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1,3-Di(2-[5-^{3}H]tolyl)guanidine: A selective ligand that labels σ-type receptors for psychotomimetic opiates and antipsychotic drugs

(*N*-allylnormetazocine/haloperidol/phencyclidine)

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ABSTRACT **Brain σ-type receptors are thought to mediate hallucinogenic effects of certain benzomorphan opiates in humans. The biochemical characterization of σ receptors has been difficult because of the lack of potent and selective ligands. We report here the synthesis and characterization of a tritiated, symmetrically substituted guanidine derivative, 1,3-di(2-[5-^{3}H]tolyl)guanidine ([^{3}H]Tol$_2$Gdn), that binds with high affinity to a single population of binding sites in guinea pig brain membrane preparations. The [^{3}H]Tol$_2$Gdn binding site displays stereoselectivity for dextrorotatory optical isomers of benzomorphan opiates known to have σ-type behavioral effects. Furthermore, the [^{3}H]Tol$_2$Gdn binding site has a high affinity for haloperidol and for phenothiazine antipsychotics, which have antihallucinatory properties in humans. The drug-selectivity profile of [^{3}H]Tol$_2$Gdn binding closely correlates with the drug-selectivity profile of tritiated (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-[^{3}H]3-PPP) binding to guinea pig brain membrane receptors. (+)-[^{3}H]3-PPP has been proposed to be a selective σ-receptor ligand [Largent, B. L., Gundlach, A. L. & Snyder, S. H. (1984) *Proc. Natl. Acad. Sci. USA* 82, 4983–4987]. Receptor autoradiography using [^{3}H]Tol$_2$Gdn on slide-mounted rat and guinea pig brain sections reveals a heterogeneous distribution pattern of enriched binding in limbic and sensorimotor structures of the brain. These results indicate that [^{3}H]Tol$_2$Gdn is a selective ligand for the σ binding site. Availability of this σ-receptor probe should greatly facilitate the physiological, biochemical, and pharmacological characterization of σ receptors in brain.**

Certain benzomorphan opiates such as *N*-allylnormetazocine (SKF 10,047) and cyclazocine, aside from analgesia, cause hallucinations, depersonalization, drunkenness, and other psychotomimetic effects in humans (1, 2). In monkeys, dogs, and rodents the psychotomimetic opiates cause behavioral and autonomic effects that are unlike those observed with administration of classical opiates such as morphine or the opioid peptides (3–8). These observations have suggested the existence of specific σ-type "opioid" receptors that mediate such atypical effects (3). It has been proposed that σ receptors also mediate the psychotomimetic effects of phencyclidine (PCP, angel dust), or alternatively, that psychotomimetic opiates act at specific PCP receptors (9–12). PCP is a drug of abuse that causes a behavioral syndrome in humans similar to that which is observed in schizophrenic psychosis (13). Because of the potent psychotomimetic effects of σ opiates and PCP, it has been suspected that σ (or PCP) receptors may play a role in mental illness, particularly schizophrenia.

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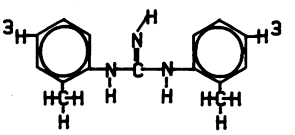

FIG. 1. Structure of [^{3}H]Tol$_2$Gdn.

A systematic investigation of the role of σ receptors in normal and abnormal brain function has been hindered by a lack of specific σ-receptor binding assays and bioassays. Development of such specific assays requires well-characterized, highly selective, and potent σ-receptor ligands. It has been shown that brain membrane σ receptors can be labeled *in vitro* with (±)-[^{3}H]SKF 10,047 (14), (±)-[^{3}H]ethylketazocine (15), or (+)-[^{3}H]SKF 10,047 (16–18), although not selectively (19, 20), and with tritiated (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine {(+)-[^{3}H]3-PPP} (21), which is apparently more selective for σ receptors than the others. In these studies it has been shown that the σ binding site has the following characteristics: (*i*) stereoselectivity toward dextrorotatory benzomorphan opiates and insensitivity to naloxone; (*ii*) high affinity for haloperidol and moderate to high affinity for phenothiazine antipsychotic drugs, which are also known to be potent dopamine-receptor blockers; and (*iii*) insensitivity to dopamine and apomorphine (14–21). This intriguing drug-selectivity profile calls for a thorough analysis of the role of σ receptors in normal and abnormal brain function. For such studies it is essential that a spectrum of highly selective and potent σ-receptor-active drugs be available.

