0026-895X/91/020222-11$03.00/0

MOLECULAR PHARMACOLOGY, 39:222-232

Labeling by [^{3}H]1,3-Di(2-tolyl)guanidine of Two High Affinity Binding Sites in Guinea Pig Brain: Evidence for Allosteric Regulation by Calcium Channel Antagonists and Pseudoallosteric Modulation by σ Ligands

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Received April 25, 1990; Accepted November 26, 1990

SUMMARY

Equilibrium binding studies with the σ receptor ligand [^{3}H]1,3-di(2-tolyl)guanidine ([^{3}H]DTG) demonstrated two high affinity binding sites in membranes prepared from guinea pig brain. The apparent K_d values of DTG for sites 1 and 2 were 11.9 and 37.6 nM, respectively. The corresponding B_{max} values were 1045 and 1423 fmol/mg of protein. Site 1 had high affinity for (+)-pentazocine, haloperidol, (*R*)-(+)-PPP, carbepentane, and other σ ligands, suggesting a similarity with the dextromethorphan/σ_1 binding site described by Musacchio *et al.* [*Life Sci.* **45**:1721–1732 (1989)]. Site 2 had high affinity for DTG and haloperidol (K_i = 36.1 nM) and low affinity for most other σ ligands. Kinetic experiments demonstrated that [^{3}H]DTG dissociated in a biphasic manner from both site 1 and site 2. DTG and haloperidol increased the dissociation rate of [^{3}H]DTG from site 1 and site 2, demonstrating the presence of pseudoallosteric interactions. Inorganic calcium channel blockers such as Cd^{2+} selectively increased the dissociation rate of [^{3}H]DTG from site 2, suggesting an association of this binding site with calcium channels.

Since the original proposition by Martin *et al.* (1) that the σ receptor is the type of opiate receptor that mediates the naloxone-reversible psychotomimetic actions of nalorphine-like agonists in humans, and delirium in dogs, the exact definition of this receptor has changed. The observations that certain opiate drugs that have high affinity for [^{3}H]PCP binding sites (2, 3) also produce PCP-like psychotomimetic effects in humans (4, 5) led to the concept of a common σ/PCP receptor (3, 6). Subsequent studies demonstrated that binding sites labeled with ligands such as [^{3}H]SKF10,047 (7, 8), [^{3}H]PPP (9), [^{3}H]DTG (10), or (+)-[^{3}H]pentazocine (11) are distinct from PCP binding sites. Currently, the σ receptor is defined as a nonopioid, nondopaminergic binding site exhibiting high affinity for haloperidol, DTG, (+)-PPP, and (+)-SKF10,047 (12). Although the functional roles of the σ receptor are not fully understood, several studies suggest a role in the regulation of motor behavior (13–16), modulation of smooth muscle contraction (17–19), enhancement of norepinephrine release (17), negative modulation of phosphoinositide metabolism (20, 21), and inhibition of neuronal firing rates (22, 23).

Previous studies of σ receptor binding reported displacement curves characterized by low Hill coefficients (9), which is evidence for the existence of multiple binding sites. Using quantitative ligand binding methods, we reported that [^{3}H]DTG labeled two binding sites in guinea pig brain (24). In the present study, we confirm these observations and provide additional evidence for pseudoallosteric modulation of these binding sites by σ ligands.

Materials and Methods

Preparation of Membranes

Forty frozen guinea pig brains, including cerebella (Pel Freeze), were homogenized with a Polytron in ice-cold 5 mM Tris·HCl, pH 8.2 (buffer), using 10 ml of buffer/brain. The homogenate was centrifuged at 39,000 × *g* for 10 min. The pellets were resuspended in the same volume of ice-cold buffer and recentrifuged. The pellets were subsequently washed four times by centrifugation, as described above, with 15-min incubations at 4° before each centrifugation. One-milliliter aliquots of the final homogenate were distributed to 1.5-ml microfuge tubes. After centrifugation at top speed for 5 min in a Beckman

ABBREVIATIONS: PCP, phencyclidine; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine; DTG, 1,3-di(2-tolyl)guanidine; *R*-(-)PPP, *R*-(-)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; HEPPSO, *N*-2-hydroxyethylpiperazine-*N*′-2-hydroxypropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BD445, (1*S*,2*R*)-(−)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; BD446, (1*R*,2*S*)-(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; BD737, (1*S*,2*R*)-(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; BD738, (1*R*,2*S*)-*cis*-(+)-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-[(1-pyrrolidnyl)]cyclohexylamine; SS, sum of squares.

microcentrifuge, the supernatants were aspirated and the pellets were stored at −70°.

Ligand Binding Assay

Assays were conducted as previously described (25). Briefly, to 12- × 75-mm polystyrene test tubes were added drugs or inorganic salts in 0.1 ml of buffer (5 mM HEPPSO, pH 8.1), [^{3}H]DTG in 0.1 ml of a protease inhibitor cocktail containing 100 μM EDTA, 100 μM EGTA, 25 μg/ml chymostatin, and 25 μg/ml leupeptin, and 0.8 ml of membranes (about 1 mg of protein) in buffer. In experiments using greater than 5 nM [^{3}H]DTG, [^{3}H]DTG (5 nM) was mixed with nonradioactive DTG to achieve the required concentration. Nonspecific binding was determined using either 50 μM haloperidol or 10 μM DTG, with identical results. After a 3–4-hr incubation at 25° (equilibrium), samples were filtered (using a Brandell cell harvester) over Whatman GF/B filters (that had been presoaked in 2% polyethylenimine) and washed with two 5-ml aliquots of ice-cold buffer. The tritium retained on the filter was determined by liquid scintillation counting, using Beckman Redi Solv scintillation fluid. Each point was determined in triplicate, with less than 10% variation. Typical total and nonspecific cpm values observed for 5 nM [^{3}H]DTG were 16,234 ± 410 and 2,310 ± 143, respectively (mean ± standard deviation, three determinations).

Experiments designed to examine the dissociation of [^{3}H]DTG from its binding sites used a dilution method. At equilibrium, three incubation conditions (see below) were terminated by centrifugation for 15 min at 27,578 × *g*. Pellets were resuspended in ice-cold buffer, and triplicate samples were filtered at various time points. The three conditions (each in triplicate) used were 1) total binding, 2) binding in the presence of 10 μM dextromethorphan, and 3) nonspecific binding. Because equilibrium binding studies demonstrated that 10 μM dextromethorphan blocked [^{3}H]DTG binding to site 1 (see Results), dissociation of [^{3}H]DTG from site 1 was calculated as condition 1 − condition 2. Dissociation of [^{3}H]DTG from site 2 was calculated as condition 2 − condition 3. These calculations were performed after the binding in each condition was corrected for the protein concentration, as determined by the method of Lowry *et al.* (26).

Experimental Design and Data Analysis

A major goal of equilibrium binding studies is to accurately determine the values of the binding parameters that describe the system. This can be difficult when the ^{3}H-ligand binds to more than one binding site. The method used in our laboratory to achieve this goal is described below.

Step 1. Drug screen. A single concentration of ^{3}H-ligand is displaced by test drugs over a broad concentration range, typically 1 nM to 10 μM. The displacement curves are fit to the two-parameter logistic equation (27) for the IC_{50} values and the slope factor, which is similar to a Hill coefficient. These parameter values are used to generate predicted displacement curves. Inspection of these curves facilitates selection of the concentration ranges over which the test drugs produce from 10% to 90% inhibition. These experiments are usually done once.

Step 2. Generation of the "primary data set." In stage 1 of this step, "binding surfaces" are generated by displacement of two concentrations of ^{3}H-ligand by the nonradioactive ligand and by various concentrations of two or three selected test drugs, which have low slope factors. Agents with low slope factors are used because they have the highest probability of distinguishing among multiple binding sites. A description of binding surface analysis appears in the next section.

The DTG versus [^{3}H]DTG data provide saturation binding information. These experiments are done twice. These data are then fit, using MLAB (28), to one-site, two-site, and three-site binding models for the best-fit parameter estimates of each model and for determination of the best-fitting model. MLAB utilizes a weighted nonlinear least squares curve-fitting algorithm to arrive at the best-fit parameter estimates.

Statistical analysis of the goodness of fit is determined using the SS. The *F* test is applied according to the following equation (29)

$$F = \frac{[SS_1 - SS_2]}{[df_1 - df_2]} \frac{SS_2}{df_2}$$

where df_1 and df_2 refer to the degrees of freedom. The *F* test provides a means of statistically testing several different hypotheses, including which binding models best fit the data, as well as whether parameter values resulting from the fitting of control and experimental groups are different to a statistically significant degree. The two-site model is selected over a one-site model if a statistically significant decrease (*F* test) in the SS is observed (30). Similarly, a three-site model is selected if fitting the data to it results in a statistically significant decrease in the SS. However, if consideration of a three-site model does not produce a statistically significant decrease in the SS, this does not mean that there are not three binding sites, only that a third site cannot be resolved. For the purpose of this discussion, we then assume that a two-site model fits the data best. The best-fit parameter estimates are used to generate predicted curves for the "cross-competition" experiments, which follow. In these experiments (stage 2), two concentrations of ^{3}H-ligand are each displaced by various concentrations of the nonradioactive ligand and test drugs, in the presence of a concentration of one or more of the test drugs sufficient to partially block the binding of the ^{3}H-ligand to one of the binding sites [i.e. (+)-pentazocine and (*S*)-(−)-PPP]. These experiments are also done twice. The data of stage 1 and stage 2 comprise the "primary data set." The combined data set is then fit to one-, two-, and three-site binding models to determine which model fits the data best. The best-fit parameter estimates are then reported in a table. In the context of the present study, the primary data set was well described by a two-site binding model.

