (0.1–0.2% formaldehyde or paraformaldehyde) of monkey striatal homogenates retained approximately 70% of specific [^{3}H]SCH 23390 or [^{3}H]spiperone binding. If rodent tissues were perfused with 4% buffered formaldehyde (and tissues were stored at 4° in 15% sucrose), the fixed sections exhibited the same pattern of labeling with PPHT-FLU as the unfixed sections treated with the probe.

Results

Binding of fluorophor- and biotin-coupled antagonists. Primary amine derivatives of SKF 83566, its desbromo analog, and spiperone were synthesized as precursors for subsequent conjugation with fluorophors or biotin (Fig. 1). As shown in Fig. 2 and Table 1, NBD, fluorescein, and biotin coupled derivatives of the D_1 antagonist SKF 83566 displaced [^{3}H]SCH 23390 from D_1 dopamine receptors with low nanomolar affinity. As is characteristic of dopamine antagonists, the pseudo-Hill coefficients approximated unity. In contrast to their high affinity at dopamine D_1 receptors, the conjugated ligands had very low affinity for dopamine D_2 receptors labeled with [^{3}H]spiperone. Based on their K_i values, the NBD, fluorescein and biotin derivatives of SKF 83566 showed at least a 130-fold selectivity for dopamine D_1 receptors. Although the fluorescein derivative of desbromo-SKF 83566 also displayed selectivity for D_1 receptors, the affinity of this compound was quite low (Table 1).

Both the NBD- and biotin-coupled derivatives of the D_2 antagonist spiperone (NAPS) displaced [^{3}H]spiperone from dopamine D_2 receptors with high affinity and had pseudo-Hill coefficients close to unity (Table 1 and Fig. 3). The two spiperone conjugates also displaced [^{3}H]SCH 23390 from dopamine D_1 receptors, but only at relatively high concentrations. Based on their K_i values, both the NBD- and the biotin-coupled derivatives of spiperone showed a greater than 100-fold selectivity for dopamine D_2 receptors. The NBD conjugate of aniline,

TABLE 1

Affinity and receptor selectivity of antagonist derivatives at D_1 and D_2 dopamine receptors

K_i and n_H values were computed by the LIGAND and EBDA programs, respectively, and (except where noted) are the means ± standard errors of two to four independent experiments, each performed in triplicate. The ratios of binding potencies (D_2/D_1, D_1/D_2) are computed from K_i values.

Compounds	D_1		D_2		Binding potency ratio[a]
	K_i	n_H	K_i	n_H	
	nM		nM		
D_1 antagonists					
SKF 83566-NH_2	2.3 ± 0.46	0.87	1,600 ± 110	0.99	690
SKF 83566-FLU	16 ± 4.0	0.94	>5,000		>300
SKF 83566-NBD	5.3 ± 2.1	1.06	710 ± 120	0.97	130
SKF 83566-biotin	3.5 ± 0.93	0.82	2,100 ± 1,500	1.1	600
Desbromo-SKF 83566-NH_2[b]	54	1.12	2,400	0.74	44
Desbromo-SKF 83566-FLU	370 ± 13	1.31	>10,000		>27
D_2 antagonists					
Spiperone (NAPS)-NBD	99 ± 19	1.30	0.66 ± 0.18	1.03	150
Spiperone (NAPS)-biotin	110 ± 26	1.09	0.58 ± 0.43	0.94	190
Control					
Aniline-NBD	>10,000		>10,000		

[a] D_1/D_2 for D_1 antagonists and D_2/D_1 for D_2 antagonists.
[b] Based on a single experiment performed in triplicate.