During a systematic search for potential σ-receptor-active compounds, we discovered that certain symmetrically substituted guanidines may have σ binding activity, as judged by their capability to displace (+)-[^{3}H]SKF 10,047 from guinea pig brain membrane binding sites (unpublished observations). We have synthesized a ^{3}H-labeled derivative of one of these compounds, 1,3-di(2-[5-^{3}H]tolyl)guanidine ([^{3}H]Tol$_2$Gdn; Fig. 1). We report here that [^{3}H]Tol$_2$Gdn binds reversibly, saturably, selectively, and with high affinity to σ binding sites in guinea pig brain membrane homogenates and slide-mounted rat and guinea pig brain sections, and we show that (+)-[^{3}H]3-PPP binds to the same sites.

MATERIALS AND METHODS

Synthesis of [^{3}H]Tol$_2$Gdn. A brominated precursor, 1,3-bis[(4-bromo-2-methyl)phenyl]guanidine, was synthesized as follows. To a stirred solution of cyanogen bromide (846 mg,

Abbreviations: [^{3}H]Tol$_2$Gdn, 1,3-di(2-[5-^{3}H]tolyl)guanidine; (+)-[^{3}H]3-PPP, tritiated (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; PCP, phencyclidine; SKF 10,047, *N*-allylnormetazocine; RP-HPLC, reversed-phase high-performance liquid chromatography.

8.06 mmol) in distilled water (70 ml) was added in small portions 2.997 g (16.11 mmol) of 4-bromo-2-methylaniline (Aldrich, recrystallized from ether/pentane). A white precipitate formed during the addition. The mixture was stirred at 80°C for 4 hr. Upon cooling at 0°C for 12 hr, a sticky yellow oil separated out and was discarded. The clear aqueous phase was concentrated to about 30 ml. The white precipitate that formed was redissolved by heating the mixture. This was then set aside at 4°C for 12 hr. Filtration gave 430 mg of white solid. A 200-mg portion was dissolved in 10 ml of hot water and treated with 5 ml of 10% KOH solution. The mixture was extracted with $CHCl_3$ and the extract was washed with brine and dried ($MgSO_4$). Evaporation of the solvent gave 171 mg of a brown solid, which was crystallized from $CHCl_3$, giving 120 mg (8%) of the brominated Tol_2Gdn derivative as small white needles: mp 209–210°C; NMR (300 MHz, $C^2H_3O^2H$, trimethylsilyl derivative) Δ 2.24 (s, 3), 7.12–7.25 (AB, 2, J = 8 Hz), 7.35 (s, 1); IR (KBr) 3460, 3340, 1630 cm^{-1}. Analysis calculated for $C_{15}H_{15}N_3Br_2$: C, 45.37; H, 3.81; N, 10.58. Found: C, 45.34; H, 3.56; N, 10.50.

Twenty-five milligrams (0.1 mmol) of the brominated derivative were submitted to Amersham for catalytic reduction in the presence of 20 Ci (1 Ci = 37 GBq) of $^3H_2$ gas. Portions (2 mCi) of the crude radioactive product were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Vydac TP218 C_{18} silica column, using a CH_3CN gradient (0–35% in 60 min) in 0.1% trifluoroacetic acid for elution. The radioactivity was eluted as a major, symmetrical peak coinciding with a major, symmetrical UV (220 nm)-absorbing peak at 41 min (Fig. 2). This is the same elution time at which authentic, unlabeled Tol_2Gdn emerges from the column in this RP-HPLC system. The specific activity of [^{3}H]Tol_2Gdn was found to be 52 Ci/mmol, based on the amount of Tol_2Gdn under the major UV-absorbing peak as determined by UV spectrophotometry and the amount of radioactivity associated with this peak as determined by liquid scintillation spectrometry.

Radioligand Binding Assays. Frozen guinea pig brains (Pel-Freeze) were homogenized in 10 volumes (wt/vol) of 0.32 M sucrose, using a Polytron (Brinkmann). The homogenate was centrifuged at 900 × g for 10 min at 4°C. The supernatant was collected and centrifuged at 22,000 × g for 20 min at 4°C. The pellet was resuspended in 10 volumes of 50 mM Tris/HCl buffer (pH 7.4), incubated at 37°C for 30 min, and centrifuged at 22,000 × g for 20 min at 4°C. The pellet was then resuspended in 10 volumes of 50 mM Tris/HCl (pH 7.4), and 10-ml aliquots were stored at −70°C until used. No effects of prolonged storage (>3 months) of the membranes at −70°C on σ receptor number or affinity for [^{3}H]Tol_2Gdn were observed.

