UV $\lambda_{max}$ ($CHCl_3$) 456 nm ($\epsilon$ 12000); NMR ($CDCl_3$) $\delta$ ($J$) 7.74 (6.0), 7.69 (6.0), [NHC=O(Thr)], 7.3–7.4 (br, Ar $NH_2$), 7.28 (s, Ar C8-H), 6.30 (7.0), 6.22 (7.0), [$\alpha$-CH(MeVal)], 4.90 (2.0), 4.86 (5.0), [$\beta$-CH(Thr)], 4.72 (6.0), 4.64 (2.0), [$\alpha$-CH(Thr)], 2.15 (s, 6-$CH_3$), 2.10 (s, 4-$CH_3$), 4.23 [m, 7-($OCH_2$)], 4.67 [br, 7-CH($NH_2$)$CH_3$].

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**Registry No.** **1b**, 50-76-0; **2a**, 57270-61-8; **2b**, 21478-73-9; **4a**, 109122-95-4; **4b**, 109122-96-5; **5a**, 109150-57-4; **5b**, 109122-97-6; **6a**, 109122-98-7; **6b**, 109122-99-8; **7a**, 109123-00-4; **7b**, 109123-01-5; **8a**, 109123-02-6; **9a**, 109123-03-7; A, 109122-91-0; B, 88419-36-7; C, 109122-92-1; D, 109122-93-2; E, 109122-94-3; $H_2C$=$CHCH_2OH$, 107-18-6; $CF_3SO_2Cl$, 421-83-0; $H_2C$=$CHCH_2I$, 556-56-9; propylene oxide, 75-56-9.

**Supplementary Material Available:** A discussion of the CD spectra and Figures 1–3 detailing UV–visible difference absorption spectra on binding to DNA, CD spectra of free drugs, and CD spectra of drug–DNA complexes, respectively (6 pages). Ordering information is given on any current masthead page.

# Charged Analogues of Chlorpromazine as Dopamine Antagonists

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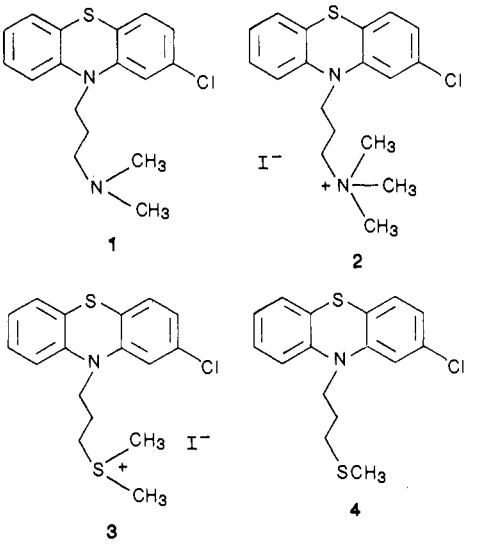
Chlorpromazine (1, CPZ) is a potent dopamine antagonist that has been used widely as an antipsychotic agent. Since dopaminergic antagonists, like dopaminergic agonists, exist in solution as the charged and uncharged molecular species, it is not clear which form of the amine is most important for interaction with the dopamine receptor. Previous work from our laboratory has indicated that a variety of permanently charged species could replace the amine/ammonium moiety of dopamine and retain dopamine agonist activity. This paper describes the synthesis and dopamine antagonist activity of both the trimethylammonium iodide (**2**) and the dimethylsulfonium iodide (**3**) analogues of chlorpromazine. The permanently uncharged methyl sulfide analogue (4) was also synthesized; however, due to its lack of aqueous solubility, its pharmacological activity could not be evaluated. Binding of both the dimethylsulfonium iodide and the trimethylammonium iodide analogues to D-2 dopamine receptors of rat striatal tissue was observed. The observed relative order of binding was CPZ > CPZ sulfonium analogue > CPZ ammonium analogue. These compounds had a similar order of activity in antagonizing the apomorphine-induced inhibition of potassium-induced release of [$^3$H]acetylcholine from mouse striatal slices.

