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New σ-Like Receptor Recognized by Novel Phenylaminotetralins: Ligand Binding and Functional Studies

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SUMMARY

Several novel phenylaminotetralins (PATs) cause functional changes in brain that are associated with binding to saturable, high affinity sites that are not identical to any known central nervous system receptor. These PATs were tested for their ability to cause receptor-mediated functional effects on tyrosine hydroxylase activity in corpus striatum from rat and guinea pig brain. (±)-(*trans*)-1-Phenyl-3-dimethylamino-6-chloro-7-hydroxy-1,2,3,4-tetrahydronaphthalene (Cl,OH-PAT) increased tyrosine hydroxylase activity (by approximately 30–40%) at 0.1 μM. Higher concentrations inhibited enzyme activity by indirect mechanisms that may include displacement of intraneuronal dopamine. The 6,7-unsubstituted congener (±)-(*trans*)-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene stimulated tyrosine hydroxylase by as much as 50–60% over basal activity, without displacement of dopamine. Similarly to certain (+)-benzomorphan σ receptor ligands, the effects of both PATs to activate tyrosine hydroxylase were blocked competitively by the putative σ antagonist BMY-14802. Radiolabeled [^{3}H]Cl,OH-PAT bound saturably and with high affinity to guinea pig brain membranes (K_d = 31 pM, B_{max} = 6.5 fmol/mg of protein). The pharmacological profile of these binding sites was inconsistent with those of known σ_1, σ_2, dopaminergic, serotonergic, adrenergic, opioid, *N*-methyl-D-aspartate, or several other characterized central nervous system recognition sites. Together, these data suggest that these PATs may be agonists at a novel σ-like site that has neuromodulatory activity that results in increases of brain catecholamine synthesis via activation of tyrosine hydroxylase.

Although generally accepted as being unrelated to opioid mechanisms, the functional role and biological significance of σ binding sites in the CNS (and even their status as "receptors") remain unclear (1, 2). Binding sites for the proposed σ ligand (+)-3-PPP occur in mammalian mid- and forebrain regions (3). Autoradiographic studies show these sites to be concentrated on cell bodies of dopamine neurons in the zona compacta of midbrain substantia nigra (4). Although still tenuous, a functional neurobiological role for σ sites in the CNS, particularly involving dopaminergic or adrenergic catecholamine systems, has been hypothesized (2). Several lines of evidence suggest that σ receptors may modulate catecholamine release (5, 6). For example, (+)-3-PPP enhanced electrically stimulated release of endogenous norepinephrine from mouse vas deferens (7) and doubled spontaneous efflux of [^{3}H]dopamine from striatal slices by a process not fully blocked by D_2 dopamine antagonists (8). The pharmacology of 3-PPP, however, is complex and includes partial agonism of dopamine autoreceptors (9). Because (+)-3-PPP has a >100-fold greater affinity for σ sites versus dopamine receptors, it has been proposed that the (+)-3-PPP-mediated inhibition of dopamine neuron firing and dopamine release may be caused, at least in part, by occupation of σ receptors (2, 10, 11).

Several other drugs are purported to be σ ligands; these include the piperazinebutanol derivative BMY-14802 (12, 13), the substituted propylpiperazine-carbazole rimcazole (BW-234U) (14), and the benzomorphan (+)-NANM (1, 15). These compounds produce complex electrophysiological alterations in rat midbrain dopamine neurons in both substantia nigra (A_9) and ventral tegmentum (A_{10}). BMY-14802 is considered to be a selective σ antagonist (10, 12, 16), although it may also have some 5-HT_{1A} agonist activity (16, 17). BMY-14802 exerts neu-

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ABBREVIATIONS: CNS, central nervous system; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; Cl,OH-PAT, (±)-(*trans*)-1-phenyl-3-*N*,*N*-dimethylamino-6-chloro-7-hydroxy-1,2,3,4-tetrahydronaphthalene; DTG, 1,3-di-(2-tolyl)guanidine; PAT, phenylaminotetralin (phenylaminotetrahydronaphthalene); NANM, (+)-*N*-allylnormetazocine; H_2-PAT, (±)-(*trans*)-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene; PCP, phencyclidene; RMS, root mean square; NMDA, *N*-methyl-D-aspartate; NPA, (*R*)-(−)-*N*-*n*-propylnorapomorphine; ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; 5-HT, 5-hydroxytryptamine.

romodulatory activity that includes blockade or reversal of (+)-3-PPP-induced inhibition of dopamine neuron firing (10) and dopamine release (11). Several σ ligands, including rimcazole (18) and BMY-14802 (16, 19), have activity in animal behavior models used to predict antipsychotic efficacy (e.g., inhibition of apomorphine-induced hyperactivity and stereotyped behavior in rats).

In light of the effects of σ ligands on dopamine neuron firing and catecholamine release, we had hypothesized that σ sites in mammalian corpus striatum may include neuromodulatory receptors affecting catecholamine (more specifically, dopamine) synthesis. Recently we reported that the benzomorphan σ ligands (+)-NANM and (+)-pentazocine (Fig. 1), but not (+)-cyclazocine, stimulated rat striatal dopamine synthesis *in vitro* by about 25% at 0.1 μM (20). Because this stimulation of dopamine synthesis by (+)-NANM and (+)-pentazocine was blocked by the σ antagonist BMY-14802, but not by the opioid antagonist naloxone, a σ receptor-mediated mechanism was suggested. The (−)-enantiomers of NANM, pentazocine, and cyclazocine, as well as the σ ligands (+)-3-PPP, DTG (21), and haloperidol (22), had no effect on dopamine synthesis at low concentration (0.1 μM) but were inhibitory at higher concentrations (>1.0 μM) by processes not blocked by BMY-14802 (20). Neither PCP ligands, which also may bind to σ sites (23), nor μ- or κ-opiate agonists affected dopamine synthesis at concentrations up to 10 μM (20).