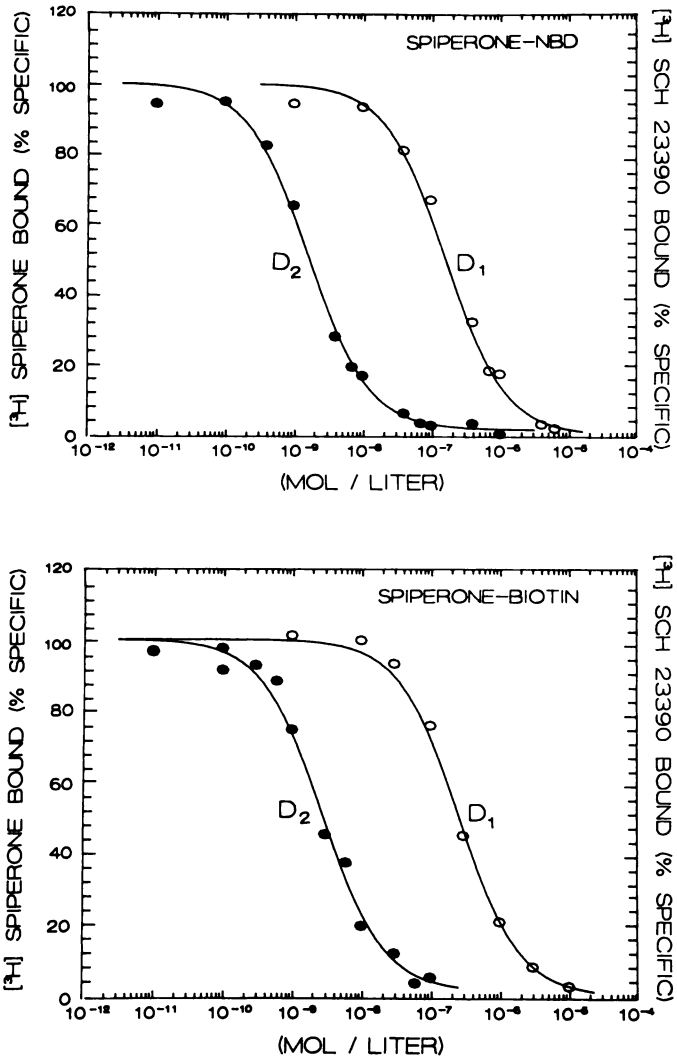

Fig. 3. Inhibition of specific binding of [^{3}H]spiperone to D_2 receptors or of [^{3}H]SCH 23390 to D_1 receptors by NBD (*top*) and biotin (*bottom*) derivatives of spiperone (NAPS). Details are given in the legend to Fig. 2.

TABLE 2

Affinity and receptor selectivity of PPHT derivatives at D_1 and D_2 dopamine receptors

Results are means ± standard errors of two to six independent experiments, each performed in triplicate. Other details are as in Table 1.

Compounds	D_1, K_i	D_2		D_2/D_1
		K_i	n_H	
	nM	nM		
(*RS*)-PPHT-NH_2	710 ± 500	6.8 ± 0.15	0.49	100
(*RS*)-PPHT-FLU	340 ± 130	7.0 ± 1.1	0.86	50
(*RS*)-PPHT-NBD	170 ± 50	0.45 ± 0.06	0.95	380
(*R*)-PPHT	6,500 ± 2,800	60 ± 2.1	0.59	110
(*R*)-PPHT-NH_2	4,900 ± 3,500	170 ± 45	0.53	30
(*R*)-PPHT-FLU	24,000 ± 14,000	110 ± 63	0.65	220
(*R*)-PPHT-NBD	3,700 ± 910	3.2 ± 0.20	0.63	1,200
(*S*)-PPHT	230 ± 33	2.1 ± 0.55	0.59	110
(*S*)-PPHT-NH_2	270 ± 21	6.7 ± 1.9	0.42	40
(*S*)-PPHT-FLU	260 ± 55	4.8 ± 0.20	0.92	54
(*S*)-PPHT-NBD	130 ± 55	0.30 ± 0.05	0.78	430
SKF 39393-FLU	>10,000	>10,000		

which was synthesized as a control compound, showed no appreciable affinity for either D_1 or D_2 dopamine receptors.

Binding of fluorophor-coupled agonists. The primary amine and fluorescein-coupled derivatives of the D_2 agonist (*RS*)-PPHT [also designated N-0434 (21)] had high D_2 receptor affinity and selectivity (Table 2). Compared with the primary amine derivative, conjugation with NBD unexpectedly resulted in a 5-fold increase in both D_2 receptor affinity and selectivity. In an attempt to further improve D_2 affinity, the stereoisomers of PPHT were resolved and the primary amines, as well as fluorescein- and NBD-coupled derivatives of the *R*- and *S*-isomers, were synthesized. In this series, NBD coupled to (*S*)-PPHT had the highest affinity (K_i, 0.3 nM) and a 400-fold selectivity for D_2 over D_1 receptors (Fig. 4). Although the NBD conjugate of (*R*)-PPHT had an even higher D_2 selectivity (about 1000-fold), its affinity for D_2 receptors was lower. A similar relationship was seen for the fluorescein-coupled derivatives of (*S*)- and (*R*)-PPHT. With the exception of fluorescein-coupled (*S*)-PPHT, all (*R*)- and (*S*)-PPHT derivatives had pseudo-Hill coefficients less than unity, as is characteristic for D_2 agonists.