Chlorpromazine (1) is a potent antipsychotic agent and is widely used in the treatment of schizophrenia and other mental disorders. The antipsychotic activity of chlorpromazine has been associated with its ability to act as a dopamine-receptor antagonist.[1] Present evidence suggests that there are at least two dopaminergic receptor subtypes.[2] The D-1 dopaminergic receptor when activated by an agonist is associated with the stimulation of adenylate cyclase activity, while the D-2 dopaminergic receptor mediates dopaminergic effects that either do not involve the stimulation of adenylate cyclase or may be related to the inhibition of this enzyme. Chlorpromazine is a nonselective dopamine antagonist, interacting with both D-1 and D-2 receptors; however, work by Seeman[3,4] indicates that most of the effects of dopaminergic drugs are mediated through their interaction with D-2 receptors.

An early issue that was addressed was the manner in which chlorpromazine, with its phenothiazine ring structure, interacted with a receptor for dopamine. Analysis of the X-ray structures of chlorpromazine and dopamine showed that the two structures can be partially superimposed.[5] When the aromatic ring of dopamine is positioned over the chlorine-containing aromatic ring of chlorpromazine, it is found that the amine nitrogens of both




compounds align. This model, therefore, has been used to support the concept that chlorpromazine, as well as other dopamine antagonists, bind to the same receptor as dopamine. In contrast, findings from other laboratories support the hypothesis that dopamine antagonists may not bind to the same receptors as dopamine.[6]

Since dopamine, at physiological pH, may exist as either an uncharged amine or a charged ammonium species, our laboratory has recently completed experiments aimed at determining which of these two species is most important for binding to the dopamine receptor.[7,8] In these studies,

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the nitrogen atom of dopamine was replaced with a neutral methyl sulfide, a neutral methyl selenide, a charged dimethylsulfonium iodide, and a charged dimethylselenonium iodide. These four analogues were tested for their ability to activate the D-2 dopaminergic receptors that inhibit the $K^+$-stimulated release of [$^3$H]acetylcholine from striatal slices. The two charged species showed significant agonist activity while the corresponding neutral species were inactive at concentrations 5–10-fold higher than those of the charged species, which produced maximum agonist effects. This suggests that it is the charged protonated form of dopamine that is important in the interaction with the receptor. Further, this shows that the nitrogen atom is not required, but may be replaced with either sulfur or selenium atoms.

Similarly, since dopaminergic antagonists, like dopaminergic agonists, also exist in solution as the charged and uncharged molecular species, it is not clear which form interacts with the dopamine receptor. Recent reports in the literature have centered on the dopamine antagonist butaclamol and the protonation state of the amine nitrogen during interaction with its receptors.[9-11] In their characterization of the dopamine receptor, Philipp et al.[9] concluded that since butaclamol has a p$K_a$ of 5.9, compared to a pH of 7.3 from homogenized rat caudate nucleus, the nitrogen atom of butaclamol exists almost exclusively in the deprotonated form during the interaction with its receptor. Therefore, they excluded any ionic interactions involving a charged protonated species and, instead, suggested that the nitrogen lone-pair electrons interact with the receptor via hydrogen-bond formation. In contrast, Chrzanowski et al.[11] independently determined the p$K_a$ for butaclamol and found it to be in the range of 7.0–7.5. They concluded that a p$K_a$ of approximately 7.0 allows for approximately 50% protonation under physiological conditions, and thus, a dopamine receptor model entailing a protonated ligand, such as that described by Olson et al.,[10] may be more appropriate.

In order to discern the protonation state of the amine nitrogen of dopamine antagonists during its receptor interaction, we have chosen to examine permanently charged and uncharged analogues of chlorpromazine. In the process, we can also learn if the nitrogen atom of dopamine antagonists, like the nitrogen atom of dopamine, can be replaced with other heteroatoms. We have synthesized and examined the dopamine antagonist activity of both the trimethylammonium iodide (**2**) and dimethylsulfonium iodide (**3**) analogues of chlorpromazine. We have also synthesized the methyl sulfide analogue (**4**); however, solubility problems have so far prevented us from evaluating it. We are currently addressing this problem.