For radioreceptor assays, aliquots of the frozen membrane suspension were thawed and diluted 1:3 with 50 mM Tris/HCl (pH 7.4). To 12 × 75-mm polystyrene or glass test tubes were added 0.8 ml of membrane suspension, 0.1 ml of [^{3}H]Tol_2Gdn or (+)-[^{3}H]3-PPP (New England Nuclear) for a final concentration of 0.9 nM, and 0.1 ml of unlabeled drugs or buffer. The protein concentration in the 1-ml final incubation volume was 800 μg, corresponding to 32 mg of brain tissue (original wet weight). Nonspecific binding was defined as that remaining in the presence of either 10 μM Tol_2Gdn or haloperidol, for both the [^{3}H]Tol_2Gdn and the (+)-[^{3}H]3-PPP binding. After incubation for 90 min at room temperature, the membrane suspension was rapidly filtered under vacuum through Whatman GF/B glass-fiber filters, using a Brandel 48-well cell harvester (Brandel, Gaithersburg, MD). The filters were washed three times with 5 ml of ice-cold 50 mM Tris/HCl buffer (pH 7.4 at room temperature). Each filter was dissolved in 10 ml of Cytoscint (Westchem Products, San Diego, CA), and radioactivity was measured by liquid scintillation spectrometry at a counting efficiency of 35–50%.

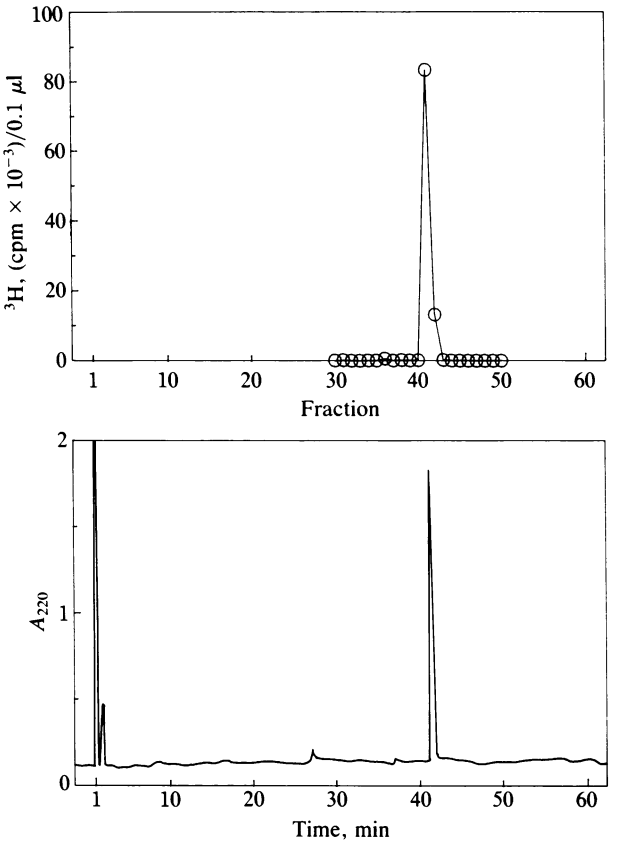

FIG. 2. RP-HPLC purification of [^{3}H]Tol_2Gdn. Crude radioactive product (2 mCi) was injected in 0.2 ml of 25% ethanol. Elution was with a CH_3CN gradient in 0.1% trifluoroacetic acid (see *Materials and Methods*). Flow rate was 1 ml/min. One-minute fractions were collected. Aliquots of the fractions were diluted 1:100 and 10-μl aliquots of the diluted fractions were dissolved in 10 ml of scintillation fluid for liquid scintillation counting. The HPLC equipment consisted of two Waters HPLC pumps, an automated electronic gradient controller, and a Kratos variable-wavelength UV spectrophotometer.

Saturation data were evaluated by Scatchard analysis using both the EBDA (22) and LIGAND (23) data analysis programs on an IBM Personal Computer-AT. IC_{50} values were determined by plotting displacement curves on semilogarithmic graph paper, followed by interpolation.