Step 3. Structure-activity study. The best-fit parameter estimates generally permit selection of a "blocking agent" to prevent, as completely and selectively as possible, the binding of the ^{3}H-ligand to one of the two binding sites. Binding surfaces are then generated for each test drug by displacement of one or two concentrations of ^{3}H-ligand by the test agent in the absence and presence of the blocking agent. Typically, the "nonblocked" and "blocked" displacement curves are generated on different days. The data are combined, and the surfaces are individually fit to the two-site binding model for the best-fit parameter estimates of the test drug, with the B_{max} values, the K_d values of the ^{3}H-ligand and the K_i values of the blocking agents fixed to the values determined in step 2. In the event that a drug is identified that can block the binding site not blocked by the first blocking agent, additional binding surfaces are generated for each test agent, using this drug as a second blocking agent. The binding surfaces for each drug are then combined and refit to the two-site model for the best-fit parameter estimates, as described above. Thus, a binding surface for a typical test drug might consist of displacement curves performed in the absence and presence of one or two blocking agents, with the data being generated in two or three separate experiments.

Step 4. Prediction experiments. Steps 1 through 3 are ideally done with the same preparation of membranes. To ensure that the results are not unique to that particular membrane preparation and to provide a quality control step, prediction experiments are conducted. For these experiments, three separate membrane preparations are made, and the inhibition of ^{3}H-ligand binding by one or two concentrations of each test drug is determined. These "observed" values are then compared with the inhibition predicted on the basis of the best-fit parameter estimates determined in step 3. The predicted result, because it is generated by an equation using the best-fit parameter estimates, does not have a corresponding standard deviation. However, each of the parameters used in the equation does, and it is often greater than 10% of the parameter value. Thus, calculation of the predicted result with appropriate propagation of the error calculations could lead to predicted standard deviation values greater than 10% of the predicted parameter value. The standard deviation of the predicted result is,

therefore, assigned a value equal to 10% of the predicted result, which is a conservative estimate. In most cases, there is good agreement between the observed and predicted values. When discrepancies are noted, the prediction experiment is rerun with the discrepant drugs, to rule out a laboratory error in the first prediction experiment. If there was no laboratory error, new binding surfaces for each discrepant drug are generated and fit to the two-site binding model for the best-fit parameter estimates. The new parameter values are then used to generate a new predicted inhibition, which in our experience is typically much closer to the observed inhibition than the first set of parameter values. We have found that prediction experiments provide for excellent quality control.

The approach used by our laboratory to define and study systems in which a ligand labels more than one binding site is potentially open to criticism as being wasteful of resources. We feel otherwise. The primary data set (step 2) provides an accurate description of the system. This is necessary to select a correct concentration of blocking agents to be used in the structure-activity study. In this study (step 3), test drugs displace ^{3}H-ligand in the absence and presence of blocking agents. A binding surface for a test agent might, therefore, be composed of between three and six displacement curves, depending on the experimental design. Investigators using more traditional approaches would repeat a single displacement curve three times and consume approximately the same amount of resources in the process.

Binding Surface Analysis

The method of binding surface analysis has been described in detail in other publications (31). Briefly, when considering the binding of a ^{3}H-ligand, L, in the presence of an inhibitor, I, the observed binding is a function of both L and I. This is mathematically described as $B = F([L],[I])$, where F is the function that relates the concentration of L and I to the observed level of binding, B. For example, if there is one binding site, then F is the equation that describes the one-site binding model, and there are three binding parameters, B_{max}, K_d, and K_i. Graphing of the equation $B = F([L],[I])$ requires three axes, one for the concentration of L, one for the concentration of I, and the third for the observed level of binding, B. Clearly, the function F describes a "surface" in three-dimensional space. Quantitative analysis of such functions is a process that has been termed binding surface analysis (31).

A major goal of ligand binding studies is to determine the functional form of F and what values of the binding parameters best fit the data. A relatively unrecognized fact is that commonly accepted methods of experimental design, such as Scatchard analysis, result in multiple sets of parameter estimates that give rise to almost equally good fits to the data, reflected by large parameter standard deviations. For examples of this, see Table 1 in the paper by Rothman (31) and Table 2 in the paper by Munson and Rodbard (29). The problem posed by this is determining which, among the various sets of parameter estimates that fit the data equally well, are the "true" or "correct" parameter estimates. The method of binding surface analysis is a tool for reducing the standard deviations associated with estimates of binding parameters, i.e., K_d values and B_{max} values. Briefly, this method requires the simultaneous analysis of multiple ligand displacement curves using two or more concentrations of radiolabeled ligand. The utility and wide applicability of the method is illustrated by its use in laboratories that specialize in quantitative ligand binding studies (32, 33).

Statistics

Statistical significance between control and experimental groups was determined by one-way analysis of variance, using the *post hoc* Scheffe F test. A difference was considered significant if $p < 0.05$.

Chemicals

[^{3}H]DTG (specific activity, 52 Ci/mmol) was purchased from New England Nuclear Corp. Protease inhibitors, Polyethylenimine, EDTA, EGTA, haloperidol, and HEPPSO buffer were purchased from Sigma Chemical Company. DTG was purchased from Aldrich Chemical Company. σ ligands structurally related to the κ-selective agonist U50,488, namely BD445 and BD446, were synthesized as described previously (25). The synthesis of BD737 and BD738 has also been described elsewhere (34). KCR-12-83.1, KCR-12-84.1, and KCR-12-69.1 were synthesized from intermediates available by the National Institutes of Health Opiate Total Synthesis (35). KCR-11-240.1 and KCR-11-239.1 were synthesized by minor modifications of previously described methods (36–38). The structures of BD445, BD446, BD737, BD738, KCR-11-240.1, KCR-12-83.1, KCR-12-84.1, KCR-11-239.1, and KCR-12-69.1 are shown in Fig. 1. Fluphenazine, perphenazine, (*R*)-(+)-PPP, (*S*)-(−)-PPP, and (−)-butaclamol were purchased from Research Biochemicals, Inc. ω-Conotoxin was purchased from Peninsula Laboratories. Buspirone and α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine butanol (BMY14802) were supplied by Bristol Meyers. Dr. Musacchio, Department of Pharmacology, New York University (New York), kindly supplied the caramiphen and carbetapentane.

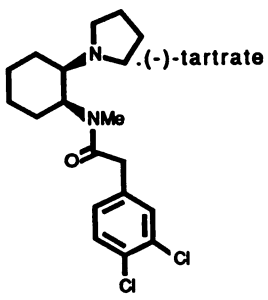
Results

Equilibrium binding studies. Binding sites labeled by [^{3}H]DTG were characterized as depicted in Fig. 2. To generate saturation binding data, two concentrations of [^{3}H]DTG were displaced by varying concentrations of DTG, in the absence or presence of blocking concentrations of (+)-pentazocine or (*S*)-(−)-PPP (Fig. 2A). These blocking concentrations were determined by preliminary analysis of the data. (+)-Pentazocine inhibition curves were also generated by displacement of two concentrations of [^{3}H]DTG by (+)-pentazocine, in the absence or presence of a blocking concentration of (*S*)-(−)-PPP. (*S*)-(−)-PPP inhibition curves were similarly generated.

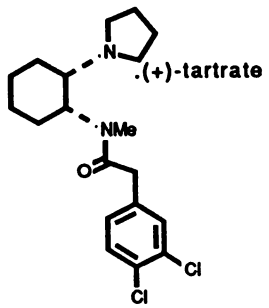
The data of Fig. 2 (140 points) were combined and fit to one-site, two-site, and three-site binding models. The two-site model fit the data best ($p < 0.001$). The best-fit parameter estimates, which were used to draw the lines in Fig. 2, are reported in Table 1. They indicate the existence of two [^{3}H]DTG binding sites, present at densities of 1044 and 1423 fmol/mg of protein. DTG had high affinity for both binding sites, with apparent K_d values of 11.9 and 37.6 nM for sites 1 and 2, respectively. (+)-Pentazocine was highly selective for site 1, having apparent K_i values of 1.97 and 456 nM for sites 1 and 2; (*S*)-(−)-PPP was also selective for site 1, with apparent K_i values of 30.5 and 1544 nM for sites 1 and 2, respectively.

The apparent K_d values of DTG for the two sites are 11.9 and 37.6 nM, respectively, implying about a 3-fold selectivity for site 1. With this low degree of selectivity between the two sites, it would not be possible to detect the presence of two binding sites if the only data available were for the saturation binding of [^{3}H]DTG. A Scatchard plot would be linear, and the Hill coefficient would be 1. However, the inclusion of more selective ligands in the data set permitted resolution of two binding sites, at which DTG happens to have about the same affinity. For example, displacement of [^{3}H]DTG by dextromethorphan yields a slope factor (pseudo-Hill coefficient) of 0.44.