Clues to the functional identity of these sites may be obtained from understanding the σ binding pharmacophore of (+)-benzomorphans. This pharmacophore is proposed to include an *N*-substituted aminotetralin moiety (24), and molecular modeling studies (25, 26) indicate that the optimal distance between the aromatic ring and the nitrogen atom for σ ligands is about 5 Å (4.3–6.4 Å). As part of our efforts to design σ receptor ligands that may modulate dopamine neuron activity, we proposed the phenylaminotetralin analog (+)-Cl,OH-PAT (Fig. 1). Molecular modeling experiments indicate that the aminotetralin moiety incorporated in this PAT analog is in the same stereochemical configuration as found in (+)-pentazocine and (+)-NANM (Fig. 1) (27). The distance between the tetrahydronaphthalene aromatic ring and the nitrogen atom in (+)-Cl,OH-PAT was calculated to be 5.2 Å (27), close to the median (5.3 Å) of the range proposed to be optimal for σ receptors (25, 26). When the minimum energy conformation of (+)-Cl,OH-PAT is superimposed by computer (27) on proposed pharmacophoric elements of (+)-NANM using a least squares fit (aligning the protonated nitrogen atoms, tetrahydronaphthalene rings, and phenyl and methyl substituents), the pharmacophoric elements of the two molecules can be superimposed (Fig. 1) extremely well (RMS fit value = 0.25 Å).

We recently reported the synthesis and chemical characterization of racemic (±)-Cl,OH-PAT and several analogs (27), as

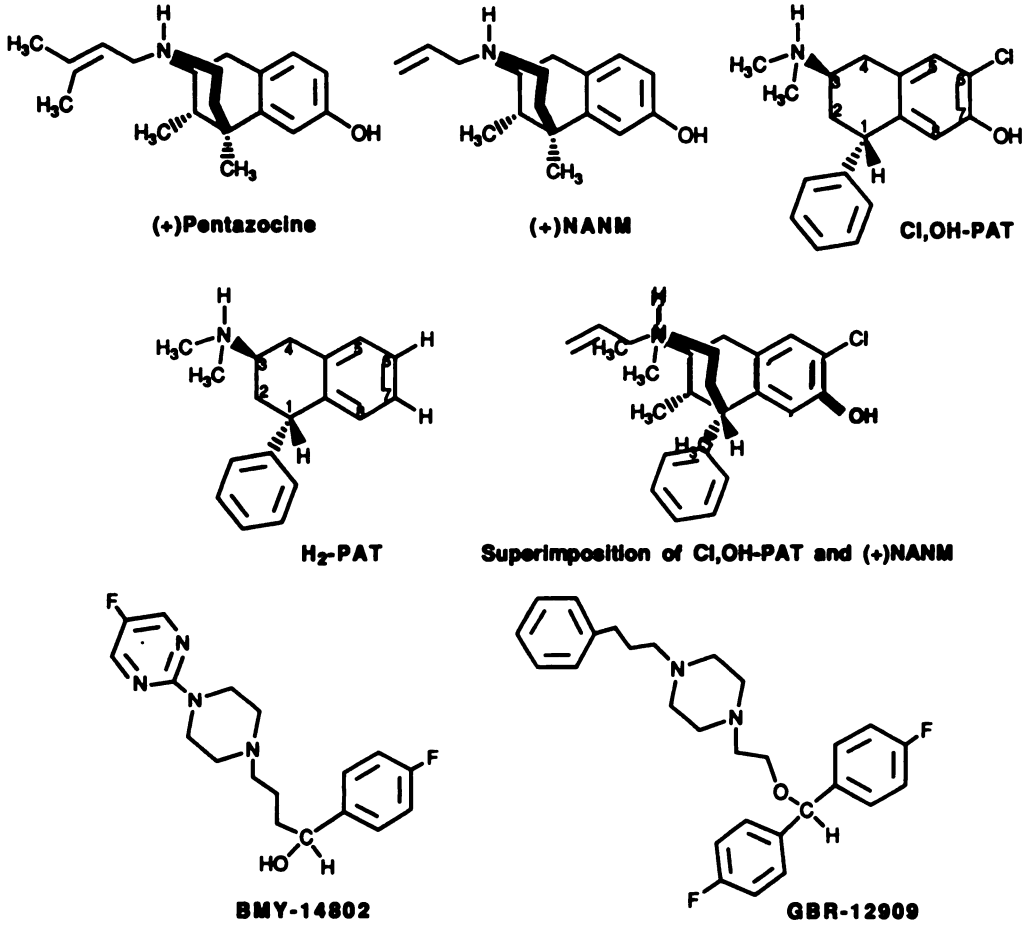

Fig. 1. Structures and proposed pharmacophoric components of compounds described in the text. Agents depicted are (+)-NANM, (+)-pentazocine, PAT compounds (+)-Cl,OH-PAT and H_2-PAT, BMY-14802 [racemic α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol], and GBR-12909.

well as the synthesis of (±)-[*N*-^{3}H-*methyl*]Cl,OH-PAT (28). Preliminary data suggested σ-like functional and pharmacological properties (27). The present experiments provide a detailed examination of the pharmacology of these compounds and test whether they affect the function of dopamine neurons, perhaps by occupation of what may be a σ-type receptor. Based on the hypothesis that the aromatic hydroxyl group present in the benzomorphans (+)-NANM and (+)-pentazocine is not required for binding to σ sites (24), the present work also examines the corresponding 6,7-unsubstituted analog of Cl,OH-PAT, i.e., H_2-PAT (Fig. 1). We report on the effects of these compounds on striatal dopamine synthesis and on binding to [^{3}H]DTG-labeled σ receptors (21) and dopamine receptors (D_1 and D_2) in rodent brain. Finally, [^{3}H]Cl,OH-PAT was used as a radioligand to characterize the apparently unique binding site(s) by which these PATs may influence dopamine nerve terminal function.

Materials and Methods

Animals. Young adult (250–300 g) male Sprague-Dawley rats or adult (300–400 g) male guinea pigs were obtained from Charles River Laboratories (Wilmington, MA). They were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities, under controlled environmental conditions (20–22°, 40–50% humidity, and lights on from 7:00 a.m. to 7:00 p.m.), for at least 1 week before use. Animals had free access to commercial food pellets and fresh tap water. Some rats were pretreated with reserpine (5 mg/kg), or an equivalent volume of physiological saline, at 20 and 2 hr before sacrifice. This regimen produces >90% depletion of striatal dopamine (29).