Autoradiographic Studies. Male Sprague–Dawley rats (200–250 g) and NIH guinea pigs (300–350 g) were killed, and their brains were rapidly removed and processed for receptor autoradiography according to the method of Herkenham and Pert (24). Slide-mounted brain sections (15 μm thick) were incubated for 45 min in 50 mM Tris/HCl (pH 8.0, 22°C) containing bovine serum albumin (1 mg/ml) and [^{3}H]Tol_2Gdn (2 nM). Adjacent sections were incubated with 10 μM haloperidol or 10 μM Tol_2Gdn to measure nonspecific binding. Incubations were terminated by four 2-min washes in 10 mM Tris/HCl (pH 7.4, 4°C) with bovine serum albumin (1 mg/ml), dried rapidly under a stream of cool air, and placed in x-ray cassettes with ^{3}H-sensitive film (^{3}H-Ultrofilm, LKB). Films were developed 6–8 weeks later (D-19, Kodak).

RESULTS

Characteristics of [^{3}H]Tol_2Gdn Binding to Guinea Pig Brain Membranes. Synthesis of [^{3}H]Tol_2Gdn resulted in a pure, homogeneous product of high specific radioactivity (52 Ci/mmol) (Fig. 2). [^{3}H]Tol_2Gdn bound specifically, saturably, reversibly, and with high affinity to guinea pig brain membranes. In a typical experiment with 0.9 nM [^{3}H]Tol_2Gdn (≈30,000 cpm, 50% counting efficiency), the total

binding was ≈2700 cpm, whereas the nonspecific binding in the presence of 10 μM Tol_2Gdn or 10 μM haloperidol was 50–150 cpm. Routinely, specific binding was 90–97% of total binding. At room temperature the binding of [^{3}H]Tol_2Gdn reached equilibrium after 60–90 min, and it was fully reversible after addition of 10 μM unlabeled Tol_2Gdn. Specific binding was linear with tissue concentration between 2 and 40 mg of tissue (original wet brain weight per assay tube). Binding of radioactivity to the glass-fiber filters in the absence of membranes was 10–20 cpm. Boiling of membranes at 100°C for 10 min prior to assay almost completely (>90%) abolished specific [^{3}H]Tol_2Gdn binding, as did treatment of the membranes with trypsin and Pronase (0.01 mg/ml for 30 min at room temperature), indicating that protein components are important for the receptors' binding ability (data not shown). Fig. 3 shows an equilibrium saturation binding curve for various concentrations of [^{3}H]Tol_2Gdn incubated with the brain membranes. Scatchard analysis (Fig. 3, *Inset*) of the saturation data shows a linear Scatchard plot with an apparent K_d of 28 nM and a maximum number of binding sites (B_{max}) of 84 pmol/g of brain tissue (original wet weight). Analysis of the binding data with the curve-fitting program LIGAND (23) showed high compatibility with a one-site binding model.

Drug Specificity of [^{3}H]Tol_2Gdn Binding. Displacement experiments were performed with drugs that are considered typical σ ligands (14–21), as well as with drugs considered to be prototypical ligands for other neurotransmitter, neuromodulator, and drug receptors. The experiments showed that the [^{3}H]Tol_2Gdn binding site was stereoselective for dextrorotatory benzomorphan opiates and for (−)-butaclamol; it did not interact significantly with drugs that have high affinities for acetylcholine, benzodiazepine, γ-aminobutyrate, or μ-, δ-, or κ-opioid receptors (Table 1). The [^{3}H]Tol_2Gdn binding site was found to have a high affinity for haloperidol and several drugs belonging to the phenothiazine class of antipsychotics (Table 1). Indeed, haloperidol had the highest displacement potency of all drugs tested (Table 1). Several other classes of psychoactive drugs had a moderate affinity for the [^{3}H]Tol_2Gdn binding site; these included several tricyclic antidepressants, PCP, and the κ-opioid-receptor ligand U50,488H (Table 1).

Drug Specificity of [^{3}H]Tol_2Gdn Binding Compared to (+)-[^{3}H]3-PPP Binding. (+)-[^{3}H]3-PPP has been described as a selective σ-receptor ligand in binding experiments with rat brain membranes (19–21). We compared the drug specificity of [^{3}H]Tol_2Gdn binding with that of (+)-[^{3}H]3-PPP binding in the guinea pig. We found that (+)-[^{3}H]3-PPP bound specifically, saturably (linear Scatchard plot), reversibly, and with high affinity to guinea pig brain membranes (K_d = 30 nM, B_{max} = 80 pmol/g of fresh brain weight; data not shown). The drug-specificity profile of (+)-[^{3}H]3-PPP binding in the guinea pig (Table 1) was found to be very similar to that reported in the rat (21). Moreover the drug-specificity profiles of typical σ-receptor-active drugs in the (+)-[^{3}H]3-PPP and [^{3}H]Tol_2Gdn binding assays were highly correlated (r = 0.95; $P < 0.00001$), suggesting that the two compounds labeled the same sites (Fig. 4).