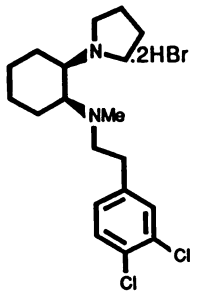
The effects of certain salts on [^{3}H]DTG binding are reported in Table 2. To permit calculation of binding to sites 1 and 2, these incubations were conducted in the absence and presence of 10 μM dextromethorphan, which other experiments demonstrated was highly selective for DTG site 1 (see next section). Therefore, the assumption inherent in these calculations is that the salts do not alter the apparent K_d values of dextromethorphan for sites 1 and 2. These data indicated that DTG binding is very sensitive to salts such as Cd^{2+}, Ln^{3+}, and Ni^{2+}, which



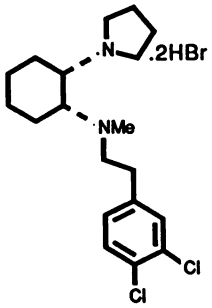
BD-446



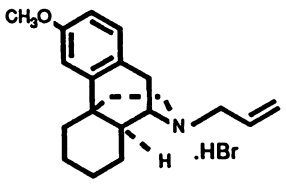
BD-445



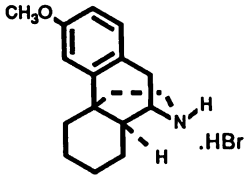
BD-737



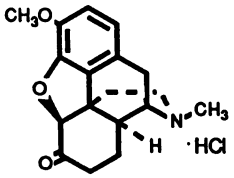
BD-738



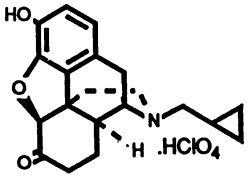
KCR-11-240.1



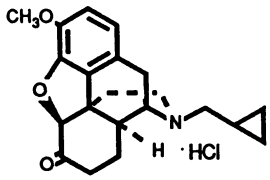
KCR-11-239.1



KCR-12-69.1



KCR-12-84.1



KCR-12-83.1

Fig. 1. Structures of selected drugs used in this study.

block calcium channels (39). None of the salts exhibited selectivity for either site 1 or 2.

Structure-activity studies. The apparent K_i values of various test drugs for DTG site 1 and site 2 were determined as described for buspirone in Fig. 3. Two concentrations of [^{3}H] DTG were displaced by the test drug, in the absence and presence of a blocking concentration of (+)-pentazocine (500 nM). These data, consisting of 24 data points, were then fit to the two-site model for the best-fit estimates of its apparent K_i, with the B_{max} values and apparent K_d values of [^{3}H]DTG and (+)-pentazocine fixed to the values reported in Table 1.

The results are presented in Table 3. DTG and buspirone were 3-fold and 8-fold selective for site 1, respectively, whereas all the other drugs were from 15- to 442-fold selective for site 1. Haloperidol was the most potent drug at both binding sites (apparent K_i values of 0.30 and 36.1 nM, respectively). The (+)-opiates, in particular dextromethorphan, were the most selective drugs for site 1. BMY14802 had moderate affinity for site 1 (apparent K_i = 41.4 nM) and low affinity for site 2 (apparent K_i = 728 nM). The *cis*-diastereomers of U50,488, namely BD445 and BD446, had moderate affinity for site 1 and very low affinity for site 2. The (*cis*)-2-(pyrrolidinyl)cyclohexylamines BD737 and BD738 were among the most potent σ ligands, having apparent K_d values of 8.0 and 6.4 nM for site 1, respectively. TCP and PCP, at concentrations of 10 μM, inhibited the binding of 5 nM [^{3}H]DTG to site 2 by 40.8% and 31.3%, respectively (data not shown).

Prediction experiments. In the prediction experiments, three separate membrane preparations were prepared. The inhibition produced by various test drugs was compared with the inhibition predicted on the basis of the binding parameters reported in Table 4. With few exceptions, there was excellent agreement between the observed and predicted data.

Kinetic studies. To further address the hypothesis of two [^{3}H]DTG binding sites, the dissociation of [^{3}H]DTG from sites 1 and 2 was determined using a dilution method. As described for Fig. 4, [^{3}H]DTG dissociated in a biphasic manner from both sites. The data were best described by a two-component dissociation model (Table 5), in which one component dissociated at an extremely slow rate. This observation suggests that one of the assumptions fundamental to the analysis of the data, i.e., the laws of mass action, might not apply to the present study.

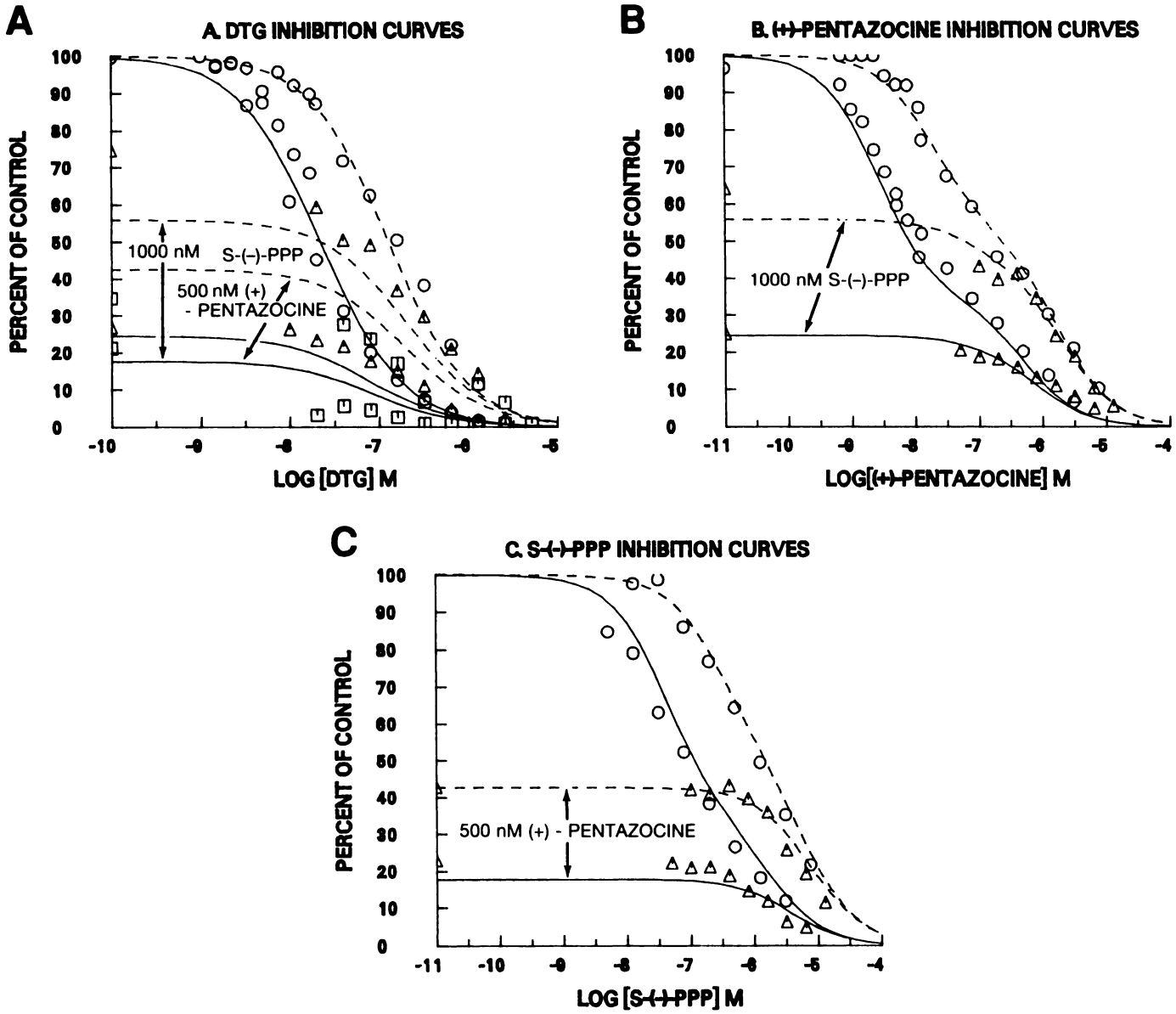


Fig. 2. [^{3}H]DTG binding data. A, Displacement of 4.09 (——) and 104.3 nM (– – –) [^{3}H]DTG by DTG in the absence (○) or presence of 500 nM (+)-pentazocine (□) or 1000 nM (*S*)-(−)-PPP (△). B, Displacement of 4.09 (——) and 104.3 nM (– – –) [^{3}H]DTG by (+)-pentazocine in the absence (○) or presence of 1000 nM (*S*)-(−)-PPP (△). C, Displacement of 4.09 (——) and 104.3 nM (– – –) [^{3}H]DTG by (*S*)-(−)-PPP in the absence (○) or presence of 500 nM (+)-pentazocine (△). Each *point* is the mean of two experiments, which differed by less than 10%. The *lines* are those predicted by the best-fit parameter estimates (Table 1). The data in A, B and C comprise 140 data points.

TABLE 1

Best-fit parameter estimates of the [^{3}H]DTG binding surfaces

The data of Fig. 2 (140 data points) were fit to a one-site binding model. The SS was 1.22. Fitting of the data to a two-site model gave a significantly better fit (SS = 0.435, r^2 = 0.96, $p < 0.001$). Fitting of the data to a three-site model did not significantly improve the goodness of fit. The best-fit estimates of the two-site model reported below generated the lines in Fig. 2. Values are mean ± standard deviation.