Chemicals. Each PAT compound was synthesized via a corresponding benzylstyrylketone (1,4-diphenyl-but-3-ene-2-one) that was cyclized to a tetralone intermediate and then reduced to the tetralol. This intermediate was tosylated and then converted to the target amine by reaction with sodium azide followed by catalytic reduction, to yield predominately the *trans*-isomer. A suitable yield of pure *trans*-racemate was obtained for each analog, as the HCl or HBr salt. Detailed syntheses of the PATs and (±)-[^{3}H]Cl,OH-PAT (85 Ci/mmol) are described elsewhere (27, 28). L-[1-^{14}C]Tyrosine (52 mCi/mmol), [^{3}H]DTG (39.4 Ci/mmol), (+)-[^{3}H]SCH-23390 (60.4 Ci/mmol), and [^{3}H]YM-09151–2 (75.0 Ci/mmol) were obtained from DuPont-NEN Corp. (Boston, MA). Sources of donated and purchased pharmaceuticals are listed in the legend to Table 2; other compounds not listed were obtained at the highest available purity from Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals Inc. (Natick, MA).

Tyrosine hydroxylase assay. Dopamine synthesis was assessed as tyrosine hydroxylase activity in rat or guinea pig striatal minces. A radiometric assay was used to measure formation of $^{14}CO_2$ evolved during the decarboxylation of L-dihydroxyphenylalanine to form dopamine, starting from L-[1-^{14}C]tyrosine, as described in detail previously (29, 30).

Radioreceptor assays. Specific high affinity radioreceptor assays of brain tissue homogenates were used to determine the affinity (K_i) of the PATs and other reference ligands for σ sites (guinea pig brain) and D_1 and D_2 receptors (rat striatum). The radioligands and agents used to define nonspecific binding were as follows: σ, 2.0 nM [^{3}H]DTG with 10 μM haloperidol (21); D_1, 0.3 nM [^{3}H]SCH-23390 with 0.3 μM (*cis*)-(*Z*)-flupenthixol (31); D_2, 0.065 nM [^{3}H]YM-09151–2 with 0.20 μM (+)-butaclamol (32, 33).

Binding of the novel radioligand [^{3}H]Cl,OH-PAT to brain membranes was examined using assay conditions similar to those reported for the σ ligand [^{3}H]DTG (21). Briefly, frozen guinea pig brain (minus cerebellum; obtained from Keystone Biologicals, Cleveland, OK) was thawed and homogenized in ice-cold 10 mM Tris buffer containing 0.32 M sucrose, pH 7.0 (10 ml/g of tissue). The homogenate was centrifuged at 1000 × *g* for 15 min at 4°, and the supernatant was recentrifuged at 31,000 × *g* for 15 min at 4°. The P_2 pellet was suspended in 10 mM Tris buffer, pH 7.4 (25°), at 3 ml/g of tissue and was incubated at room temperature for 15 min before recentrifugation at 31,000 × *g* for 15 min at 4°. The resulting pellet was stored at −70° in 10 mM Tris, pH 7.4, at approximately 20 mg of protein/ml. A saturation isotherm was obtained using at least six concentrations (0.02–2.0 nM) of free ligand in triplicate in 50 mM Tris buffer, pH 7.4 (1.0 mg of protein in 2.0-ml total volume, in glass tubes). Excess BMY-14802 (5.0 μM) was used to define nonspecific binding. Tubes were incubated for 60 min at 30° and incubation mixtures were then filtered in a Brandel cell harvester through glass fiber sheets (GF/B), which were cut and counted for tritium by liquid scintillation counting at 50% efficiency. Results were first analyzed as Scatchard-Rosenthal plots (B/F versus B) to provide an estimate of apparent affinity (K_d) and density of binding sites (B_{max}). These values were used as starting values for analysis with the LIGAND program (34) adapted for the Macintosh microcomputer (35). For competitive binding assays, tubes were incubated (60 min at 30°) with 50 pM (approximately K_d) [^{3}H]Cl,OH-PAT and four to eight concentrations (10 pM to 10 μM) of test compounds. The resulting competition data were analyzed using the ALLFIT program for the Macintosh computer to determine IC_{50} values (36), which were converted to corresponding K_i values using the equation $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioligand having affinity K_d (37).

Results

Effects of PATs on dopamine synthesis. In striatum from saline-pretreated control rats, 0.1 μM Cl,OH-PAT significantly increased tyrosine hydroxylase activity over basal levels, by 28 ± 3% (Fig. 2A). Lower concentrations of Cl,OH-PAT (0.01 μM) did not significantly affect tyrosine hydroxylase activity (108 ± 6%; data not shown). The stimulation observed at 0.1 μM was blocked completely by a 1.0 μM concentration of the σ antagonist BMY-14802 (Fig. 2A) but not by the opioid antagonist naloxone or the dopamine antagonist fluphenazine (up to 10 μM; data not shown). BMY-14802 at 0.1 μM also blocked the effect of 0.1 μM Cl,OH-PAT (enzyme activity, 107 ± 5%; data not shown). At concentrations greater than 1.0 μM, the effect of Cl,OH-PAT on tyrosine hydroxylase activity was reversed, so that at 10 μM Cl,OH-PAT inhibited enzyme activity by 50 ± 6% (Fig. 2A). This inhibitory effect was not blocked by BMY-14802 (Fig. 2A), however, suggesting that effects of high concentrations of Cl,OH-PAT occur either through a receptor system unrelated to σ receptors or through non-receptor-mediated mechanisms (e.g., involving dopamine release or direct inhibitory actions on tyrosine hydroxylase).