## Chemistry

The synthesis of the target compounds was completed in a straightforward manner.[12] Treatment of chlorpromazine with methyl iodide gave the known[13] trimethylammonium iodide (**2**). 2-Chlorophenothiazine (**5**, Scheme I) was sequentially reacted with NaH and 1-chloro-3-iodopropane. After flash column chromatography to remove the elimination side product, the chloride **6** was isolated in 71% yield. This was then reacted with methanethiol in basic solution to afford the methyl sulfide analogue **4**. Finally, treatment with methyl iodide gave the dimethylsulfonium iodide **3**. While this last reaction appears trivial, it initially presented us with some difficulty. Formation of methyl iodide salts is usually quick and efficient; however, the reaction of methyl sulfide **4** with methyl iodide proceeded very slowly. We are only able to obtain a 58% yield after 7 days. Further, the dimethylsulfonium iodide **3** is unstable at room temperature. Within 48 h at room temperature, the dimethylsulfonium iodide **3** completely reverted back to the methyl sulfide **4** and methyl iodide. The compound is, however, stable at 0 °C. NMR studies showed that, within 24 h, a solution of **3** in $CDCl_3$ also decomposed to **4** and methyl iodide. Fortunately, however, NMR studies also showed that **3** is stable in aqueous solution; therefore, decomposition was not a problem during testing of the compound. The charged compound, although freely soluble in $CHCl_3$, must be destabilized by the nonpolar environment and revert back to the neutral compounds **4** and methyl iodide. In contrast, the polar aqueous environment provides stabilization for the salt and is a more suitable media for the compound.

**Scheme I**

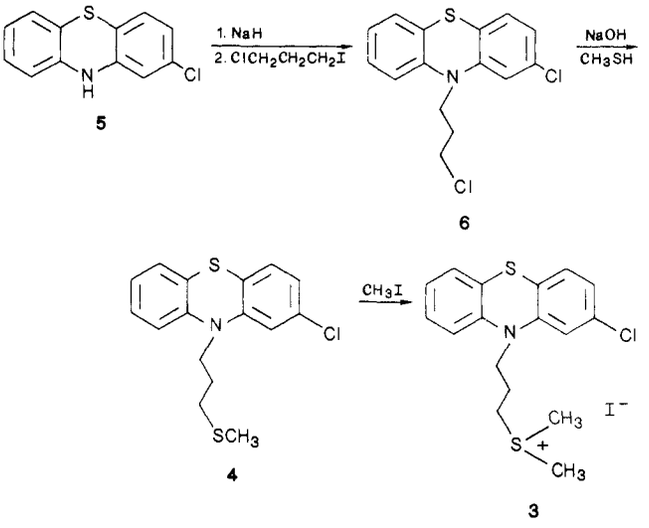


**Table I.** Binding Characteristics of Chlorpromazine and Chlorpromazine Analogues (Mean ± SEM)[a]

| | CPZ | CPZ-N$^+$ | CPZ-S$^+$ |
|---|---|---|---|
| $K_i$, nM | 1.2 ± 0.3 | 2150 ± 230 | 280 ± 18 |

[a] The order of potency is: CPZ > CPZ-S$^+$ > CPZ-N$^+$.

## Biological Results and Discussion

To ascertain whether permanently charged analogues of chlorpromazine interact with the D-2 dopamine receptor, we determined the abilities of these compounds to bind to the D-2 receptor and to antagonize the effects of apomorphine (a dopaminergic agonist) at the D-2 site.

Binding to the D-2 dopamine receptor was evaluated by measuring the abilities of chlorpromazine and the permanently charged analogues of chlorpromazine to inhibit [$^3$H]spiperone binding to rat striatal membranes. In our

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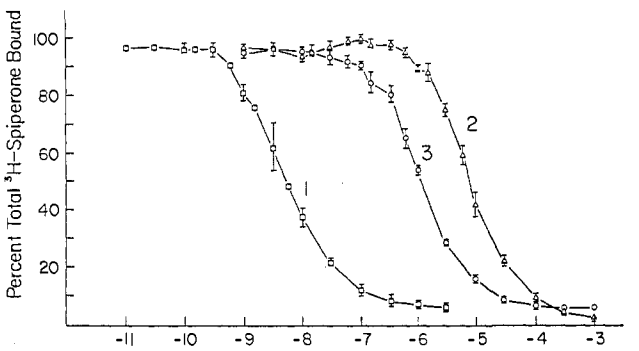