The D_2 dopamine receptor has been shown to exist in high

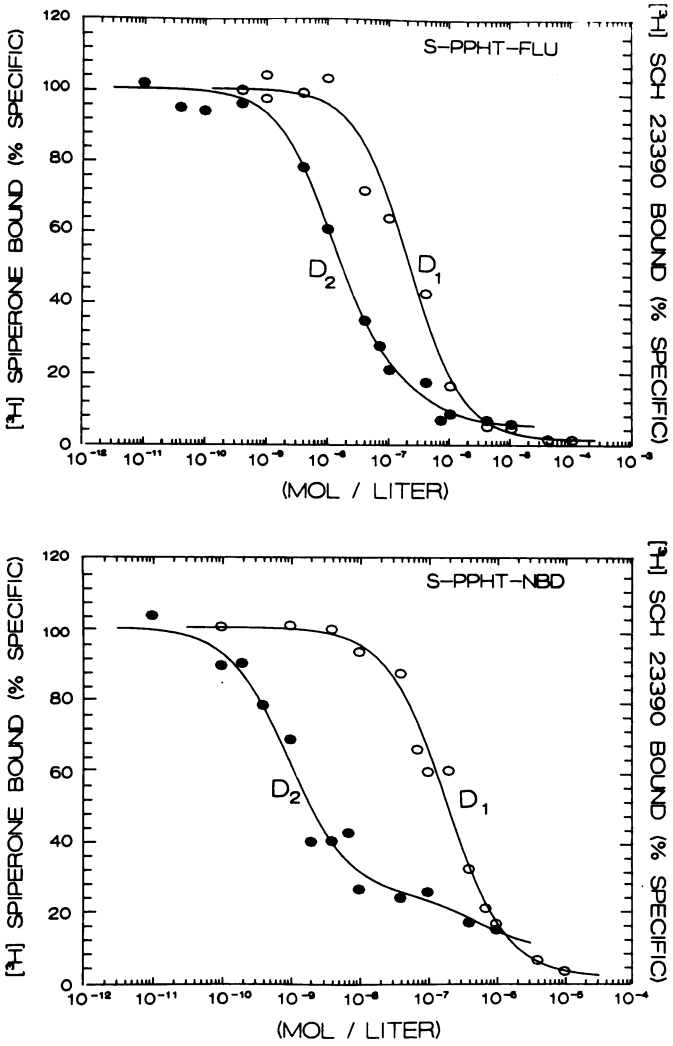

Fig. 4. Inhibition of specific binding to [^{3}H]spiperone to D_2 receptors or of [^{3}H]SCH 23390 to D_1 receptors by fluorescein (*top*) and NBD (*bottom*) derivatives of (*S*)-PPHT. Details are given in the legend to Fig. 2.

TABLE 3

Affinities of fluorescent and biotin probes at 5-HT_2 serotonin receptors labeled by [^{3}H]ketanserin (0.5 nM) in monkey frontal cortex

Data are means ± standard deviations, based on triplicate determinations from two independent experiments. Other details are described under Materials and Methods.

Compounds	IC_{50}	K_i	Binding potency ratio[a]
	nM		
D_1-selective drugs			
SKF 83566-FLU	160 ± 91	120 ± 70	7.5
SKF 83566-NBD	45 ± 1.5	35 ± 10	6.6
D_2-selective drugs			
Spiperone (NAPS)-NBD	23 ± 7.4	18 ± 5.3	27
Spiperone (NAPS)-biotin	110 ± 5.5	53 ± 35	91
(*S*)-PPHT-FLU	>4,000		≧800
(*S*)-PPHT-NBD	>4,000		≧10,000

[a] D_1/S_2 for D_1-selective drugs and D_2/S_2 for D_2-selective drugs.