	Parameter value	
	Site 1	Site 2
B_{max} (fmol/mg of protein)	1045 ± 100	1423 ± 136
DTG, apparent K_d (nM)	11.9 ± 1.3	37.6 ± 7.8
(+)-Pentazocine, apparent K_i (nM)	1.97 ± 0.71	456 ± 132
(*S*)-(−)-PPP, apparent K_i (nM)	30.5 ± 7.1	1544 ± 444

To demonstrate mass action, the following experiment was performed. Two sets of total and nonspecific binding tubes were incubated to equilibrium (3 hr) with 5 nM [^{3}H]DTG. The first set of total and nonspecific points were then filtered, for determination of the specific binding at equilibrium. Unlabeled DTG was added to the second set of total and nonspecific binding tubes, to achieve a final concentration of 10 nM. These tubes were then filtered 4 hr later. If the laws of mass action did not apply, then the specific binding of [^{3}H]DTG, representing the prebound ligand, would not be altered by the addition of unlabeled DTG. If mass action did apply, then the addition of unlabeled DTG would perturb the equilibrium, and the system would reequilibrate to a lower level of specific binding. The latter occurred. The specific binding decreased from 13,104 cpm to 4,865 cpm. One likely reason for this is

TABLE 2

Effect of salts on [^{3}H]DTG binding

As described in Materials and Methods, [^{3}H]DTG incubations took place in the presence of the indicated concentrations of various salts. Specific binding to DTG site 1 was calculated as total binding minus binding in the presence of 10 μM dextromethorphan. Specific binding to DTG site 2 was calculated as binding in the presence of 10 μM dextromethorphan minus nonspecific binding. Each value is the mean ± standard error of three experiments.

Salt	Concentration	Binding	
		Site 1	Site 2
	mM	% of control	
NaCl	1.0	101.7 ± 2.5	88.2 ± 5.8
	4.0	93.3 ± 8.2	73.5 ± 1.4*
	16	79.9 ± 6.1*	64.7 ± 2.1*
	64	62.5 ± 4.4*	49.2 ± 0.7*
	250	54.2 ± 6.6*	30.9 ± 3.0*
NiCl	0.1	100.3 ± 7.8	95.8 ± 1.2
	0.3	78.9 ± 3.5*	58.6 ± 1.8*
	0.9	49.9 ± 1.5	33.6 ± 3.2*
	2.7	34.2 ± 2.1*	23.0 ± 5.3*
	8.1	20.0 ± 1.9*	22.6 ± 10.6*
$CdCl_2$	0.1	92.1 ± 13.3	68.5 ± 10.2*
	0.3	42.1 ± 5.5*	8.6 ± 0.8*
	0.9	18.5 ± 2.3*	1.0 ± 0.6*
	2.7	12.3 ± 1.9*	4.1 ± 3.0*
	8.1	9.4 ± 1.7*	2.4 ± 1.2*
$LaCl_3$	0.1	94.4 ± 7.8	68.2 ± 13.7*
	0.3	62.7 ± 9.2*	35.6 ± 8.9*
	0.9	19.2 ± 3.8*	34.9 ± 16.6*
	2.7	11.1 ± 3.6*	23.3 ± 15.9*
	8.1	6.8 ± 3.5*	23.8 ± 16.8*
$MgCl_2$	0.1	87.1 ± 14.2	77.5 ± 7.8*
	0.3	82.8 ± 6.2	60.9 ± 5.2*
	0.9	75.0 ± 5.8*	47.3 ± 7.0*
	2.7	63.5 ± 6.2*	37.9 ± 6.1*
	8.1	56.8 ± 5.8*	31.2 ± 5.7*
$ZnSO_4$	0.1	100.6 ± 13.3	66.0 ± 21.4
	0.3	54.4 ± 7.8*	37.5 ± 10.3*
	0.9	18.0 ± 1.7*	8.7 ± 1.33*
	2.7	9.7 ± 3.4	0 ± 0.1*
	8.1	5.0 ± 1.9*	1.6 ± 1.1*
$FeCl_3$	0.01	95.2 ± 7.1	89.2 ± 5.9
	0.05	70.8 ± 18.8	88.2 ± 6.1
	0.25	75.2 ± 9.1*	68.9 ± 5.9*
	1.25	1.6 ± 1.4*	1.8 ± 1.6*
	6.25	0.10 ± 0.06*	1.7 ± 0.9*
	31.25	0.13 ± 0.03*	2.1 ± 1.1*

*$p < 0.05$, compared with control (one-way analysis of variance, *post hoc* Scheffe *F* test).

that the addition of DTG speeds the dissociation of prebound [^{3}H]DTG (see Table 8), allowing for exchange of bound and unbound ligand.

Other experiments determined the effect of various agents on the dissociation of [^{3}H]DTG from sites 1 and 2. Because of the number of agents involved, binding was monitored only at the 2.0-hr time point. Thus, the effect of an agent on dissociation is expressed as a percentage of the specific binding present in controls 2 hr after initiation of dissociation by dilution. As reported in Table 6, the inorganic calcium channel blockers Mg^{2+}, Ni^{2+}, La^{3+}, and Cd^{2+} selectively accelerated the dissociation of [^{3}H]DTG from site 2, in a dose-dependent manner. Na^+, which nonselectively accelerated the dissociation of [^{3}H]DTG from both binding sites, had a greater effect on site 2 then site 1.

Several organic calcium channel modulators (bepridil, amidirone, and verapamil) accelerated the dissociation of [^{3}H]DTG (Table 7). These agents tended to have a greater effect on site 1 than site 2 at 1 μM concentrations and nonselective effects at

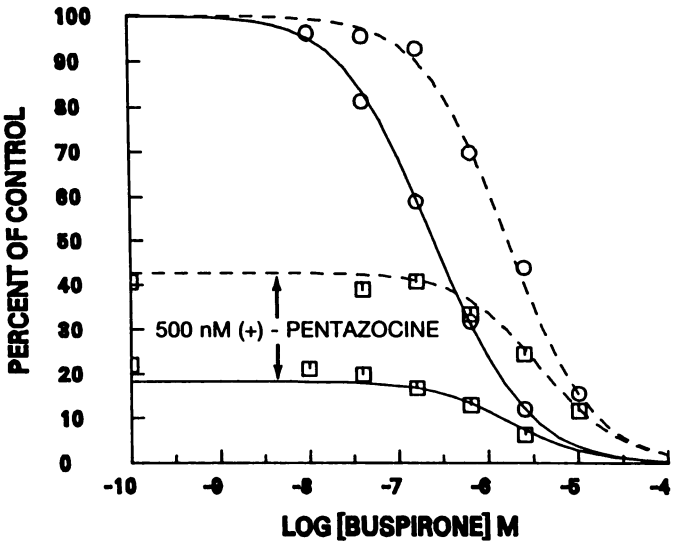


Fig. 3. Buspirone inhibition curves. Two concentrations of [^{3}H]DTG [5 nM (——) and 105 nM (- - -)] were each displaced by five concentrations of buspirone in the absence (○) or presence of a blocking concentration of (+)-pentazocine (500 nM) (□). Each *point* is the mean of two determinations, each assayed in triplicate. The binding surface was fit to the two-site model for the best-fit estimates of the apparent K_i values reported in Table 3, with the B_{max} values and apparent K_d values of DTG and (+)-pentazocine fixed to the values reported in Table 1. The *lines* are those predicted by the best-fit parameter estimates.

10 μM concentrations. Nifedipine, amiloride, and BAYK-8644 tended to slow the dissociation of [^{3}H]DTG, whereas ω-conotoxin had no significant effect.

DTG, caramiphen, and haloperidol increased the dissociation of [^{3}H]DTG from both site 1 and site 2, in a dose-dependent manner (Table 8). Dextromethorphan selectively enhanced the dissociation of [^{3}H]DTG from site 1; quinidine had similar effects but was tested at a single concentration.

Discussion

Evidence for multiple [^{3}H]DTG binding sites. Equilibrium binding studies readily resolved two high affinity binding sites for [^{3}H]DTG (Table 1), with different structure-activity profiles. It is important to point out that, although consideration of a three-site model did not result in a statistically significant improvement in the goodness of fit, this does not mean that there are not three sites, merely that three sites were not resolved. Site 1 has the characteristics expected of the σ receptor, as it is currently defined (12). It has high affinity for haloperidol (0.3 nM), (*R*)-(+)-PPP (5.1 nM), and (+)-SKF10,047 (41.4 nM). Site 2 has low affinity for most agents, with the exception of DTG (37.6 nM) and haloperidol (36.1 nM). In general terms, the structure-activity relationship of site 1 is very close to that reported by investigators using [^{3}H]PPP (9) and (+)-[^{3}H]pentazocine (11), which our data predict will selectively label DTG site 1.

Because of the high selectivity of dextromethorphan for site 1, it was possible in the kinetic experiments to independently measure the dissociation of [^{3}H]DTG from site 1 and site 2. These experiments demonstrated that the dissociation of [^{3}H]DTG from both site 1 and site 2 was best described by a two-component model (Table 5). The observation that certain salts selectively increase the dissociation of [^{3}H]DTG from site 2,

TABLE 3

Inhibitory dissociation constants of test drugs for DTG site 1 and site 2 in guinea pig brain

Inhibition curves for test drugs were generated as described for buspirone in Fig. 3. Two concentrations of [^{3}H]DTG (5 and 105 nM) were each displaced by five concentrations of test drug, in the absence or presence of a blocking concentration of (+)-pentazocine (500 nM). Each point was the mean of two determinations, each assayed in triplicate. The inhibition curves were fit to the two-site model for the best-fit estimates of the apparent K_i values, reported below, with the B_{max} values and apparent K_d values of DTG and (+)-pentazocine fixed to the values reported in Table 1.