**Figure 1.** Inhibition of [³H]spiperone binding by various concentrations (M) of chlorpromazine (1), the trimethylammonium analogue of chlorpromazine (2), and the dimethylsulfonium analogue of chlorpromazine (3). Each value is the mean of at least three experiments. Vertical lines represent SEM.

system, [³H]spiperone bound to a single class of sites with an equilibrium binding dissociation constant ($K_d$) of 0.03 ± 0.008 nM and a $B_{max}$ of 25.3 ± 0.7 pmol/g of tissue, both of which agree with previously published values.[14] Specific binding of [³H]spiperone, calculated as the difference between the total binding and the binding in the presence of (+)- butaclamol (1 μM), represented 80–90% of total binding.

As expected, chlorpromazine inhibited the binding of [³H]spiperone (Figure 1), and the inhibition curve was consistent with a single class of binding sites and an apparent equilibrium binding dissociation constant ($K_i$) of 1.2 ± 0.3 nM (Table I). The permanently charged analogues of chlorpromazine (2, 3) were also found to bind to the D-2 dopaminergic site as shown by their abilities to maximally inhibit [³H]spiperone binding to the same extent as was observed with chlorpromazine (Figure 1). The $K_i$'s for the permanently charged trimethylammonium and dimethylsulfonium analogues were 2150 ± 230 and 280 ± 18 nM, respectively (Table I). The lower affinities of the permanently charged analogues may be due to their lack of a hydrogen atom at the ammonium center. Thus, these analogues are only capable of ionic binding, but not of reinforced ionic binding involving a hydrogen bond.

In addition to determining whether the permanently charged chlorpromazine analogues bind to the D-2 dopamine receptor, we also determined whether these compounds were able to act as antagonists at the D-2 dopaminergic receptor. This was done by evaluating the abilities of chlorpromazine and the permanently charged chlorpromazine analogues to antagonize the apomorphine-induced inhibition of the potassium-evoked release of [³H]acetylcholine from striatal slices (another D-2 dopamine receptor system).

Chlorpromazine was found to antagonize the ability of apomorphine to decrease the potassium-evoked release of [³H]acetylcholine as shown by the shift of the apomorphine concentration response curve to the right (Figure 2). The antagonist equilibrium binding dissociation constant ($K_B$) for chlorpromazine was 72 nM. The permanently charged analogues of chlorpromazine were also able to antagonize the effect of apomorphine at the striatal D-2 receptor regulating the release of [³H]acetylcholine. Similar to chlorpromazine, the trimethylammonium and dimethylsulfonium analogues of chlorpromazine shifted the apomorphine concentration–response curve to the right. The $K_B$'s for antagonizing the apomorphine-induced inhibition

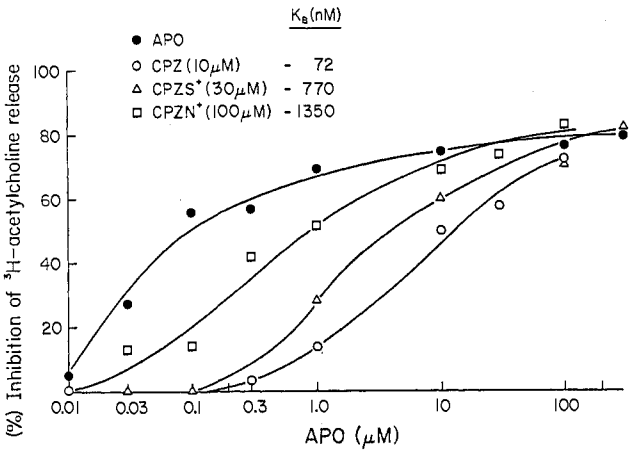


**Figure 2.** Antagonism by chlorpromazine and chlorpromazine analogues of the apomorphine-induced inhibition of the evoked release of [³H]acetylcholine. Each value is the mean of at least three experiments.

of [³H]acetylcholine release were 1350 nM (trimethylammonium analogue) and 770 nM (dimethylsulfonium analogue). The order of potencies for inhibiting [³H]-spiperone binding ($K_i$'s) and for antagonizing apomorphine-induced inhibition of [³H]acetylcholine release ($K_B$'s) were the same, chlorpromazine (1) > dimethylsulfonium analogue (3) > trimethylammonium analogue (2), arguing that both systems are measures of interaction at the same receptor, the D-2 dopaminergic site. The fact that the binding affinities are reduced by factors of 1800 (2) and 230 (3) while the potencies on acetylcholine release are reduced by much lower factors of 19 and 11, respectively, may be due to the more complex nature of the functional release system.