and low affinity states, to which agonists (but not antagonists) bind with differing affinities. Conversion of the high to the low affinity state is thought to be mediated by guanine nucleotides, and binding of agonists to D_2 receptors often is characterized

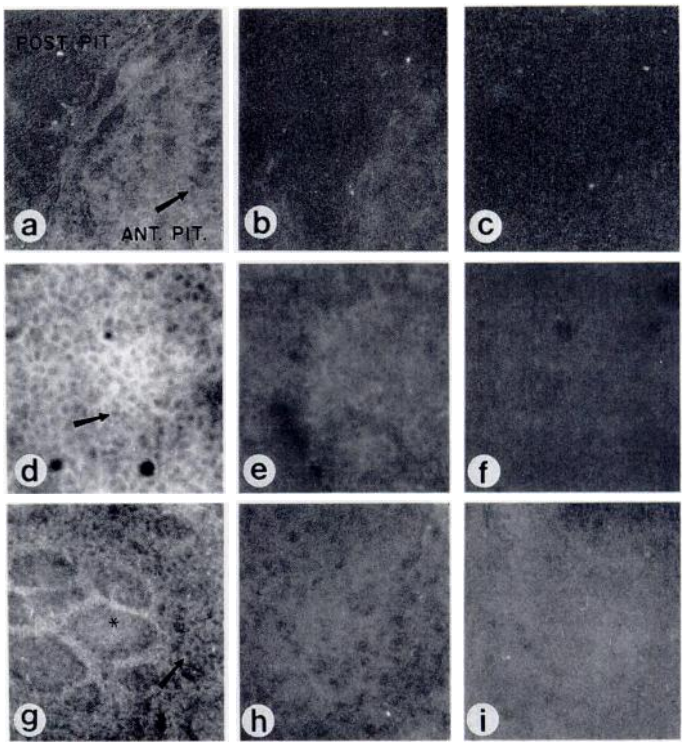

Fig. 5. Fluorescent distribution of (*S*)-PPHT-FLU in tissue sections of monkey pituitary gland (*a–c*), rodent pituitary gland (*d–f*), and rodent striatum (*g–i*). Shown are total binding of (*S*)-PPHT-FLU (200 or 500 nM) in monkey anterior and posterior pituitary (*a*), rodent pituitary (*d*), and rodent striatum (*g*), (*S*)-PPHT-FLU binding in the presence of (*RS*)-PPHT (50 μM) (*b*, *e*, and *h*), and tissue sections incubated in the absence of fluorophor (*c*, *f*, and *i*). *Arrows*, cell rimming by fluorophor in monkey pituitary (*a*), rodent pituitary (*d*) and rodent striatum (*g*). *Asterisk*, a fiber bundle in rodent striatum (*g*).

by reduced affinity in the presence of guanine nucleotides (20, 22). In a side-by-side comparison, the addition of GTP and NaCl to the assay medium reduced the affinity of the fluorescein-coupled derivative of (*S*)-PPHT, with a corresponding increase in K_i from 4.6 ± 0.3 nM to 8.4 ± 2.9 nM (mean ± SD, $n = 2$). A parallel experiment with the NBD derivative of (*S*)-PPHT also showed a reduction in affinity (K_i, 0.3 ± 0.04 nM versus 1.24 ± 0.3 nM). In contrast, an increase rather than decrease in affinity was seen for the NBD derivative of the D_2 antagonist spiperone when GTP and NaCl were added to the assay medium in a single experiment (K_i, 0.66 nM versus 0.35 nM).

Binding at 5-HT_2 serotonin receptors. Because both SKF 83566 and spiperone have nanomolar affinities for 5-HT_2 serotonin receptors (23, 24), the potencies of these compounds and fluorophor-coupled (*S*)-PPHT were determined at these receptors. The NBD and fluorescein derivatives of SKF 83566 were 7 times more potent at D_1 receptors, respectively, than at 5-HT_2 serotonin receptors, whereas spiperone-NBD was 30 times more potent at D_2 receptors than at 5-HT_2 receptors (Table 3). In contrast, spiperone-biotin was 90-fold more potent at D_2 than at 5-HT_2 receptors, suggesting that the biotin derivatives conferred a higher degree of dopamine receptor selectively on spiperone than the fluorophor derivative. The D_2 receptor agonist derivatives (*S*)-PPHT-NBD and (*S*)-PPHT-FLU were at least 800 times more potent at D_2 than at 5-HT_2 receptors.