Drug	Apparent K_i		Site 2/site 1
	Site 1	Site 2	
	nM		
Haloperidol	0.30 ± 0.01	36.1 ± 1.6	120
(+)-Pentazocine[a]	2.0 ± 0.06	456 ± 11	228
(*R*)-(+)-PPP	5.1 ± 0.3	442 ± 34	86
Carbetapentane	5.2 ± 0.3	1,523 ± 85	292
BD738	6.4 ± 0.1	188 ± 6	29.4
BD737	8.0 ± 0.3	502 ± 29	62.8
Fluphenazine	7.6 ± 0.5	440 ± 30	58
Dextrallorphan	8.4 ± 0.4	1,861 ± 94	221
Perphenazine	8.9 ± 0.5	429 ± 27	48
DTG[a]	11.9 ± 0.1	37.6 ± 0.6	3
(*S*)-(−)-PPP[a]	30.5 ± 0.6	1,544 ± 37	50
KCR-11-240.1	33.5 ± 1.4	1,399 ± 68	42
BMY14802	41.4 ± 2.0	728 ± 52	18
(+)-SKF10047	44.8 ± 1.6	4,263 ± 190	95
BD446	47.4 ± 3.0	1,383 ± 96	29
(−)-Butaclamol	47.4 ± 2.9	3,646 ± 312	77
Caramiphen	65.3 ± 3.8	2,864 ± 224	44
(+)-Cyclazocine	77.6 ± 6.4	1,238 ± 108	16
Buspirone	95.7 ± 2.3	744 ± 19	8
Dextromethorphan	121 ± 6	53,503 ± 3,962	442
BD445	126 ± 7	4,144 ± 265	33
KCR-12-83.1	154 ± 4	18,245 ± 657	118
KCR-12-84.1	179 ± 11	29,258 ± 2,081	163
Dextrorphan	202 ± 10	11,386 ± 719	56
Levallorphan	721 ± 20	13,686 ± 422	19
KCR-11-239.1	1,245 ± 85	18,705 ± 1,373	15
KCR-12-69.1	9,064 ± 1,289	>1 mM	~110

[a] Values for these drugs are from Table 1.

and not site 1 (Table 6), provides independent evidence for the existence of two high affinity [^{3}H]DTG binding sites.

The observations that the salts inhibited binding to site 1 (Table 2) but did not apparently alter the dissociation of [^{3}H]DTG from site 1 (Table 6) are paradoxical. That is, how can a salt inhibit the binding of a ligand without altering its dissociation rate? 1) A salt could decrease the on-rate without changing the off-rate, which would act to increase the apparent K_d of a drug for the binding site. 2) The salt binding site might be inaccessible when the ligand is bound to its site; thus, there would be no discernable effect in a dissociation experiment, where the [^{3}H]DTG is prebound, although there would be an inhibition in an equilibrium binding assay.

In a recent study, Walker *et al.* (16) showed that microinjection of certain σ ligands into the red nucleus of the rat produces dystonic reactions. Matsumoto *et al.* (15) extended these findings, demonstrating a high correlation of behavioral potency with the affinity of various agents [haloperidol, DTG, BD614 (the racemic mixture of BD737 and BD738), (+)-SKF10,047, (+)-pentazocine, and dextrallorphan] for [^{3}H]DTG binding sites in rat brain. The correlation coefficients of the behavioral potencies of these drugs with their apparent K_i values at DTG sites 1 and 2 are $r = 0.69$ (not significant) and $r = 0.89$ ($p <$ 0.02), respectively.[1] Matsumoto *et al.* (15) also noted that the (+)-opiates have lower affinity for rat brain [^{3}H]DTG binding sites than for σ binding sites labeled by either [^{3}H]PPP or (+)-[^{3}H]pentazocine and that the σ site mediating the dystonic effects of the σ ligands has relatively low affinity for (+)-opiates. Our data, which suggest that [^{3}H]PPP and (+)-[^{3}H]pentazocine should selectively label DTG site 1, permit the speculation that in rat brain [^{3}H]DTG mainly labels DTG site 2 and the σ site mediating the dystonic effects of the σ ligands may be DTG site 2. The high potency of (+)-opiates in inhibiting phosphoinositide turnover in rat brain (20, 21) suggests that this effect may be mediated via DTG site 1.

Moreover, the recent report that rat pheochromocytoma

TABLE 4

Prediction experiments

Three batches of guinea pig brain membranes were prepared, as described in Materials and Methods. The inhibition produced by the indicated concentrations of test drugs are reported as a percentage of control (mean ± standard deviation, three experiments). The observed percentage of control was compared, using the Student's *t* test, with the percentage of control predicted on the basis of the binding parameters reported in Tables 1 and 3.

Drug	Concentration	Inhibition	
		Observed	Predicted
	nM	% of control	
Haloperidol	0.5	88.6 ± 2.5	80.2
	25	30.5 ± 4.9	33.5
(+)-Pentazocine	25	59.5 ± 5.8	54.1
	1,000	24.3 ± 1.8	19.6
(*R*)-(+)-PPP	25	71.5 ± 4.3	64.6
	500	26.4 ± 3.1	28.2
Dextrallorphan	25	80.8 ± 3.6	72.5
	1,000	44.3 ± 4.1	35.1[a]
Perphenazine	25	96 ± 3.2	92.3
	500	56.0 ± 5.4	49.6
(*S*)-(−)-PPP	50	82.6 ± 2.3	79.9
	5,000	9.7 ± 2.1	16.2[b]
DTG	50	59.4 ± 4.4	48.3[b]
KCR-11-240.1	50	75.1 ± 2.7	81.0
	1,000	39.1 ± 4.8	36.4
(+)-SKF10,047	50	77.8 ± 2.7	85.0
	1,000	37.8 ± 4.0	45.8
BD446	50	84.7 ± 7.1	84.9
	1,000	41.2 ± 2.8	38.2
(+)-Cyclazocine	100	61.2 ± 5.4	61.0
	1,000	39.6 ± 0.6	38.3
Buspirone	100	77.6 ± 3.2	82.7
	1,000	38.8 ± 3.6	37.3
Dextromethorphan	100	85.1 ± 6.7	88.3
	20,000	34.8 ± 2.1	37.4
BD445	100	79.8 ± 2.9	88.0
	1,000	39.5 ± 2.9	54.5[b]
KCR-12-83.1	100	82.6 ± 3.3	90.2
	1,000	34.9 ± 0.8	36.1
KCR-12-84.1	100	85.0 ± 3.4	91.4
	20,000	31.2 ± 1.6	33.1
Dextrorphan	100	86.8 ± 3.8	92.1
	10,000	23.8 ± 0.3	32.4[b]
Levallorphan	5,000	45.5 ± 2.3	53.6
KCR-11-239.1	1,000	77.2 ± 7.9	87.2
	10,000	53.2 ± 5.8	49.1
KCR-12-69.1	10,000	74.6 ± 1.1	81.1
Fluphenazine	25	85.0 ± 7.1	81.1
	1,000	22.9 ± 1.2	21.4
(−)-Butaclamol	50	82.9 ± 9.7	77.6
	1,000	44.7 ± 6.8	41.5

[a] $p < 0.05$ (Student's *t* test, two-tailed).
[b] $p < 0.01$.

[1] J. M. Walker, personal communication.

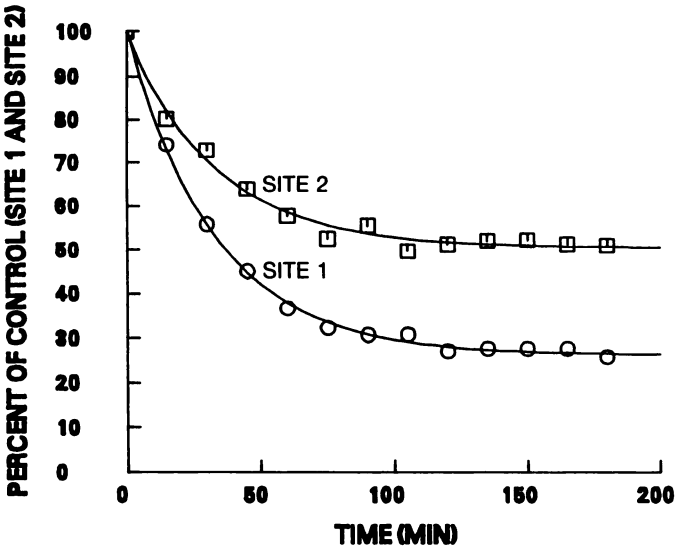


Fig. 4. Dissociation of [^{3}H]DTG. As described in Materials and Methods, guinea pig membranes were incubated to equilibrium with 5 nM [^{3}H]DTG. Incubations were terminated by centrifugation. The membrane pellets were resuspended in fresh buffer, and samples were filtered at the indicated times after resuspension. The dissociation of [^{3}H]DTG from site 1 (○) and site 2 (□) was calculated and was well described by a two-component dissociation model. The *lines* are those predicted by the best-fit parameter estimates (see Table 5). Each *point* is the mean of three experiments, with less than 5% coefficient of variation.

TABLE 5

Dissociation kinetics of [^{3}H]DTG binding sites

The dissociation of [^{3}H]DTG from site 1 and 2, as a function of time, was monitored as described for Fig. 4. The data were fit to one- or two-component dissociation models. The two-component models fit the data significantly better ($p < 0.001$). The best-fit parameter estimates ± standard deviation are reported above. The A_1 and A_2 constants represent the proportions of slow and fast components, respectively, that are present under the specified experimental conditions.

	Component 1		A_2	k_{-2}
	A_1	k_{-1}		
		min^{-1}		min^{-1}
DTG site 1	26.4 ± 3.2	<0.0001	74.2 ± 3.1	0.031 ± 0.002
DTG site 2	50.5 ± 5.8	<0.0001	49.4 ± 5.6	0.030 ± 0.005

(PC12) cells (40) possess a σ binding site with characteristics similar to those of DTG site 2 suggests that the PC12 σ binding site and DTG site 2 may similar.