These studies show that a nitrogen atom on the side chain of chlorpromazine is not required for dopamine antagonist activity. In addition, a permanently charged dopamine antagonist can bind to the dopamine receptor, and the antagonist activity is retained when the side chain of chlorpromazine contains a permanent positive charge. These last two findings support the hypothesis that chlorpromazine and other dopamine antagonists, such as butaclamol, interact with their receptor via an ionic interaction involving a protonated amine species.

From the previous studies involving permanently charged and uncharged sulfur and selenium analogues of dopamine,[8] it seems reasonable that the charged protonated form of chlorpromazine should be important in receptor interactions if it is binding to the same receptor as dopamine. If, instead, it is the uncharged form that is important for receptor interactions, then this would suggest that chlorpromazine and other dopamine antagonists bind to a different recognition site than dopamine. The ability of these permanently charged molecules to interact with the D-2 dopamine receptor supports the concept that both agonist and antagonist compounds have a common site of action.

Although these studies do not show that uncharged analogues cannot interact with the dopamine D-2 receptor, the essential question is which molecular species, charged or uncharged, is most important for the binding of dopamine antagonists. In contrast to our work with dopamine agonists,[8] these studies did not allow us to compare the relative activities of a permanently charged and permanently uncharged dopamine antagonist due to the insolubility of the methyl sulfide analogue (4) in aqueous media. To overcome this problem, we are currently focusing our attention on the synthesis and activity of permanently

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charged and uncharged analogues of more aqueous soluble dopamine antagonists.

## Experimental Section

Melting points (uncorrected) were determined on a Thomas-Hoover melting point apparatus. IR spectral data were obtained with a Beckman 4230 infrared spectrophotometer, and NMR spectral data were obtained with a Bruker HX-90E NMR spectrophotometer (90 MHz) in the pulse mode. Chemical shifts are expressed in parts per million (ppm) on the $\delta$ scale relative to $Me_4Si$. Mass spectra were obtained at the Ohio State University Chemical Instrumentation Center, by use of a Kratos MS-30 mass spectrometer. The spectroscopic data for all new compounds were consistent with the assigned structures. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and were within ±0.4% of the theoretical values for the elements indicated. [$^3$H]Spiperone (23.2 Ci/mmol) and [$^3$H]choline (80.0 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

**2-Chloro-10-(3-chloropropyl)phenothiazine (6).** Commercially available 2-chlorophenothiazine (6.8 g, 29.1 mmol) was dissolved in 10 mL of dry THF with the aid of heat. This solution was added to a suspension of NaH (1.15 g, 29.1 mmol) in 50 mL of dry $Me_2SO$ and 25 mL of dry THF. The mixture was stirred under argon at 0 °C for 30 min and then added to a solution of 1-chloro-3-iodopropane (11.9 mL, 30.6 mmol) in 10 mL of $Me_2SO$. The reaction mixture was stirred at room temperature under argon for 4 h. The reaction mixture was then poured into 50 mL of ice water and was extracted with $CH_2Cl_2$ (3 × 75 mL). The extracts were pooled, washed with water (3 × 75 mL), dried ($MgSO_4$), and concentrated to a yellowish oil. This oil was purified by flash chromatography (10:1 hexane/$CH_2Cl_2$) to provide 6.4 g (70.8%) of **6** as a yellowish oil. IR (neat): 1590 cm$^{-1}$ (aromatic). NMR ($CDCl_3$): $\delta$ 2.23 (m, 2 H, $NCH_2CH_2CH_2Cl$), 3.67 (t, $J$ = 6.04 Hz, 2 H, $CH_2Cl$), 4.06 (t, $J$ = 6.36 Hz, 2 H, $NCH_2$), 6.86–7.30 (m, 7 H, Ar H). Mass spectrum, $m/e$ 309 (2 × $^{35}$Cl), 3.11 ($^{35}$Cl, $^{37}$Cl), 313 (2 × $^{37}$Cl). Anal. ($C_{15}H_{13}Cl_2NS$), C, H, N, Cl, S.