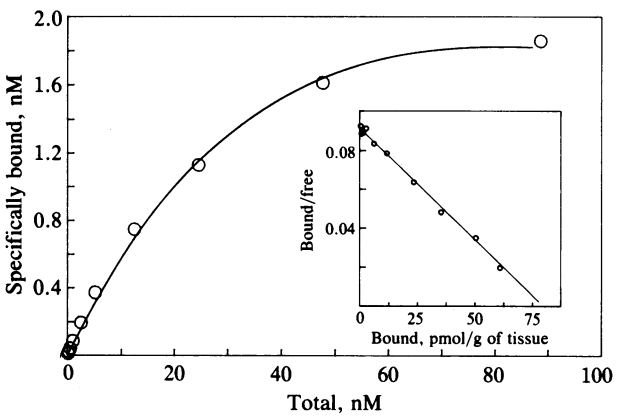

FIG. 3. Equilibrium saturation binding of [^{3}H]Tol_2Gdn to guinea pig brain membranes. Membranes were incubated with [^{3}H]Tol_2Gdn at various concentrations from 0.3 nM to 90 nM in 1 ml of 50 mM Tris/HCl buffer (pH 7.4) for 120 min at room temperature. Values are the means of quadruplicate determinations. (*Inset*) Scatchard plot of saturation binding data.

Table 1. Ability of various drugs to displace [^{3}H]Tol_2Gdn and (+)-[^{3}H]3-PPP from guinea pig brain membrane binding sites

Drug	IC_{50}, nM Against [^{3}H]Tol_2Gdn	IC_{50}, nM Against (+)-[^{3}H]3-PPP
Haloperidol	5.0 ± 0.3	17 ± 1
Tol_2Gdn	28 ± 1	53 ± 9
Perphenazine	42 ± 10	21 ± 3
(+)-Pentazocine	43 ± 2	8 ± 3
(−)-Pentazocine	135 ± 3	81 ± 1
(±) Pentazocine	69 ± 1	ND
(+)-3-PPP	76 ± 4	33 ± 12
(−)-3-PPP	280 ± 21	235 ± 60
(+)-Cyclazocine	365 ± 25	47 ± 12
(−)-Cyclazocine	2600 ± 210	1000 ± 0
Spiperone	690 ± 21	ND
(−)-Butaclamol	530 ± 49	183 ± 5
(+)-Butaclamol	2150 ± 250	2100 ± 71
(+)-SKF 10,047	625 ± 88	93 ± 5
(−)-SKF 10,047	4000 ± 566	2850 ± 390
PCP	1050 ± 106	1000 ± 71
U50,488H	1350 ± 106	ND
Trifluoperazine	345 ± 4	ND
Triflupromazine	605 ± 67	ND
Chlorpromazine	1475 ± 265	ND
Amitriptyline	300 ± 7	ND
Imipramine	520 ± 14	ND
Desipramine	4000 ± 212	ND
Nortriptyline	2000 ± 640	ND
Guanabenz	4600 ± 283	ND
Clonidine	>10,000	ND
Cocaine	>10,000	ND

The IC_{50} values represent the mean ± SEM from 2–4 separate experiments (in triplicate). ND, not determined. The following compounds caused no significant displacement at 10 μM concentration: scopolamine, 5-hydroxytryptamine, diazepam, bicuculline, picrotoxin, hexamethonium, dopamine, apomorphine, γ-aminobutyric acid, γ-guanidinobutyric acid, morphine, metorphamide, dynorphin A, [Leu^5]enkephalin, β-endorphin, naloxone, guanidinoacetic acid, creatine, creatinine, 1,1-dimethylguanidine, methylguanidine, β-guanidinopropionic acid, and cimetidine.