Relationship of [^{3}H]DTG binding sites and [^{3}H]dextromethorphan binding sites. Musacchio and associates (41–46) have extensively studied a [^{3}H]dextromethorphan binding site that has high affinity for certain centrally acting antitussive and anticonvulsant drugs, as well as classical σ agents such as haloperidol, (+)-pentazocine, and (+)-SKF10,047. In light of these findings, Musacchio *et al.* (47) proposed that [^{3}H]dextromethorphan and [^{3}H]PPP label a common high affinity binding site, termed the DM_1/σ_1 receptor.

The data of the present study support this concept. Carbetapentane, which has an apparent K_i of 11 nM at the DM_1/σ_1 (42) binding site, has an apparent K_i of 5.2 nM at DTG site 1. Moreover, caramiphen and dextromethorphan are reasonably potent at DTG site 1. The apparent K_i of dextromethorphan at the DM_1/σ_1 binding site is 57 nM (42) and for DTG site 1 is 121 nM. Moreover, the apparent K_i values observed in this study for many drugs at site 1 are similar to the apparent K_i values reported at [^{3}H]dextromethorphan binding sites (42).

TABLE 6

Effect of salts on [^{3}H]DTG dissociation

Guinea pig membranes were incubated to equilibrium with 5 nM [^{3}H]DTG under three conditions. The three incubation conditions (see below) were terminated by centrifugation for 15 min at 27,578 × *g*. Pellets were resuspended in ice-cold buffer. One-milliliter aliquots were distributed to test tubes that were prefilled with either buffer (control) or salts at the concentrations reported above. Samples were filtered (in triplicate) at time 0 and 2 hr after the addition of membranes. The three conditions used were 1) total binding, 2) binding in the presence of 10 μM dextromethorphan (to block binding to site 1), and 3) nonspecific binding. Dissociation of [^{3}H]DTG from site 1 was calculated as condition 1 − condition 2. Dissociation of [^{3}H]DTG from site 2 was calculated as condition 2 − condition 3. These calculations were performed after the binding in each condition was corrected for the protein concentration. The results are reported as a percentage of the 2-hr control (standard deviation, three experiments).

Salt	Concentration	Dissociation	
		Site 1	Site 2
	mM	% of 2-hr control	
$MgCl_2$	0.1	92.0 ± 4.8	80.4 ± 0.8*
	0.2	86.8 ± 4.3	70.2 ± 4.7*
	0.4	90.1 ± 2.6	66.3 ± 7.7*
	0.8	90.7 ± 8.0	56.5 ± 6.7*
	1.6	91.0 ± 7.5	48.5 ± 5.4*
	5.0	93.8 ± 3.5	39.9 ± 6.4*
$NiCl_2$	0.1	102.6 ± 5.9	88.5 ± 1.0*
	1.0	96.9 ± 6.8	46.0 ± 5.5*
	5.0	97.8 ± 6.7	36.3 ± 8.5*
$LaCl_3$	0.1	108.8 ± 6.2	52.5 ± 4.6*
	0.5	103.5 ± 2.4	32.6 ± 4.6*
	1.0	98.4 ± 1.6	32.2 ± 2.2*
$CdCl_2$	0.01	111.3 ± 8.8	94.8 ± 9.9
	0.1	100.1 ± 6.6	59.3 ± 11.9*
	0.5	103.4 ± 2.4	19.7 ± 0.9*
	1.0	93.9 ± 5.2	23.1 ± 2.2*
NaCl	1.0	104.6 ± 10	98.1 ± 9.6
	10	93.2 ± 4.5	80.5 ± 6.2*
	100	77.8 ± 2.0*	43.0 ± 1.3*
	250	70.8 ± 8.8*	37.9 ± 7.4*

*$p < 0.05$, compared with control (one-way analysis of variance, *post hoc* Scheffe *F* test).

TABLE 7

Effect of calcium channel modulators on dissociation of [^{3}H]DTG from site 1 and site 2

The effects of the indicated calcium channel modulators on dissociation of [^{3}H]DTG were determined as described in the legend to Table 6. Data are reported as a percentage of the 2-hr control (mean ± standard deviation, three experiments).

Agent	Concentration	Dissociation	
		Site 1	Site 2
	μM	% of 2-hr control	
Control	0	99.9 ± 1.1	100 ± 1.9
Bepridil	1	57.9 ± 2.2*	91.9 ± 1.2*
	10	54.8 ± 1.1*	49.7 ± 2.3*
Amiloride	1	100 ± 0.9	117.2 ± 1.6*
	10	99.8 ± 2.7	110.9 ± 1.7*
Amidirone	1	41.1 ± 2.1*	92.0 ± 1.9*
	10	54.1 ± 1.1*	52.2 ± 2.4*
Nifedipine	1	104.7 ± 4.1	126.2 ± 4.8*
	10	109.1 ± 1.3*	109.6 ± 1.1*
BAYK-8644	1	108.0 ± 1.4*	116.9 ± 2.2*
	10	110 ± 2.9*	100.5 ± 1.8
Verapamil	1	92.3 ± 1.5*	102.3 ± 1.5
Verapamil	10	64.6 ± 2.6*	55.7 ± 1.3*
ω-Conotoxin	0.1	102.8 ± 1.6	96.9 ± 3.4

*$p < 0.05$, compared with control (one-way analysis of variance, *post hoc* Sheffe *F* test).

Differences in the apparent K_i values probably reflect the different assay conditions used to characterize [^{3}H]dextromethorphan and [^{3}H]DTG binding sites.

Interaction of calcium channel modulators with [^{3}H]

TABLE 8

Effect of various agents on the dissociation of [^{3}H]DTG from site 1 and site 2

The effects of the indicated agents on dissociation of [^{3}H]DTG were determined as described in the legend to Table 6. Data are reported as a percentage of the 2-hr control (mean ± standard deviation, three experiments).

Agent	Concentration	Dissociation	
		Site 1	Site 2
	nM	% of 2-hr control	
Control	0	96.9 ± 3.4	100 ± 0.6
DTG	10	63.3 ± 2.3*	93.4 ± 1.3*
	100	24.8 ± 0.8*	66.7 ± 0.7*
	1,000	17.0 ± 1.8*	30.6 ± 0.4*
Haloperidol	10	23.3 ± 1.6*	91.2 ± 2.5*
	100	8.2 ± 1.1*	64.1 ± 2.5*
	1,000	11.4 ± 2.6*	26.3 ± 1.1*
Caramiphen	10	50 ± 10.3	84.8 ± 6.4
	100	8.1 ± 5.8*	82.2 ± 1.4*
	1,000	8.8 ± 4.4*	42.1 ± 0.8*
	10,000	77.7 ± 1.7	42.9 ± 0.5*
Dextromethorphan	10,000	17.2 ± 1.7*	101.5 ± 3.7

*$p < 0.05$, compared with control (one-way analysis of variance, *post hoc* Scheffe *F* test).

DTG binding sites. In view of the data that inorganic calcium channel blockers such as Cd and La inhibit the equilibrium binding of [^{3}H]DTG to site 1 and site 2 (Table 2), we examined the ability of these agents to alter the dissociation of [^{3}H]DTG from sites 1 and 2 (Table 6). It is important to realize that in these dissociation experiments we monitored the effect of agents at a single time point, i.e., 2 hr after the addition of the agent. Therefore, we did not determine the effect of the agents on the kinetic parameters that describe the system (A_1, A_2, k_{-1}, and k_{-2}). Given that by 2 hr of dissociation the [^{3}H]DTG had completely dissociated from the fast component (A_2), it is likely that the effect of the agents was to decrease the value of A_1. Interestingly, although these agents inhibited the equilibrium binding of [^{3}H]DTG to both site 1 and site 2, in the kinetic experiments they affected only site 2.

For this reason we studied the effect of organic calcium channel modulators on dissociation of [^{3}H]DTG (Table 7). Bepridil, amidirone, and verapamil had selective effects on DTG site 1 at 1 μM, but not at 10 μM. Amiloride slowed the dissociation of [^{3}H]DTG from site 2 and had no effect on site 1. Nifedipine had a slowing effect on both sites 1 and 2. ω-Conotoxin had no effect on either site.

The selective effects of the inorganic calcium channel antagonists on DTG site 2 occurred at concentrations that block calcium channels (39), suggesting that DTG site 2 may be associated with a calcium channel. The results obtained using the organic calcium channel modulators are more difficult to interpret, because the effects occurred at high (micromolar) concentrations. Further experiments will be necessary to clarify the observations that certain calcium channel antagonists appeared to slow the dissociation of [^{3}H]DTG from both site 1 and site 2.

Evidence for pseudoallosteric modulation of DTG site 1 and site 2 by σ ligands. The selective and potent effects of Cd^{2+} and Ln^{3+} on the dissociation of [^{3}H]DTG from site 2 prompted us to examine the effect of DTG and other σ ligands on the dissociation of [^{3}H]DTG. DTG, haloperidol, dextromethorphan, and caramiphen all produced a dose-dependent increase in the dissociation rate of [^{3}H]DTG from site 1 and site 2. The drugs were more potent at site 1, consistent with the selectivity of these agents for site 1.