**2-Chloro-10-[3-(methylthio)propyl]phenothiazine (4).** 2-Chloro-10-(3-chloropropyl)phenothiazine (3.00 g, 9.67 mmol) was dissolved in 50 mL of ether and then added to a solution of $CH_3SH$/KOH (7 mL of $CH_3SH$, 4.2 g of KOH) in 150 mL of MeOH which had been cooled to 0 °C. The reaction mixture was stirred for 30 min at 0 °C and 24 h at room temperature. Water (100 mL) was then added to the reaction mixture, and the solution was extracted with $CH_2Cl_2$ (3 × 100 mL), dried ($MgSO_4$), and evaporated to afford a yellow oil. During evaporation, a Chlorox trap was used between the aspirator and the rotavapor to prevent mercaptan escape. The resulting oil was purified by flash chromatography (15% $CH_2Cl_2$/petroleum ether) to give 2.64 g (84.8%) of **4** as a yellow oil. IR (neat): 1595 cm$^{-1}$ (aromatic). NMR ($CDCl_3$): $\delta$ 1.92–2.21 (m, 2 H, $NCH_2CH_2CH_2S$), 2.06 (s, 3 H, $SCH_3$), 2.62 (t, $J$ = 6.68 Hz, 2 H, $CH_2S$), 3.98 (t, $J$ = 6.68 Hz, 2 H, $NCH_2$), 6.78–7.19 (m, 7 H, Ar H). Mass spectrum, $m/e$ 321 ($^{35}$Cl), 323 ($^{37}$Cl). Anal. ($C_{16}H_{16}ClNS_2$) C, H, N, Cl, S.

**3-[(2-Chlorophenothiazin-10-yl)propyl]dimethylsulfonium Iodide (3).** The methyl sulfide **4** (100 mg, 0.311 mmol) was dissolved in 2 mL of freshly distilled $CH_3I$ and cooled to −20 °C. The reaction mixture was placed under an argon atmosphere, the flask was sealed, and the reaction was kept at −20 °C for 7 days. The excess $CH_3I$ was evaporated, leaving a yellow oil, which was recrystallized from $CH_2Cl_2$/ether to give 84 mg (58%) of **3** as light yellow needles. Interestingly, the product crystallized with 0.5 mol of $CH_2Cl_2$ which could be quantitated by NMR and verified by analysis. NMR ($CDCl_3$): $\delta$ 2.29–2.55 (m, 2 H, $NCH_2CH_2CH_2S$), 3.14 (s, 6 H, $S(CH_3)_2$), 3.87 (t, $J$ = 6.99 Hz, 2 H, $CH_2S$), 4.16 (t, $J$ = 5.72 Hz, 2 H, $NCH_2$), 5.30 (s, 1 H, $CH_2Cl_2$, 50 mol %), 6.92–7.36 (m, 7 H, Ar H). Mass spectrum, $m/e$ 321 (M − $CH_3I$, $^{35}$Cl), 323 (M − $CH_3I$, $^{37}$Cl). Anal. ($C_{17}H_{19}ClINS_2 \cdot {}^1/_2CH_2Cl_2$) C, H, N, S.

**Preparation of Striatal Homogenates.** Male Sprague–Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), 300–400 g, were killed by decapitation. The brains were removed, and the striata were dissected, weighed, and placed in 50 volumes of ice-cold buffer (50 mM Tris-base, 2 mM $MgSO_4$, pH 7.7 @ 25 °C). The striatal tissue was homogenized (nine complete strokes) with a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. After homogenization, the tissue suspension was centrifuged for 10 min at 48000$g$. The supernatant was discarded and the pellet was resuspended in 50 volumes of buffer (same as above) and centrifuged again for 10 min at 48000$g$. The pellet was then resuspended in 200 volumes of ice-cold assay buffer (50 mM Tris-base, 1 mM $MgSO_4$, 125 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1 mM ascorbic acid, 0.1 $\mu$M cinanserin, 10 $\mu$M pargyline, pH 7.7 @ 25 °C), resulting in a final concentration of 5 mg of original tissue wet weight/mL of buffer. The tissue homogenate was stored on ice until addition to the incubation tubes.