These possibilities were addressed first by adding spiperone (1.0 μM) to block D_2-type autoreceptors (thus preventing inhibition of tyrosine hydroxylase caused by release of endogenous dopamine). Although the stimulatory effect of 0.1 μM Cl,OH-PAT was not affected significantly ($p > 0.05$), spiperone abolished the inhibitory effects of 10 μM Cl,OH-PAT, returning levels to control values but not to the stimulated levels seen with 0.1 μM Cl,OH-PAT (Fig. 2A). One explanation for such a result is that, in addition to inducing dopamine release into the synapse, high concentrations of Cl,OH-PAT may displace dopamine from vesicular or other intraneuronal storage pools, thus inhibiting tyrosine hydroxylase directly. This hypothesis was tested by using reserpine pretreatment to deplete dopamine from striatum (29). As predicted, in the absence of endogenous dopamine Cl,OH-PAT produced only concentration-dependent increases of tyrosine hydroxylase activity (26 ± 8, 30 ± 7, and 38 ± 8% at 0.1, 1.0, and 10 μM, respectively) (Fig. 2A). These

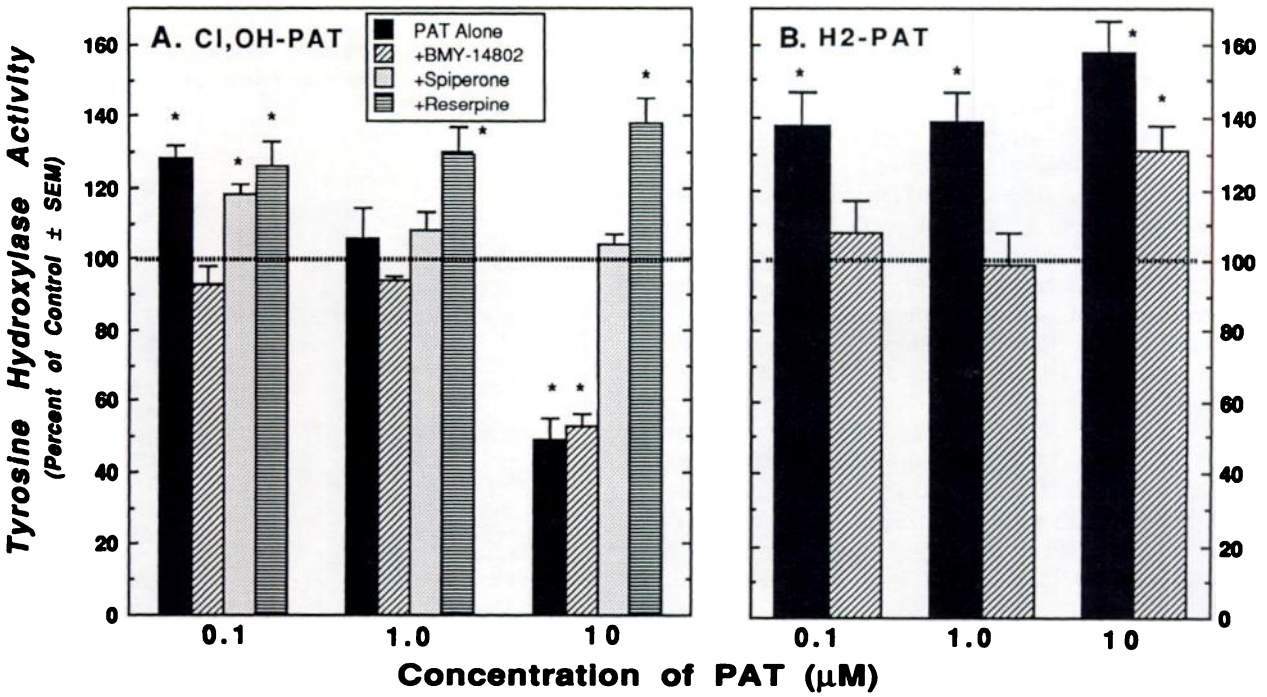

Fig. 2. Effects of PATs on striatal tyrosine hydroxylase activity. The effect of (A) Cl,OH-PAT or (B) H_2-PAT on dopamine synthesis was assayed by measuring tyrosine hydroxylase activity in striatal minces from saline- or reserpine-pretreated rats, as described in Materials and Methods; data are mean ± standard error of percentage of control for at least six replications. Basal (control) enzyme activity (100%) was 16.9 ± 1.5 (saline) or 10.1 ± 1.0 (reserpine) pmol of $^{14}CO_2$/30 min/mg of protein. BMY-14802 (1.0 μM) and spiperone (0.1 μM) were included in assays of tissue from saline-pretreated rats; at these concentrations, neither drug alone significantly altered tyrosine hydroxylase activity. *, $p < 0.005$, versus control, by t test.

functional effects are similar to those of the σ ligands (+)-NANM and (+)-pentazocine (20) and suggest that Cl,OH-PAT and the (+)-6,7-benzomorphans may act through similar sites to stimulate dopamine synthesis in mammalian brain. In addition, Cl,OH-PAT, like (+)-amphetamine (29), appears to have a dopamine-releasing effect at concentrations greater than 1 μM.

Unlike Cl,OH-PAT, the 6,7-unsubstituted PAT congener H_2-PAT (Fig. 1) produced only concentration-dependent increases of tyrosine hydroxylase activity in control tissue (38 ± 9, 39 ± 8, and 58 ± 9% at 0.1, 1.0, and 10 μM, respectively) (Fig. 2B). H_2-PAT did not appear to have a dopamine-releasing effect, because it did not inhibit tyrosine hydroxylase activity, even at 10 μM. The stimulation of dopamine synthesis caused by 0.1 μM and 1.0 μM H_2-PAT was fully blocked by 1.0 μM BMY-14802 (Fig. 2B), as noted above with Cl,OH-PAT and as previously reported for the σ ligands (+)-NANM and (+)-pentazocine (20). At 1.0 μM, BMY-14802 only partially blocked the stimulatory effect of 10 μM H_2-PAT on tyrosine hydroxylase activity (Fig. 2B); at 0.1 μM it did not block the PAT effect at all. The ability of H_2-PAT to surmount the blockade suggests that the antagonism by BMY-14802 of PAT functional effects is competitive. As observed with other hydroxyl group-containing σ ligands, such as benzomorphans (20) and haloperidol (38), higher concentrations of BMY-14802 (e.g., ≥10 μM) inhibit tyrosine hydroxylase activity, thus confounding antagonism experiments. It is believed that compounds with hydroxyl moieties compete with biopterin cofactor for the active site of tyrosine hydroxylase (39).