Slide-mounted tissue sections. Incubation of monkey pi-

tuitary sections with (*S*)-PPHT-FLU revealed high fluorescent labeling in the anterior pituitary and considerably less in the posterior pituitary (Fig. 5a). If the fluorophor probe was coincubated with (*RS*)-PPHT, a significant reduction in fluorescent labeling was observed, particularly in anterior pituitary (Fig. 5b). Tissue sections incubated in buffer alone were somewhat darker (5c) than those incubated in the presence of the unlabeled probe (*RS*)-PPHT (data not shown), suggesting that high concentrations of the blocking agent (*RS*)-PPHT may generate a weak fluorescent signal. Fluorescence labeling of tissue sections was not visibly reduced if ethanol was coincubated with PPHT-FLU at the concentration (1.25%) required to maintain the blocking agent in solution (data not shown). Comparable fluorescence was observed if PPHT-FLU was coincubated with (*RS*)PPHT in rodent anterior pituitary and striatum (Fig. 5, d and g) and fluorescence was attenuated by (*RS*)-PPHT (Fig. 5, e and h). Most of the fluorescence was ligand induced, inasmuch as incubation of tissue alone showed only sparse autofluorescence (Fig. 5, c, f, and i).

Intense fluorescent labeling "rimmed" the cells in monkey and rat anterior pituitary treated with PPHT-FLU at concentrations ≧200 nM (Fig. 5, a and d). In the caudate nucleus, the neuropil was brightly fluorescent and in some cases the neurons appeared to be outlined. The fiber bundles passing through the caudate nucleus were unlabeled. Similarly, the corpus callosum showed little or no fluorescence. The cortex was less fluorescent than the caudate neuropil, except for the cingulate cortex, which was generally brightly fluorescent.

Discussion

Fluorescent and biotin probes for peripheral hormone receptors have been used successfully in a number of applications, including localizing receptors at cellular and subcellular levels (12–14), monitoring receptor mobility and internalization (16), and cell sorting (25). The absence of corresponding probes for D_1 and D_2 dopamine receptors has impeded comparable research on dopamine receptor systems. In order to establish the suitability of dopamine receptor probes for these applications, essential binding criteria must be met. First, the compounds should retain low nanomolar affinity, to ensure that the receptor-ligand complex is preserved during washing and other procedures. Second, D_1/D_2 receptor selectivity also must be sufficiently high, so that the ligand concentration needed for detection of a particular receptor subtype is below the level that would cause substantial binding to the other subtype. This criterion is particularly important for localizing receptors in brain regions containing high densities of both D_1 and D_2 receptors, such as caudate putamen or nucleus accumbens. Third, binding affinity should be invested primarily in the drug and not in the fluorescent moiety. Finally, the compounds should retain binding properties that are generally comparable to those of the parent agonist or antagonist, such as appropriate pseudo-Hill coefficients and, in the case of agonists, sensitivity to guanine nucleotides.

The present study demonstrates that selective ligands for D_1 or D_2 dopamine receptors can be coupled to fluorophors or to biotin and retain high affinity and receptor selectivity. The most promising compounds derived from antagonist drugs, SKF 83566-NBD or SKF 83566-biotin (for D_1 receptors) and spiperone-biotin or spiperone-NBD (for D_2 receptors), had low nanomolar affinity and at least 130-fold selectivity for the appropriate receptor subtype. Steep competition curves characteristic of antagonist binding to dopamine receptors also were observed for each of these conjugates. Furthermore, in the case of the NBD analogs, binding appeared to be a property of the ligand and not the coupled fluorophor, because no binding was seen with the NBD conjugate of aniline. Fluorescent probes based on dopamine antagonists such as these may be useful for cell sorting and for receptor localization at the light microscopic level (26). Additionally, biotin-coupled ligands may be suitable probes for subcellular localization of receptors, because biotin forms a tight complex with avidin (15) that can be visualized using electron microscopy (13, 14).