**[$^3$H]Spiperone Binding Assays.** The buffer used in the assays was the same as the buffer in which the tissue was finally suspended as described above. Binding assays were done in duplicate in disposable glass test tubes (16 × 125 mm). For saturation assays, the tubes received in order: [$^3$H]spiperone (diluted and added in such a volume as to give a final concentration of 0.1–1 nM); 50 $\mu$L of (+)-butaclamol (to give a final concentration of 1 $\mu$M) added to some samples to determine nonspecific binding; assay buffer (sufficient to bring the total assay volume to 5 mL) and 1.0 mL of striatal homogenate (final concentration of 1 mg of original wet tissue weight/mL). Specific binding of [$^3$H]spiperone was defined as the difference between total [$^3$H]spiperone bound and [$^3$H]spiperone bound in the presence of 1 $\mu$M (+)-butaclamol. For competition assays, the tubes received in order: [$^3$H]spiperone (added to give a final concentration of 0.1 nM); varying concentrations of cold competitor; assay buffer sufficient to yield a final assay volume of 5 mL and 1 mL of striatal homogenate (with a final concentration of 1 mg of original tissue wet weight/mL). Cinanserin (0.1 $\mu$M) was included in the assay buffer to eliminate the serotonergic component of [$^3$H]spiperone binding. This concentration (0.1 $\mu$M) of cinanserin has been previously reported to saturate S-2 serotonergic sites without affecting [$^3$H]spiperone binding to D-2 dopaminergic sites.[14]

All assays were carried out at room temperature (23–25 °C).[14,15] The tubes were incubated for 100 min, a time at which equilibrium had been established. The reaction was terminated by separation of the free radioligand from bound by rapid vacuum filtration (Whatman B glass fiber filters) with a 12-well cell harvester (Brandel, Gaithersburg, MD). The filters were washed with 20 mL (4 × 5 mL washes) of assay buffer at room temperature; the duration of the washing was approximately 30 s. The filters were then transferred to liquid scintillation vials (20 mL) and 10 mL of scintillation cocktail (Formula 963, New England Nuclear, Boston, MA) was immediately added. The vials were then shaken for 30 min in a mechanical shaker after which time the bound radioactivity was counted in a Beckman LS 6800 liquid scintillation counter at 40% efficiency.

**Measurement of the K$^+$-Induced Release of [$^3$H]Acetylcholine from Striatal Slices.** Male Swiss–Webster mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with reserpine (5 mg/kg) and $\alpha$-methyl-$p$-tyrosine 20 h and 2 h, respectively, before decapitation. The brains were removed, and the striatal tissue rostral to the anterior commissures was dissected.[16] The tissue was cut into 0.6 mm × 0.6 mm with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY) and dispersed into a Krebs–Ringer bicarbonate medium. The medium contained (mM): NaCl, 118; KCl, 4.8; $CaCl_2$ 1.3; $MgSO_4$, 1.2; $NaHCO_3$, 25; $KH_2PO_4$, 1.2; ascorbic acid, 0.6; $Na_2EDTA$, 0.03; and glucose, 11. The medium was stored on ice, bubbled with a 95% $O_2$/5% $CO_2$ mixture and adjusted to a pH of 7.2 with NaOH. The slices were incubated for 20 min with [$^3$H]choline at a final concentration of 0.1 $\mu$M, a low concentration that favors the selective uptake of choline into cholinergic neurons through a high-affinity uptake system. After incubation, the slices were rinsed with cold medium and transferred to a plastic tube with nylon mesh (size) attached to one end. This tube was placed into a water jacketed tissue chamber maintained at 37 °C, and the slices were superfused with normal medium at a constant rate of 0.5 mL/min for 45 min. The superfusion medium and the subsequent incubation medium contained hemicholinium (10 $\mu$M) and $\alpha$-methyl-$p$-tyrosine (250