Two methods are commonly used to quantitate the rate which ^{3}H-ligands dissociate from their binding sites, isotopic dilution and chemical dilution (48). In the former method, the addition of a large excess of nonradioactive ligand at equilibrium blocks the association of ^{3}H-ligand with the receptor, permitting quantitation of the dissociation rate. In the latter method, the dilution of the assay medium (>100-fold) decreases the concentration of ^{3}H-ligand and receptor, which slows the association of ^{3}H-ligand with the receptor to the point that the dissociation of the ^{3}H-ligand can be quantitated. The approach used in the present study is a variation of the chemical dilution method used by Chang and Cuatrecacas (49). At equilibrium, the membranes are centrifuged, and the supernatant, which contains the free ^{3}H-ligand, is discarded. The membranes are resuspended in fresh buffer, and the rate at which the ^{3}H-ligand dissociates from the receptor is quantitated. ^{3}H-Ligand that dissociates into the medium is essentially infinitely diluted, so that it does not interfere with the quantification of the dissociation rate. The chief advantage of this method is that it avoids the filtering of large volumes necessitated by the chemical dilution method.

As pointed out by Boeynaems and Dumont (48), an increase in the dissociation rate of a ^{3}H-ligand produced by the addition of a nonradioactive ligand (i.e., isotopic dilution being more effective than chemical dilution) is consistent with a negative cooperativity model, in which the nonradioactive ligand binds to a site not occupied by ^{3}H-ligand and, thereby, induces a conformational change in binding sites occupied by ^{3}H-ligand, producing a configuration characterized by lower affinity and an increased dissociation rate.

The demonstration that DTG and other ligands enhance the dissociation of [^{3}H]DTG from both DTG site 1 and DTG site 2 could indicate the presence of negatively cooperative interactions. However, equilibrium binding studies with [^{3}H]DTG in which the binding to site 1 and that to site 2 are separately measured (including the present study) do not provide compelling evidence for negative cooperativity, such as curvilinear Scatchard plots or low Hill coefficients. Thus, alternative hypotheses must be sought.

One possibility is the conceptualization of a DTG binding site composed of several "attachment points," which are the portions of the receptor domain to which a ligand binds. For the purpose of this discussion, we will assume that there are five such attachment points. The data of the present study are compatible with the notion that DTG binds preferentially to a subset of these attachment points (points 1, 2 and 3), leaving other attachment points (points 4 and 5) unoccupied. When a binding site is already occupied by one ligand (L1, DTG), a second ligand (L2) can interact only with attachment points 4 and 5. A consequence of this interaction is a conformational change in the binding site, resulting in the dissociation of L1 from attachment points 1, 2, and 3 and the subsequent association of L2 with attachment points 1, 2, and 3. This model, which does not postulate distinct allosteric sites, is consistent with both a lack of negative cooperativity in equilibrium binding studies and the kinetic data reported here.

We term this the "pseudoallosteric model." Recently, Reid *et al.* (50) reported similar data for the PCP receptor, demonstrating that (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohep-

ten-5,10-imiine maleate [(+)-MK801] enhanced the dissociation of [^{3}H]TCP from the NMDA-associated PCP binding site. The pseudoallosteric model can potentially explain the observation that caramiphen was more potent in accelerating the dissociation of [^{3}H]DTG from site 1 than would be predicted on the basis of its apparent Ki for site 1. Because attachment points 1, 2, and 3 are already occupied with [^{3}H]DTG, the potency of an agent in promoting dissociation must reflect its affinity for attachment points 4 and 5, not attachment points 1, 2, and 3. It is hypothetically possible, therefore, that the affinity of an agent might be higher for attachment points 4 and 5 then for attachment points 1, 2, and 3.

The pseudoallosteric model differs from that proposed by Bowen *et al.* (51), who reported the presence of a classical allosteric binding site associated with σ binding sites. Ropazine and phenytoin, which allosterically modulate [^{3}H]dextromethorphan (45) and [^{3}H]PPP binding sites (44), failed to alter the dissociation of [^{3}H]DTG from either site 1 or site 2 (data not shown). This finding could argue against the similarity of DTG site 1 and the DM_1/σ_1 binding site. However, Musacchio *et al.* (45) use membranes prepared from the pons and medulla, whereas we use membranes prepared from whole guinea pig brain. A possible explanation of the discrepancy is that the positive allosteric effects of ropazine are confined to the hindbrain.

Evidence for complex intertactions. Several pieces of data in the present study indicate that the system we are attempting to characterize is complex. These include the biphasic dissociation of [^{3}H]DTG from each site (Fig. 4) and the ability of σ ligands and inorganic calcium channel blockers to accelerate the dissociation of [^{3}H]DTG from each site (Tables 6 and 8). Moreover, the data of Bowen *et al.* (51), which suggest the existence of classical allosteric binding sites, represent another layer of complexity.

Clearly, this is not a system characterized by simple kinetics, where the K_d equals the dissociation rate divided by the association rate. Therefore, fitting of the data to the two-site model, or any model that assumes simple kinetic interactions, is, strictly speaking, not correct. Nevertheless, this is often done by many investigators. The usually unwritten assumption is that analysis of data in this way provides a first approximation of the system. The K_d and K_i values are "apparent" and in fact are complex functions of the various kinetic constants that characterize the system. With these caveats in mind, we feel that the data obtained in the present study, as reviewed in Evidence for multiple [^{3}H]DTG binding sites, do support the hypothesis that [^{3}H]DTG labels two binding sites. However, experiments at the biochemical and anatomical level will be required to test the hypothesis that DTG site 1 and DTG site 2 are physically distinct binding sites.

Conclusions. The major finding of this paper is that [^{3}H] DTG labels two high affinity binding sites in guinea pig brain. DTG site 1 corresponds to the site termed DM_1/σ_1 by Musacchio *et al.* (47). DTG site 2, which has high affinity for DTG and haloperidol, may be associated with a calcium channel, and may mediate the dystonic effects of σ ligands. The dissociation of [^{3}H]DTG from both site 1 and site 2 is pseudoallosterically modulated by σ ligands. The similarity between DTG site 1 and the DM_1/σ_1 site reinforces the concept that the σ receptor, as it currently defined, is most likely not a psychotomimetic binding site. Rather, as suggested by Musacchio (52), given that drugs with antipsychotic activity, such as haloperidol and BMY14802 (53), bind to it with high affinity, it may be that high affinity σ ligands exert antipsychotic effects in humans. Finally, the observations that certain high affinity σ ligands functionally antagonize behaviors thought to be mediated by mesolimbic dopamine (53) suggest that high affinity σ ligands might functionally antagonize the behavioral effects of cocaine, which has been proposed to produce its addictive effects by elevating synaptic levels of mesolimbic dopamine (54).

References

1. Martin, W. R., C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert. The effect of morphine and nalorphine-like drugs in the nondependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517–532 (1976).
2. Zukin, S. R., and R. S. Zukin. Specific ^{3}H-phencyclidine binding in rat central nervous system. *Proc. Natl. Acad. Sci. USA* **76**:5372–5376 (1979).
3. Quirion, R., R. P. Hammer, M. Herkenham, and C. B. Pert. Phencyclidine (angel dust)/*sigma* "opiate" receptor: visualization by tritium-sensitive film. *Proc. Natl. Acad. Sci. USA* **78**:5881–5885 (1981).
4. Haertzen, C. A. Subjective effects of narcotic antagonists cyclazocine and nalorphine on the addiction research center inventory. *Psychopharmacologia* **18**:366–377 (1970).
5. Telford, J., C. N. Papadopoulos, and A. S. Keats. Studies of analgesic drugs. VII. Morphine antagonists as analgesics. *J. Pharmacol. Exp. Ther.* **133**:106–116 (1961).
6. Zukin, R. S., and S. R. Zukin. Demonstration of [^{3}H]cyclazocine binding to multiple opiate receptor sites. *Mol. Pharmacol.* **20**:246–254 (1981).
7. Tam, S. W., and L. Cook. *Sigma* opiates and certain antipsychotic drugs mutually inhibit (+)-[^{3}H]SKF10,047 and [^{3}H]haloperidol binding in guinea pig brain membranes. *Proc. Natl. Acad. Sci. USA* **81**:5618–5621 (1984).
8. Su, T. P. Evidence for *sigma* opioid receptor: binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *J. Pharmacol. Exp. Ther.* **223**:284–290 (1982).
9. Largent, B. L., A. L. Gundlach, and S. H. Snyder. Psychotomimetic opiate receptors labeled and visualized with (+)-[^{3}H]3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine. *Proc. Natl. Acad. Sci. USA* **81**:4983–4987 (1984).
10. Weber, E., M. Sonders, M. Quarum, S. McLean, S. Pou, and J. F. Keana. 1,3-Di(2-[5-^{3}H]tolyl)guanidine: a selective ligand that labels *sigma*-type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. USA* **83**:8784–8788 (1986).
11. de Costa, B. R., W. D. Bowen, S. B. Hellewell, J. M. Walker, A. Thurkauf, A. E. Jacobson, and K. C. Rice. Synthesis and evaluation of optically pure [^{3}H]-(+)-pentazocine, a highly potent and selective radioligand for *sigma* receptors. *FEBS. Lett.* **251**:53–58 (1989).
12. Quirion, R., R. Chicheportiche, P. C. Contreras, K. M. Johnson, D. Lodge, S. W. Tam, J. H. Woods, and S. R. Zukin. Classification and nomenclature of phencyclidine and *sigma* receptor sites. *Trends Neurosci.* **10**:444–446 (1987).
13. Bowen, W. D., J. M. Walker, A. G. Yashar, R. R. Matsumoto, F. O. Walker, and J. F. Lorden. Altered haloperidol-sensitive *sigma* receptors in the genetically dystonic (dt) rat. *Eur. J. Pharmacol.* **147**:153–154 (1988).
14. Goldstein, S. R., R. R. Matsumoto, T. L. Thompson, R. L. Patrick, W. D. Bowen, and J. M. Walker. Motor effects of two *sigma* ligands mediated by nigrostriatal dopamine neurons. *Synapse* **4**:254–258 (1989).
15. Matsumoto, R. R., M. K. Hemstreet, N. L. Lai, A. Thurkauf, B. R. de Costa, K. C. Rice, S. B. Hellewell, W. D. Bowen, and J. M. Walker. Drug specificity of pharmacological dystonia. *Pharmacol. Biochem. Behav.* **36**:151–155 (1990).
16. Walker, J. M., R. R. Matsumoto, W. D. Bowen, D. L. Gans, K. D. Jones, and F. O. Walker. Evidence for a role of haloperidol-sensitive *sigma*-'opiate' receptors in the motor effects of antipsychotic drugs. *Neurology* **38**:961–965 (1988).
17. Campbell, B. G., D. H. Bobker, F. M. Leslie, I. N. Mefford, and E. Weber. Both the *sigma* receptor-specific ligand (+)-PPP and the PCP receptor-specific ligand TCP act in the mouse vas deferens via augumentation of electrically evoked norepinephrine release. *Eur. J. Pharmacol.* **138**:447–449 (1987).
18. Campbell, B. G., M. W. Scherz, J. F. Keana, and E. Weber. *Sigma* receptors regulate contractions of the guinea pig ileum longitudinal muscle/myenteric plexus preparation elicited by both electrical stimulation and exogenous serotonin. *J. Neurosci.* **9**:3380–3391 (1989).
19. Vaupel, D. B., and T. P. Su. Guinea-pig vas deferens preparation may contain both *sigma* receptors and phencyclidine receptors. *Eur. J. Pharmacol.* **139**:125–128 (1987).
20. Bowen, W. D., B. N. Kirschner, A. H. Newman, and K. C. Rice. *Sigma* receptors negatively modulate agonist-stimulated phosphoinositide metabolism in rat brain. *Eur. J. Pharmacol.* **149**:399–400 (1988).
21. Bowen, W. D., P. J. Tolentino, B. N. Kirschner, P. Varghese, B. R. de Costa, and K. C. Rice. *Sigma* receptors and signal transduction: negative modulation of signalling through phosphoinositide-linked receptor systems. *Natl. Inst. Drug Abuse Res. Monogr. Ser.*, in press.
22. Ceci, A., M. Smith, and E. D. French. Activation of the A10 mesolimbic system by the *sigma* receptor agonist (+)-SKF10,047 can be blocked by