We also evaluated the effects of the PAT compounds on tyrosine hydroxylase activity in fresh minced guinea pig striatum, because σ binding assays commonly use membranes prepared from brain tissue of this species rather than rat (21). Cl,OH-PAT and H_2-PAT had stimulatory actions on tyrosine hydroxylase activity (21 ± 2 and 32 ± 3%, respectively, at 0.1 μM) in striatal tissue from guinea pig, similar to those observed in rat brain, and the effects were fully blocked by 1.0 μM BMY-14802.

Binding to [^{3}H]DTG-labeled σ binding sites and D_1 and D_2 receptors. The observed functional effects of Cl,OH-PAT and H_2-PAT could involve either "traditional" σ sites (i.e., σ_1 or σ_2) or dopamine receptors. To test these possibilities, the ability of Cl,OH-PAT and H_2-PAT to compete for binding to [^{3}H]DTG-labeled σ sites in guinea pig brain and to [^{3}H]SCH-23390-labeled D_1 or [^{3}H]YM-09151-2-labeled D_2 receptors in rat striatum was tested (Table 1). As expected (21, 22), DTG and haloperidol had relatively high affinity for [^{3}H]DTG-la-

TABLE 1

Affinity of test compounds at [^{3}H]DTG-labeled σ binding sites and D_1 and D_2 dopamine receptors in rodent brain tissue

The σ assay used whole guinea pig brain (minus cerebellum) and 2.0 nM [^{3}H]DTG, and the dopamine receptor assays used rat striatum and 0.3 nM [^{3}H]SCH-23390 (D_1) or 0.065 nM [^{3}H]YM-09151-2 (D_2), as described in Materials and Methods. Sources of compounds are provided in Table 2. Values are means ± standard errors.

Compound	K_i		
	σ	D_1	D_2
	nM		
Haloperidol	6.4 ± 1.5	47.1 ± 5.0	1.1 ± 0.2
DTG	28.5 ± 5.7	>5000	>5000
BMY-14802	41.5 ± 1.9	>5000	>5000
(+)-Pentazocine	380 ± 63	>5000	>5000
(+)-NANM	1000 ± 220	>5000	>5000
Cl,OH-PAT	2000 ± 190	950 ± 98	1900 ± 50
H_2-PAT	3100 ± 220	1500 ± 75	690 ± 18

beled σ sites (K_i = 28.5 nM and 6.4 nM, respectively) and, consistent with other reports (12, 16), the putative σ antagonist BMY-14802 had moderate affinity for these sites (K_i = 41.5 nM). Both (+)-pentazocine (K_i = 380 nM) and (+)-NANM (K_i = 1000 nM) had moderate affinity for [^{3}H]DTG-labeled σ sites, albeit somewhat lower than that reported by Weber *et al.* (21) (K_i = 43 and 625 nM, respectively); this relatively lower affinity may represent binding to σ_2-like sites (40) or technical differences in our assay. K_i values for Cl,OH-PAT and H_2-PAT binding to the [^{3}H]DTG-labeled σ sites (≥2000 nM) were much higher than those for all of the preceding σ_1 or σ_2 ligands.

Whereas haloperidol showed expected (31, 32) high affinity for D_2 receptors (K_i = 1.1 nM) (Table 1), DTG, BMY-14802, (+)-pentazocine, and (+)-NANM had little affinity for D_1 or D_2 dopamine receptors (K_i > 5000 nM) (Table 1). The affinity of Cl,OH-PAT and H_2-PAT for dopamine receptors also was low (K_i = 690–1900 nM). Taken together, these results suggest that the dopamine synthesis-modulating effects of the PATs, which occur at 0.1 μM and are blocked by similarly low concentrations of the σ ligand BMY-14802 (Fig. 2), are unlikely to be mediated by agonism of traditional σ receptors (i.e., defined by binding of [^{3}H]DTG) or by antagonism of dopamine autoreceptors.

[^{3}H]Cl,OH-PAT binding site. Under assay conditions similar to those developed for σ radioligands (e.g., [^{3}H]DTG), [^{3}H] Cl,OH-PAT bound saturably with very high affinity to guinea pig brain membranes (Fig. 3A), with a B_{max} of 6.5 fmol/mg of protein and an apparent K_d of 31 pM, as determined by Scatchard-Rosenthal analysis (Fig. 3B). Kinetic analysis gave rates of association (k_1 = 3.44 dpm/min/nM) and dissociation (k_{-1} = 0.086 dpm/min) that yielded a calculated K_d of 25 pM; this result is consistent with the observed K_d of 31 pM obtained from the Scatchard analysis. Having determined that [^{3}H] Cl,OH-PAT labeled a saturable high affinity site, we performed experiments to characterize the nature of these sites. The affinity of selected CNS receptor-active drugs for [^{3}H]Cl,OH-PAT-labeled sites is shown in Table 2.

As expected, both unlabeled Cl,OH-PAT (K_i = 0.1 nM) and

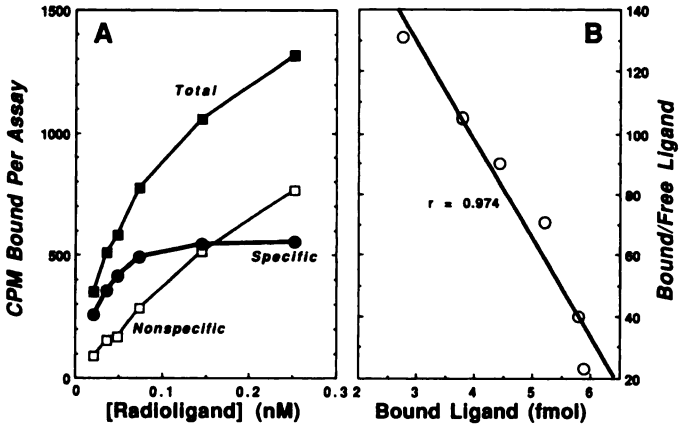

Fig. 3. Saturation isotherm and Scatchard-Rosenthal plot of [^{3}H]Cl,OH-PAT binding to guinea pig cerebral membranes. A, Saturation curves showing total, nonspecific (cpm with 5 μM BMY-14802 as blank), and specific (difference) counts bound, based on free radioligand concentrations ranging from 0.02 to 0.25 nM, with each point based on triplicate assays pooled from three separate experiments. B, Scatchard-Rosenthal plot of specific counts bound in A as the ratio of bound/free versus fmol of [^{3}H]Cl,OH-PAT bound/assay containing 1 mg of protein. Linear regression (r = 0.975) indicates B_{max} of 6.5 fmol/mg of protein (x-intercept) and K_d of 31 pM (1/slope).