Autoradiographic Visualization of [^{3}H]Tol_2Gdn Binding. Receptor autoradiography studies on guinea pig and rat brain sections showed a low density of specific [^{3}H]Tol_2Gdn binding diffusely distributed throughout the gray matter of the rat and guinea pig brain. Superimposed on this homogeneous binding pattern was a heterogeneous distribution of enriched binding in limbic and sensorimotor structures. The pattern of binding was more distinct in the guinea pig than in the rat [similar observations for (+)-[^{3}H]3-PPP autoradiography have been reported (20)]; thus, description of [^{3}H]Tol_2Gdn binding was drawn primarily from the guinea pig. In the forebrain, limbic structures moderately to densely labeled by [^{3}H]Tol_2Gdn were the diagonal band of Broca, septum, hypothalamus (especially the paraventricular nucleus), anterodorsal thalamic nucleus, and zona incerta (Fig. 5 *A* and *B*). Sensorimotor thalamic nuclei moderately to densely labeled included the thalamic taste relay and reticular

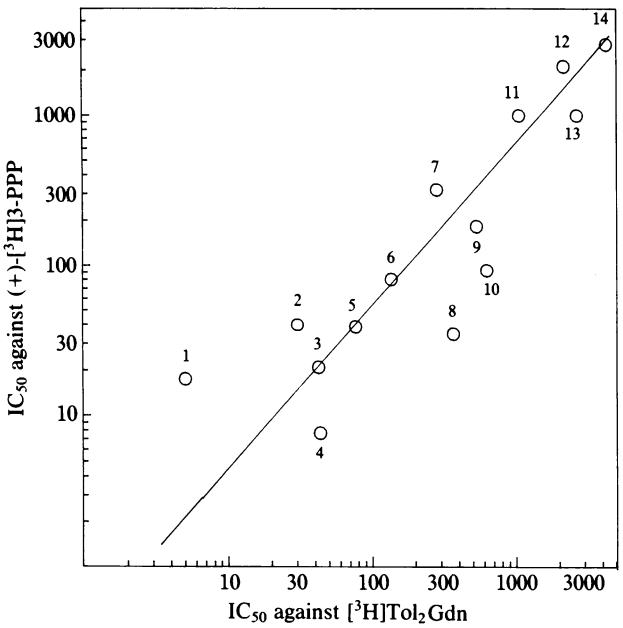

FIG. 4. Correlation of the IC_{50} values (nM) of typical σ-receptor-active drugs in the [^{3}H]Tol_2Gdn and (+)-[^{3}H]3-PPP binding assays. The correlation coefficient is 0.95 ($P < 0.00001$). 1, Haloperidol; 2, Tol_2Gdn; 3, perphenazine; 4, (+)-pentazocine; 5, (+)-3-PPP; 6, (−)-pentazocine; 7, (−)-3-PPP; 8, (+)-cyclazocine; 9, (−)-butaclamol; 10, (+)-SKF 10,047; 11, PCP; 12, (+)-butaclamol; 10, (+)-SKF 10,047; 11, PCP; 12, (+)-butaclamol; 13, (−)-cyclazocine; 14, (−)-SKF 10,047.

nuclei (Fig. 5*B*). Other thalamic nuclei labeled were the paraventricular and habenular nuclei. Very dense binding was seen in the choroid plexus. In the cortex, dense [^{3}H]Tol_2Gdn labeling occupied layer III/IV of retrosplenial (Fig. 5*C*), piriform, and entorhinal cortices. The rest of the cortex exhibited a low level of homogeneous binding. The hippocampal formation exhibited discrete binding in the pyramidal and granular cell layers (Fig. 5*B*). Sensorimotor areas of the midbrain were selectively labeled by [^{3}H]Tol_2-Gdn. The oculomotor nucleus, and more caudally, the trochlear nucleus were very densely labeled, and the superior colliculus and red nucleus had moderate levels of binding (Fig. 5*C*). Other midbrain nuclei labeled were the dorsal raphe, interpeduncular nucleus, central gray, and the substantia nigra, pars compacta. The selective labeling of the pars compacta in the guinea pig contrasted with the low to moderate density of labeling present throughout the substantia nigra of the rat. In addition, very dense binding was found in the subcommissural organ. In the hindbrain, the locus coeruleus was the most densely labeled nucleus. Sensorimotor nuclei enriched in [^{3}H]Tol_2Gdn binding sites were the trigeminal motor nucleus, nucleus of the facial nerve, nucleus of the solitary tract, dorsal motor nucleus of the vagus, and hypoglossal nucleus. Moderate to dense binding was also found throughout the gray matter of the cerebellum, and in the pontine reticular nuclei.