rimcazole, a novel putative antipsychotic agent. *Eur. J. Pharmacol.* **154**:53–57 (1988).
23. Matsumoto, R. R., and J. M. Walker. Inhibition of rubral neurons by a specific ligand for *sigma* receptors. *Eur. J. Pharmacol.* **158**:161–165 (1988).
24. Reid, A. A., R. B. Rothman, C. H. Kim, A. E. Jacobson, and K. C. Rice. Evidence for two populations of haloperidol-sensitive *sigma* binding sites in guinea pig brain. *Soc. Neurosci. Abstr.* 703 (1988).
25. de Costa, B. R., W. D. Bowen, S. B. Hellewell, C. George, R. B. Rothman, A. A. Reid, J. M. Walker, A. E. Jacobson, and K. C. Rice. Alterations in the stereochemistry of the *kappa*-selective opioid agonist U50,488 result in high-affinity *sigma* ligands. *J. Med. Chem.* **32**:1996–2002 (1989).
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
27. Rodbard, D., R. H. Lenox, L. Wray, and D. Ramseth. Statistical characterization of the random errors in the radioimmunoassay dose-response variable. *Clin. Chem.* **22**:350–358 (1976).
28. Knott, G. D., and D. K. Reece. MLAB: a civilized curve fitting system. *Proc. Online 1972 Int. Conf.* **1**:497–526 (1972).
29. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
30. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
31. Rothman, R. B. Binding surface analysis: an intuitive yet quantitative method for the design and analysis of ligand binding studies. *Alcohol Drug Res.* **6**:309–325 (1986).
32. McGonigle, P., K. A. Neve, and P. B. Molinoff. A quantitative method of analyzing the interaction of slightly selective radioligands with multiple receptor subtypes. *Mol. Pharmacol.* **30**:329–337 (1986).
33. Rovati, G. E., D. Rodbard, and P. J. Munson. DESIGN: computerized optimization of experimental design for estimating K_d and B_{max} in ligand binding experiments. II. Simultaneous analysis of homologous and heterologous competition curves and analysis blocking and of "multiligand: dose-response surfaces. *Anal. Biochem.* **184**:172–183 (1990).
34. de Costa, B. R., K. C. Rice, W. D. Browen, A. Thurkauf, R. B. Rothman, L. Band, A. E. Jacobson, L. Radesca, P. C. Contreras, N. M. Gray, I. Daly, S. Iyengar, D. T. Finn, S. Vazirani, and J. M. Walker. Synthesis and evaluation of *N*-substituted *cis*-*N*-methyl-2-(1-pyrrolidnyl)cyclohexylamines as high affinity *sigma* receptor ligands: identification of a new class of highly potent and selective *sigma* receptor probes. *J. Med. Chem.*, **33**:3100–3110 (1990).
35. Rice, K. C. The development of a practical total synthesis of natural and unnatural codeine, morphine and thebaine, in *The Chemistry and Biology of Isoquinoline Alkaloids* (J. D. Phillipson, M. F. Roberts, and M. H. Zenk, eds.). Springer-Verlag, New York, 191–203 (1985).
36. Peet, N. P. *N*-Demethylation of dextromethorphan. *J. Pharm. Sci.* **69**:1447–1448 (1980).
37. van Schnider, O., and A. Grussner. Oxymorphinane: optisch aktive 3-oxymorphinane. *Helv. Chemica Acta* **34**:2211–2217 (1951).
38. von Hellerbach, J., A. Grussner, and O. van Schnider. Hydroxymorphane: (−)-3-hydroxy-*N*-allyl-morphinan und verwandte verbindungen. *Helv. Chem. Acta* **39**:429–440 (1956).
39. Bean, B. P. Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* **51**:367–384 (1989).
40. Hellewell, S. B., and W. D. Bowen. A *sigma*-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different *sigma* receptor form from that of guinea pig brain. *Brain Res.* **527**:244–253 (1990).
41. Canoll, P. D., P. R. Smith, S. Gottesman, and J. M. Musacchio. Autoradiographic localization of [^{3}H]dextromethorphan in guinea pig brain: allosteric enhancement by ropizine. *J. Neurosci. Res.* **24**:311–328 (1989).
42. Klein, M., and J. M. Musacchio. High affinity dextromethorphan binding sites in guinea pig brain: effect of *sigma* ligands and other agents. *J. Pharmacol. Exp. Ther.* **251**:207–215 (1989).
43. Klein, M., J. J. Paturzo, and J. M. Musacchio. The effect of prototypic *sigma* ligands on the binding of [^{3}H]dextromethorphan to guinea pig brain. *Neurosci. Lett.* **97**:175–180 (1989).
44. Musacchio, J. M., M. Klein, and J. J. Paturzo. Effects of dextromethorphan site ligands and allosteric modifiers on the binding of (+)-[^{3}H]3-(-3-hydroxyphenyl)-*N*-(1-propyl)piperidine. *Mol. Pharmacol.* **35**:1–5 (1989).
45. Musacchio, J. M., M. Klein, and L. J. Santiago. High affinity dextromethorphan binding sites in guinea pig brain: further characterization and allosteric interactions. *J. Pharmacol. Exp. Ther.* **247**:424–431 (1988).
46. Musacchio, J. M., and M. Klein. Dextromethorphan binding sites in the guinea pig brain. *Cell. Mol. Neurobiol.* **8**:149–156 (1988).
47. Musacchio, J. M., M. Klein, and P. D. Canoll. Dextromethorphan and *sigma* ligands: common sites but diverse effects. *Life Sci.* **45**:1721–1732 (1989).
48. Boeynaems, J. M., and J. E. Dumont. Quantitative analysis of the binding of ligands to their receptors. *J. Cyclic Nucleotide Res.* **1**:123–142 (1975).
49. Chang, K. J., and P. Cuatrecasas. Multiple opiate receptors. Enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
50. Reid, A. A., J. A. Monn, A. E. Jacobson, K. C. Rice, and R. B. Rothman. Pseudoallosteric modulation by (+)-MK801 of NMDA-coupled phencyclidine binding sites. *Pharmacol. Lett.* **47**:PL77–PL82 (1990).
51. Bowen, W. D., S. B. Hellewell, and K. A. McGarry. Evidence for a multi-site model of the rat brain *sigma* receptor. *Eur. J. Pharmacol.* **163**:309–318 (1989).
52. Musacchio, J. M. The psychotomimetic effects of opiates and the *sigma* receptor. *Neuropsychopharmagology* **3**:191–200 (1990).
53. Taylor, D. P., and J. Dekleva. BMY 14802: a potential antipsychotic agent that selectively binds to *sigma* receptors, in *Sigma and Phencyclidine-like Compounds as Molecular Probes in Biology* (E. F. Domino and J. M. Kamenka, eds.). NPP Books, Ann Arbor, MI, 345–355 (1988).
54. Ritz, M. C., R. J. Lamb, S. R. Goldberg, and M. J. Kuhar. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science (Washington, D. C.)* **237**:1219–1223 (1987).

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