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μM). At the end of the superfusion, the tube containing the slices was removed from the tissue chamber, and two slices were placed in each of 12 tubes with nylon-mesh attachments. The slices in each tube were then transferred at 5-min intervals into six different 10-mL beakers, which contained 3 mL of fresh medium at 37 °C. The first four beakers contained normal medium, while the fifth and sixth beakers contained medium in which the concentration of $K^+$ was increased to 13.8 mM (the concentration of sodium was reduced to maintain isotonicity). The dopaminergic agonists, when present, were added to the high-$K^+$ medium. After the last incubation, the tubes containing the slices were removed from the beaker, and the slices were homogenized in 0.4 N perchloric acid. The radioactivity in the medium that remained in the beakers and the perchloric acid extracts was determined by liquid scintillation counting. The tritium that was released into the medium by the high $K^+$ was not further characterized. Under the conditions of the present study, the $K^+$-induced release of tritium was completely dependent on the presence of calcium ions in the medium.

The amount of tritium released from the tissue into the medium in each 5-min incubation period is expressed as a percentage of the total tritium content of the tissue at the start of the incubation period (fractional release × 100). This was calculated by correcting the tissue content of tritium for the tritium released into the medium. The $K^+$-evoked increase in tritium release is the mean percentage release of tritium obtained when the slices were incubated in the beakers with high $K^+$ medium above the base line of spontaneous release. The latter is the percentage fractional release in the incubation of slices in normal medium preceding their incubation in high $K^+$ medium.

**Analysis of Data.** All binding data were analyzed using an iterative nonlinear least-squares curve-fitting program. For [$^3$H]spiperone saturation studies, the data were fitted to a model assuming either one ligand and one binding site or one ligand and two binding sites. The equilibrium dissociation constant for [$^3$H]spiperone was derived from the analysis of the [$^3$H]spiperone saturation studies and was used in the subsequent analysis of the [$^3$H]spiperone competition studies. For the [$^3$H]spiperone competition studies, the data were fitted to a model assuming either two ligands and one binding site or two ligands and two binding sites. From these analyses, the apparent equilibrium dissociation constants of the competing drugs were determined.

To determine whether a one-site or two-site model more appropriately described the data, the generalized form of the logistic function was initially fitted to the binding data.[17] This analysis yields a slope factor that describes the steepness of the curve and represents the slope of the logit–log plot when the concentration of the cold competing drug is expressed in terms of natural logarithms. When the slope factor equals one, the logistic equation becomes identical with the law of mass action equation which describes the interaction of one binding site with one ligand (saturation experiments) or with two competing ligands (competition experiments). Therefore, binding curves with slope factors equal to one were assumed to represent the case in which ligands interact with one class of binding sites. Binding curves with slope factors significantly less than one as determined by Student's $t$ test were considered justification to further analyze the data by using the model that describes interactions with two classes of binding sites. A partial $F$ statistic, used to determine whether the two-site model fit the data better than the one-site model, was calculated from the following equation:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}$$

where $SS_1$ and $df_1$ and $SS_2$ and $df_2$ represent the residual sum of squares and degrees of freedom associated with the one-site and two-site models, respectively.[18] Only when the two-site binding model resulted in a significant reduction in the residual sum of squares, as determined by the partial $F$ test, was the binding data considered to represent the binding of the ligand to two classes of receptors.

ANOVA and Duncan's multiple-range test were used to determine whether significant differences existed among the binding dissociation constants for the compounds tested. Before statistical analyses, the data were transformed by converting the binding dissociation constants to negative logarithms in order to obtain data that are normally distributed.

Apomorphine concentration–response curves for inhibiting the potassium-evoked release of [$^3$H]acetylcholine were generated in the presence and absence of a given concentration of antagonist. The antagonist equilibrium dissociation constants ($K_B$'s) were then calculated from the equation:

$$K_B = [B]/(DR - 1)$$

where $B$ is the concentration of antagonist used and DR is the ratio of the concentrations of apomorphine required to produce a half-maximal inhibitory effect on [$^3$H]acetylcholine release in the presence and absence of antagonist.[19]

The level of significance employed for all statistical tests was $P < 0.05$.

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