DISCUSSION

It has been suggested that σ-type receptors mediate the psychotomimetic effects of SKF 10,047 and related benzomorphan opiates (1–3). The characterization of these receptors *in vitro* has been difficult because of the lack of selective drug ligands. Most benzomorphan opiates crossreact with other (μ, δ, κ) opioid receptors and are therefore of only limited value for characterizing and isolating the receptors (9, 25, 26). We report here the synthesis of a tritiated drug, [^{3}H]Tol_2Gdn, that binds specifically and with high affinity to a single class of binding sites in guinea pig brain membranes. The binding characteristics and the drug-specificity profile of these sites are concordant with those proposed for the σ receptor (14–21), including (*i*) naloxone insensitivity and stereoselectivity for dextrorotatory isomers of benzomorphan opiates such as (+)-SKF 10,047, (+)-cyclazocine, and (+)-pentazocine; (*ii*) high affinity for haloperidol and certain phenothiazine antipsychotic drugs; and (*iii*) stereoselectivity for (−)-butaclamol. [^{3}H]Tol_2Gdn is one of only two known drugs that are selective for the σ site. The other, (+)-[^{3}H]3-PPP, originally proposed to be a dopamine autoreceptor agonist (27–29), has been shown to be selective for σ sites in rat brain membrane binding assays (21). In the present study, we have confirmed these findings in the guinea pig and have shown that [^{3}H]Tol_2Gdn and (+)-[^{3}H]3-PPP have virtually identical receptor binding characteristics and drug-selectivity profiles. σ sites can also be labeled with (±)-[^{3}H]SKF 10,047 (14), (±)-[^{3}H]ethylketazocine (15), and (+)-[^{3}H]SKF 10,047 (16–18); however, these ligands are not selective for the σ site and require the presence of appropriate drugs in binding assays to mask crossreacting non-σ binding sites (14, 15, 19, 20).

[^{3}H]Tol_2Gdn has a number of advantages as a σ ligand: it is highly selective for the σ site (unlike [^{3}H]SKF 10,047 and (±)-[^{3}H]ethylketazocine), it has a high degree of specific binding (90–97% of total binding), and it has a relatively simple chemical structure that is not chiral (unlike (+)-[^{3}H]3-PPP and the benzomorphan opiates). These characteristics make it a good starting compound for the synthesis of analogs for

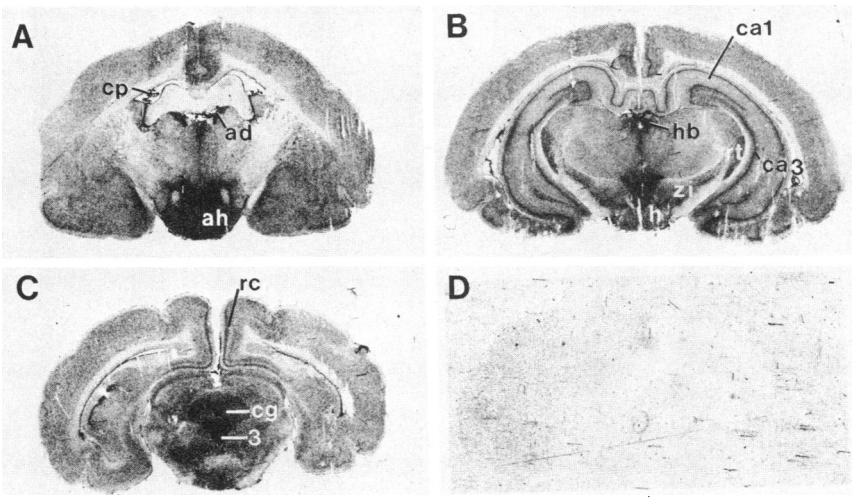

FIG. 5. Coronal sections (15 μm) of guinea pig brain with 2 nM [^{3}H]Tol_2Gdn, apposed to ^{3}H-sensitive film and developed 6–8 weeks later. (*A*) Dense binding in the choroid plexus (cp) and moderate to dense binding in the anterodorsal (ad) and paraventricular thalamus, piriform cortex, and anterior hypothalamus (ah). (*B*) Moderate to dense binding in hypothalamus (h), zona incerta (zi), medial and reticular thalamic nuclei (rt), and habenula (hb). In the hippocampus, peaks of dense binding are localized to the pyramidal and granule cell layers. (*C*) Dense binding in the central gray (cg), oculomotor nucleus (3), and layer IV of the retrosplenial cortex (rc). (*D*) Adjacent section incubated with 10 μM haloperidol.

structure–activity studies and for the design of irreversible σ-receptor ligands.