TABLE 2

Affinities at [^{3}H]Cl,OH-PAT binding sites in guinea pig brain

Values are means ± standard errors. Sources of compounds are indicated. Methods are described in the text.

Compound	K_i
	nM
Cl,OH-PAT	0.11 ± 0.02
H_2-PAT	0.20 ± 0.02
GBR-12909[a]	0.38 ± 0.07
Ketanserin[b]	0.38 ± 0.01
(*cis*)-Flupenthixol[c]	0.86 ± 0.17
(*trans*)-Flupenthixol[c]	5.73 ± 0.85
Buspirone[d]	18.5 ± 10.1
(+)-Butaclamol[e]	20.4 ± 9.2
Spiperone[a]	23.5 ± 18.1
BMY-14802[e]	34.6 ± 11.1
Cinanserin[e]	70.4 ± 5.8
Haloperidol[f]	81.5 ± 21.1
Mazindol[g]	90.8 ± 16.9
SCH-23390[a]	151 ± 23
(+)-3-PPP[a]	166 ± 13
(+)-Phentolamine[h]	213 ± 38
Rimcazole[e]	228 ± 30
Fluoxetine[i]	326 ± 130
Methysergide[g]	353 ± 150
(−)-3-PPP[a]	370 ± 29
YM-09151-2[a]	597 ± 170
SKF-525A[e]	615 ± 208
(+)-Pentazocine[j]	~3000
Dextromethorphan[j]	~3000
(−)-Apomorphine[k]	~4000
DTG[a]	~4000
(+)-NANM[a]	~4000
(−)-NPA[a]	~4000
Yohimbine[k]	~4000
(±)-ADTN[l]	>5000
(+)-Amphetamine[k]	>5000
(−)-Butaclamol[e]	>5000
Cocaine[a]	>5000
(+)-Cyclazocine[j]	>5000
(−)-Cyclazocine[j]	>5000
Dopamine[k]	>5000
Histamine[a]	>5000
(+)-MK-801[m]	>5000
Naloxone[k]	>5000
(−)-Norepinephrine[k]	>5000
Pargyline[k]	>5000
Prazosin[k]	>5000
Serotonin[i]	>5000
(+)-SKF-38393[a]	>5000

[a] Research Biochemicals, Inc. (Natick, MA).
[b] Janssen Pharmaceuticals (Beerse, Belgium).
[c] Lundbeck Laboratories (Copenhagen, Denmark).
[d] Mead & Johnson (Evansville, IN).
[e] Bristol-Meyers Squibb Corp. (Wallingford, CT).
[f] McNeil Laboratories (Fort Washington, PA).
[g] Sandoz Research Institute (Berne, Switzerland).
[h] CIBA-Geigy Pharmaceuticals (Basel, Switzerland).
[i] Eli Lilly Laboratories (Indianapolis, IN).
[j] National Institute on Drug Abuse (Baltimore, MD).
[k] Sigma Chemical Co. (St. Louis, MO).
[l] Burroughs-Wellcome Corp. (Research Triangle, NC).
[m] Merck, Sharp & Dohme (Harlow, UK).

H_2-PAT (K_i = 0.2 nM) had very high affinity for [^{3}H]Cl,OH-PAT-labeled sites. Although a few ligands [e.g., the 5-HT_2 antagonist ketanserin and the D_1 and D_2 antagonist (*cis*)-flupenthixol] had significant affinity for the [^{3}H]Cl,OH-PAT-labeled site, most serotonergic (e.g., the 5-HT_{1A} ligand buspirone, cinanserin, methysergide, and serotonin itself) and dopaminergic [e.g., spiperone, haloperidol, (±)-YM-09151-2, (±)-SCH-23390, (±)-SKF-38393, (±)-6,7-ADTN, (*R*)-(−)-apomorphine, (*R*)-(−)-NPA, and dopamine itself] ligands had little

affinity, relative to the PATs (Table 2). Taken together, these data appear to rule out all of the known CNS dopamine or serotonin receptors as binding sites for [^{3}H]Cl,OH-PAT.

The neuronal dopamine transporter antagonist GBR-12909 [1-(2-[bis(fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine] also competed potently (K_i ~ 0.4 nM) for the [^{3}H]Cl,OH-PAT binding site in guinea pig brain tissue. It is interesting that GBR-12909 was reported recently to bind with high affinity to (+)-[^{3}H]3-PPP-labeled σ sites (41). Conversely, other blockers of the dopamine neurotransporter that do not have σ activity (e.g., cocaine and mazindol) (2) were virtually inactive. The evident dissimilarity between the [^{3}H]Cl,OH-PAT site and the dopamine transporter suggests that the apparent dopamine-releasing effect of Cl,OH-PAT (see below) is unlikely to involve altered neuronal uptake of dopamine. Negative results were also obtained with (+)-amphetamine, the selective serotonin uptake blocker fluoxetine, histamine, several adrenoreceptor ligands (norepinephrine, phentolamine, prazosin, and yohimbine), the NMDA/PCP receptor antagonist (+)-MK-801, the opioid antagonist naloxone, and two enzyme inhibitors, SKF-525-A (for the cytochrome P-450 oxidase system) and pargyline (for monoamine oxidase) (Table 2). Thus, [^{3}H]Cl,OH-PAT labels a site in brain whose pharmacological profile differs from those of known dopaminergic, serotonergic, adrenergic, NMDA/PCP, and opioid receptors, as well as cytochrome P-450 and monoamine oxidase enzymes.