In contrast to antagonists, dopamine receptor agonist generally recognize two affinity states of the receptor, which are thought to be interconvertible during association and dissociation with secondary messengers (20, 22). As such, agonist binding appears to be a dynamic process, which may alter the mobility of dopamine receptors within the membrane, as has been previously demonstrated for other receptor systems (16). Fluorescent probes based on dopamine agonists might, therefore, be particularly useful for monitoring receptor translocation or other dynamic processes such as receptor internalization that may be initiated by agonist binding. In equilibrium binding assays, agonist binding to D_2 dopamine receptors often is characterized by shallow competition curves and reduced affinity in the presence of guanine nucleotides. Of the agonist probes we synthesized, (*S*)-PPHT-NBD had the highest binding affinity and a 320-fold selectivity for D_2 receptors. In the presence of GTP and NaCl, a reduction in affinity was observed. Similar results were obtained with fluorescein-coupled (*S*)-PPHT. Additional experiments are needed to determine whether these (*S*)-PPHT derivatives retain biological properties of D_2 agonists, such as capacity to inhibit adenylate cyclase activity or prolactin release.

Because both spiperone and SKF 83566 are known to bind to 5-HT_2 serotonin receptors (23, 24), it was of interest to determine the affinities of these compounds at [^{3}H]ketanserin binding sites in frontal cortex. The fluorescent derivatives of both spiperone and SKF 83566 demonstrated nanomolar affinity for these receptors, suggesting that fluorescence labeling of dopamine receptors with these compounds in tissue slices requires the presence of ketanserin to mask 5-HT_2 serotonin receptors. Interestingly, the biotin derivative of spiperone had lower affinity at these sites than did the NBD derivative. On the other hand, the NBD and fluorescein derivatives of (*S*)-PPHT bound to 5-HT_2 sites with low (micromolar) affinity.

The utility of fluorophor-coupled compounds as molecular probes in organized tissue requires fulfillment of several criteria. First, the probes must fluoresce after association with slide-mounted tissue or cultured cells, a criterion that was readily fulfilled in the present investigation. In preliminary studies with slide-mounted caudate and pituitary tissue sections from monkey and rodent brain, we were able to detect dose-dependent fluorescence of NBD derivatives of spiperone, SKF 83566, and (*S*)-PPHT and of the fluorescein derivatives of (*S*)-PPHT and SKF 83566. A second criterion is that fluorescence should be of sufficiently high energy to be detectable at appropriately low ligand concentrations. In the present study, bright fluorescence of PPHT-FLU was detectable at 200 nM and at lower concentrations with spiperone-NBD (not shown).

A third criterion requires that the fluorescent signal be

attenuated in the presence of excess concentrations of nonfluorescent dopamine receptor ligands and that the solvent used to dissolve high concentrations of these drugs not contribute to the masking effect. Fluorescence on tissue sections incubated with the fluorescein derivative of PPHT was blocked with excess concentrations of (*RS*)-PPHT. Furthermore, the ethanol concentrations required to dissolve the (*RS*)-PPHT did not contribute to the signal reduction. Dopamine receptor antagonists such as (*S*)-butaclamol, spiperone, and YM 09151-2 (≧50 μM) were not used because they required solvents at concentrations shown to decrease neuroleptic binding to D_1 or D_2 receptors. (*S*)-Sulpiride, which required only ineffectual levels of solvent, however, did not attenuate fluorescence in the present study. Clearly, additional studies are needed to confirm the receptor-selective binding of the probes, using either water-soluble dopaminergic drugs or fluorophors with higher emission energy.

Finally, the distribution of the fluorescent signal should match the known distribution of dopamine receptors in brain and pituitary. A considerably greater degree of fluorescence was observed in the anterior than in the posterior pituitary of monkey, which corresponds to the known distribution of D_2 dopamine receptors in these regions. In tissue sections of rodent brain, fluorescent labeling of striatum, which has the highest concentration of both D_1 and D_2 receptors, was more pronounced than was the labeling in the cortex, with the exception of the cingulate cortex. Similar results have been reported recently using rhodamine derivatives of D_1 and D_2 dopamine receptor antagonists (19). Additional studies in organized tissue are required to evaluate adequately the specificity of fluorescence and the potential for practical applications. Nonetheless, several novel compounds described in this study retain characteristic binding properties, suggesting the feasibility of developing useful probes. This approach to dopamine receptor detection may help to answer questions about receptor localization and regulation that cannot be addressed using conventional radioligand probes.

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