The σ site labeled with [^{3}H]Tol_2Gdn is clearly not related to conventional (μ, δ, κ) opioid receptors, as it is naloxone-insensitive and shows stereoselectivity for dextrorotatory isomers of benzomorphan drugs (Table 1). This is a reversed stereoselectivity compared to naloxone-sensitive opioid receptors, which are selective for levorotatory isomers of opiates (30). σ receptors should therefore not be referred to as σ "opioid" receptors. The drug selectivity of σ sites for dextrorotatory isomers of psychotomimetic opiates does, however, correlate well with the pharmacological profile of dextrorotatory vs. levorotatory opiates in animal tests designed to differentiate between conventional opioid-receptor activity and σ (behavioral) activity of benzomorphan drugs (4, 7, 8).

The autoradiographic studies using [^{3}H]Tol_2Gdn to visualize the σ site in slide-mounted brain sections also confirm that σ sites are different from μ-, δ-, and κ-opioid receptors, as the distribution of [^{3}H]Tol_2Gdn binding is rather distinct from the distribution of μ-, δ-, and κ-opioid receptors (31–33). The anatomical distribution of [^{3}H]Tol_2Gdn binding sites is, however, very similar if not identical to the distribution of (+)-[^{3}H]3-PPP binding sites (20, 21), further confirming the notion that the two radioligands label identical binding sites. The high affinity of the [^{3}H]Tol_2Gdn binding site for haloperidol and for certain phenothiazine antipsychotics (Table 1) that are also dopamine D_2-receptor antagonists raises the question as to the relation of σ receptors to dopamine D_2 receptors (34). Our results show that the [^{3}H]Tol_2Gdn site is clearly distinct from dopamine D_2 receptors, since the autoradiographic distribution of [^{3}H]Tol_2Gdn binding does not parallel the distribution of dopamine receptors, and dopamine and apomorphine do not interact with the [^{3}H]Tol_2Gdn binding site (ref. 34; Fig. 3, Table 1). Further, the σ site labeled with [^{3}H]Tol_2Gdn is stereoselective for (−)-butaclamol, whereas dopamine D_2 receptors are stereoselective for (+)-butaclamol (34).

The haloperidol-sensitive σ site labeled with [^{3}H]Tol_2Gdn was found to have a moderate affinity for the potent hallucinogen PCP in competition experiments (Table 1). This is in agreement with findings by others who used (+)-[^{3}H]SKF 10,047, (±)-[^{3}H]SKF 10,047, or (+)-[^{3}H]3-PPP to label σ sites (14–21). In PCP-receptor binding assays, however, [^{3}H]PCP labels predominantly (but not exclusively) a haloperidol-insensitive PCP binding site [termed PCP/σ (opiate) receptor by Zukin and colleagues (9, 12)] that is separate from the haloperidol-sensitive σ site labeled with [^{3}H]Tol_2Gdn or (+)-[^{3}H]3-PPP (19, 20). In contrast, [^{3}H]Tol_2Gdn appears to label exclusively the haloperidol-sensitive σ site, since all specific binding is inhibited by haloperidol and the anatomical distribution of [^{3}H]Tol_2Gdn binding (Fig. 5) is distinct from the distribution of PCP receptors (20, 35). Furthermore, unlabeled Tol_2Gdn is virtually inactive in a [^{3}H]PCP binding assay (unpublished observation). There is some controversy as to which of the two binding sites is responsible for causing the behavioral effects of PCP and psychotomimetic benzomorphan opiates and would therefore correspond to the σ receptor postulated by Martin *et al.* (3). Zukin and collaborators (12) have argued that the behavioral effects of both PCP and psychotomimetic benzomorphan opiates are mediated by the haloperidol-insensitive PCP site to which benzomorphan opiates bind with moderate affinity (20, 26). Largent *et al.* (20) cited circumstantial evidence suggesting that it is equally likely that the behavioral effects of both PCP and psychotomimetic opiates are mediated through the haloperidol-sensitive σ site. As [^{3}H]Tol_2Gdn exclusively labels the haloperidol-sensitive σ site and does not interact significantly with the haloperidol-insensitive PCP site, we suggest that behavioral studies using Tol_2Gdn as a prototype σ ligand may help to resolve this issue.

Perhaps the most important aspect of the findings on the drug specificity of σ sites that have emerged from this and other studies is that they interact with certain very potent psychotomimetic drugs (PCP, benzomorphans), as well as with antipsychotic drugs (haloperidol, phenothiazines) that are used clinically to treat schizophrenia. The use of Tol_2Gdn as a high selective σ-receptor ligand should facilitate investigations on a possible role of σ receptors in both abnormal brain function and in the pharmacological action of psychotomimetic and antipsychotic drugs.

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