Although the [^{3}H]Cl,OH-PAT binding site clearly is not a D_1- or D_2-like dopamine receptor, it is interesting to note that it has the same stereoselectivity as dopamine receptors for (*cis*)- versus (*trans*)-flupenthixol and for (+)- versus (−)-butaclamol. However, the relative selectivity of (*cis*)- versus (*trans*)-flupenthixol and (+)- versus (−)-butaclamol at the [^{3}H]Cl,OH-PAT binding site (approximately 7-fold and at least 245-fold, respectively) (Table 2) was much lower than the stereoselectivity observed at dopamine receptors (approximately 400-fold and 20,000-fold, respectively) (42). Interestingly, the stereoselectivity observed for butaclamol at the [^{3}H]Cl,OH-PAT site [(+) > (−)] is opposite that observed at [^{3}H]DTG-labeled σ sites in guinea pig brain, where (−)-butaclamol is reported to have about 4-fold greater affinity than (+)-butaclamol (21). Both isomers of the benzomorphan cyclazocine were inactive at the [^{3}H]Cl,OH-PAT site (and both isomers also are inactive in stimulating tyrosine hydroxylase) (20). The stereoselectivity observed at the [^{3}H]Cl,OH-PAT site for 3-PPP [(+) > (−); 2.2-fold] is similar to that observed at [^{3}H]DTG-labeled σ sites (3.7-fold) in guinea pig brain (21). The σ ligand rimcazole and the σ/dopaminergic ligand haloperidol had modest affinity for the [^{3}H]Cl,OH-PAT binding site, whereas the σ ligands (+)-pentazocine, (+)-NANM, dextromethorphan, and DTG all had relatively low affinity. Interestingly, the affinity of the putative selective σ antagonist BMY-14802 for the [^{3}H]Cl,OH-PAT site (K_i ~ 35 nM) (Table 2) was similar to its affinity for the [^{3}H]DTG σ site (K_i ~ 40 nM) (Table 1).

Discussion

In the present experiments, we have shown that low concentrations of Cl,OH-PAT and H_2-PAT can stimulate tyrosine hydroxylase activity in dopamine-rich tissue of mammalian forebrain. These stimulatory events were blocked by BMY-14802, an accepted σ receptor (antagonist) ligand. These functional changes are associated with the presence of a high affinity, saturable binding site for [^{3}H]Cl,OH-PAT in brain. Competition studies with numerous ligands from a variety of classes ruled out all of the known neurotransmitter receptors from several systems (including all known dopamine, serotonin, adrenergic, and opioid receptors) as the locus of [^{3}H]Cl,OH-PAT binding. Recent preliminary experiments have examined the effects of H_2-PAT and Cl,OH-PAT in competition for 30 different CNS recognition sites labeled with selective radioligands (National Institute of Mental Health NovaScreen Program) (43). With the exception of the histamine H_1 receptor (a receptor not known to modulate striatal dopamine synthesis), these receptor-screening results indicate that the PATs have negligible affinity for tested reference neurotransmitter (including acetylcholine and γ-aminobutyric acid), neuromodulatory, and ion channel receptors (43). Histamine itself had no affinity for the [^{3}H]Cl,OH-PAT site (Table 2). Thus, the high affinity [^{3}H]Cl,OH-PAT binding site and the parallel functional changes induced by these PATs in brain tissue raise the question of the identity and intrinsic function of the sites at which these compounds act. An obvious possibility is that these PATs may act at a previously uncharacterized member of the σ receptor family.

The available literature has suggested that there may be multiple types of σ receptors (2, 40, 44). For example, σ-like sites in cultured PC12 pheochromocytoma cells [having low affinity for (+)-benzomorphans but high affinity for the σ ligands DTG, (+)-3-PPP, and haloperidol] were named "σ_2" sites to distinguish them from "σ_1" sites characterized in homogenates of guinea pig brain [which have high affinity for (+)-benzomorphans] (45). Even within guinea pig brain, there may be at least two classes of binding sites for [^{3}H]DTG, with (+)-benzomorphans having high and low affinity for σ sites designated 1 and 2, respectively. Furthermore, Musacchio and co-workers (46, 47) have proposed multiple types of [^{3}H]dextromethorphan binding sites (apparently representing σ sites) in guinea pig liver, kidney, and adrenal medulla, as well as high and low affinity sites within guinea pig brain. Recently, it has been proposed that sites that bind the (+)-benzomorphans with moderate to high versus low affinity be classified as sites σ_1 and σ_2, respectively (40). The (−)-benzomorphans have been designated as low affinity, nonselective, σ_1/σ_2 ligands, whereas (+)-3-PPP, haloperidol, and DTG all have been designated as high affinity, nonselective, σ_1/σ_2 ligands (40). As expected, because the σ receptor is nonopioid in nature, the potent and selective opioid antagonist naloxone is inactive at both sites (40).

As noted above, the present findings support the hypothesis that (±)-Cl,OH-PAT and (±)-H_2-PAT modulate striatal dopamine synthesis in rodent brain by a mechanism that may involve interactions with a receptor that recognizes, and can be antagonized by, BMY-14802. The binding of [^{3}H]Cl,OH-PAT to rodent brain membranes occurs with high affinity and stereoselectivity. Not only is the pharmacological profile inconsistent with that of any known CNS receptor, but the pharmacology also is inconsistent with the characteristics of reported [^{3}H]DTG-labeled σ sites. Thus, one might explain the functional effect of PATs on striatal dopamine synthesis via action at a novel CNS binding site or receptor. In light of the functional effects (Fig. 2) of BMY-14802 (Fig. 2) and the moderate to high affinity of some (but not all) σ ligands at this

site (Table 2), perhaps the [^{3}H]Cl,OH-PAT site is a previously uncharacterized σ receptor subtype. We hypothesize that the second of these possibilities should be examined first, for the following reasons. *A priori*, it must be remembered that, until recently, identification of new receptor subtypes occurred almost solely on the basis of pharmacological and functional data. Thus, one should not dismiss out of hand the possibility that the PAT site involved may be a σ-like receptor. Moreover, subtypes of the same receptor class may show vastly different structure-activity requirements for both binding and functional effects; D_1- and D_2-like dopamine receptors and μ-, δ-, and κ-opioid receptors are but two examples of this phenomenon (1, 5, 31, 32, 42).

Several points pertain to a σ receptor subtype hypothesis. First, the PATs caused stimulatory effects on dopamine synthesis in rat and guinea pig striatum qualitatively similar to the effects of the benzomorphan σ ligands (+)-pentazocine and (+)-NANM. Second, the effects of both types of compounds on tyrosine hydroxylase activity could be blocked by the σ antagonist BMY-14802. It has already been noted that BMY-14802 may also possess 5-HT_{1A} agonist activity (12, 16, 17). In the present tyrosine hydroxylase assay, however, 1 μM BMY-14802 showed evidence only of receptor antagonism (i.e., no functional effect was observed with BMY-14802 alone) (Fig. 2). Third, pharmacophoric elements of the PATs, hypothesized to be relevant to σ-type sites, can be closely superimposed on those of (+)-NANM and (+)-pentazocine (Fig. 1). Preliminary molecular modeling studies show that the PAT pharmacophore also aligns closely with proposed σ pharmacophoric elements of BMY-14802 (protonated piperazine-N^1, fluorophenyl, and hydroxyl groups) (RMS fit value = 0.70 Å) (27). Finally, it should be noted that other aminotetralin derivatives recently have been reported to have affinity for σ receptors. For example, (*RS*)-(*trans*)-7-hydroxy-2-(*N*-*n*-propyl-*N*-3′-[^{125}I]iodo-2′-propenyl)aminotetralin (a radioligand for dopamine D_3 receptors) has high affinity (K_d = 10 nM) for σ receptors in rat striatum (48). Moreover, several 6,7-dichloro-*N*-pyrrolidinylethylaminotetralins, which are conformationally analogus to the (*trans*)-PATs described here, also have high affinity (K_i = 1.3–4 nM) for guinea pig brain σ receptors (49).

Although the [^{3}H]Cl,OH-PAT site may represent a σ receptor subtype, it clearly is not identical to the proposed σ-type site(s) through which (+)-benzomorphans increase firing of ventral tegmental (A_{10}) dopamine neurons (15) or stimulate striatal dopamine synthesis (20). Thus, whereas the (+)-benzomorphans stimulate dopamine synthesis at low (i.e., 0.1 μM) concentrations by a mechanism that is blocked by BMY-14802 (20), their affinity for the [^{3}H]Cl,OH-PAT site was negligible. Interestingly, the benzomorphan (+)-cyclazocine was inactive both at the [^{3}H]Cl,OH-PAT binding site and as a modulator of striatal tyrosine hydroxylase activity (20). It may be that there are different neuromodulatory σ-type receptors on dopamine neurons, one type that binds (+)-benzomorphans and another type that binds PATs. BMY-14802 would have affinity for both sites because, as already noted, the K_i values for BMY-14802 at [^{3}H]Cl,OH-PAT- and [^{3}H]DTG-labeled sites are similar. In this regard, it is important to note that the dopamine-uptake blocker GBR-12909 (Fig. 1) is structurally related to BMY-14802 and also has affinity comparable to that of BMY-14802 for (+)-[^{3}H]3-PPP-labeled σ sites (41). However, GBR-12909 has nearly 100-fold higher affinity than does BMY-14802 for the [^{3}H]Cl,OH-PAT site (Table 2), suggesting that it may be possible to design piperazine-substituted congeners with selectivity for the [^{3}H]Cl,OH-PAT site. Molecular modeling studies show that hypothesized PAT pharmacophoric elements align very closely with proposed σ-type pharmacophore groups for GBR-12909 (protonated piperazine-N^6 and each of the fluorophenyl rings) (RMS fit value = 0.54 Å) (27).

Of the two PATs evaluated here, the 6,7-unsubstituted compound H_2-PAT was somewhat more potent and showed monophasic, concentration-dependent stimulation of tyrosine hydroxylase of up to 60%. Unlike H_2-PAT, Cl,OH-PAT also produced nonspecific effects that we ascribe to altered storage or release of dopamine, resulting in D_2 autoreceptor-mediated inhibition of tyrosine hydroxylase (38). Moreover, catechols such as dopamine compete with biopterin cofactor for the active site of tyrosine hydroxylase, to inhibit the enzyme (39). The 6,7-Cl,OH substituents on the tetrahydronaphthalene ring of Cl,OH-PAT may mimic a catechol functional group, thus directly inhibiting tyrosine hydroxylase.

In conclusion, we believe that chemical, functional, and pharmacological data suggest that Cl,OH-PAT and H_2-PAT bind to, and act as agonists at, a common site that may be a new type of neuromodulatory σ receptor in mammalian brain. We hypothesize that, in the basal ganglia and perhaps other neural tissues, this novel receptor is linked to stimulation of tyrosine hydroxylase activity and catecholamine synthesis. Furthermore, receptor-mediated effects on tyrosine hydroxylase activity in brain catecholamine nerve terminals can provide a useful and convenient method for evaluating σ-type or other receptor-mediated neuromodulatory activities. It is worthwhile to note that the PATs tested here were racemic mixtures. Thus, most of their proposed neuromodulatory agonist activity may reside in one enantiomer, with the other isomer having "diluting" or even antagonistic effects. This remains to be tested in detail as suitable quantities of the purified enantiomers become available. Preliminary results indicate that the [^{3}H]Cl,OH-PAT site does indeed show stereoselectivity, at least for the H_2-PAT analog (27).

Although these receptors ultimately may be found not to belong to the σ family, the concordance of functional changes (mediated in a receptor-like manner) and the very high affinity of certain PATs for these binding sites suggest that these loci will be of great interest. For reasons detailed earlier, we have focused on the functional changes caused in striatum. It is imperative that the σ-like sites we hypothesize be mapped autoradiographically. Such data not only will be useful in ensuring that this site is not a previously characterized receptor but also can direct future functional studies. It may be that such functions are of greater importance than the effects on dopamine synthesis we report here.

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