ELSEVIER

European Journal of Pharmacology 285 (1995) 127–134

ejp

Discrepancies in characterization of σ sites in the mouse central nervous system

Katalin J. Kovács, Alice A. Larson *

Department of Veterinary Pathobiology, University of Minnesota, St. Paul, MN 55108, USA

Received 26 January 1995; revised 7 June 1995; accepted 20 June 1995

Abstract

The characteristics of [^{3}H](+)-pentazocine and [^{3}H]1,3-di(2-tolyl)guanidine (DTG) binding to mouse whole brain, cortex, cerebellum and spinal cord membranes were investigated in radioreceptor assays. [^{3}H](+)-Pentazocine bound to a single, high affinity site (K_d = 1.2–1.6 nM) with increasing density along the neuraxis from the cortex (B_{max} = 543 fmol/mg protein) to the spinal cord (B_{max} = 886 fmol/mg protein). Hot saturation studies resolved the presence of one binding site for [^{3}H]DTG showing no tissue variations in terms of density (B_{max} = 1075–1264 fmol/mg protein) or affinity (K_d = 16.6–22.3 nM). Incubation with 100 nM (+)-pentazocine revealed two classes of high affinity [^{3}H]DTG labeled binding sites corresponding to σ_1 and σ_2 subtypes. A preponderance of σ_2 sites was revealed in all investigated tissues. Different pharmacological profiles were demonstrated for the σ_2 sites in mouse whole brain compared to mouse spinal cord. However, competition studies indicated that the whole brain and spinal [^{3}H](+)-pentazocine labeled σ_1 binding sites exhibited similar pharmacological properties. The density of [^{3}H](+)-pentazocine labeled σ_1 population was found not to match that of [^{3}H]DTG labeled σ_1 site throughout the mouse central nervous system. The presence of low affinity [^{3}H]DTG labeled sites was demonstrated in cold saturation experiments. Equilibrium binding data for the low affinity [^{3}H]DTG binding site resulted in an increasing density (B_{max} = 1973–11369 fmol/mg protein) with a decreasing affinity (K_d = 242–943 nM) in mouse cortex through the spinal cord.

Keywords: σ Binding site, heterogeneity; Radioreceptor assay; DTG (1,3-di(2-tolyl)guanidine); (+)-Pentazocine; Brain; Spinal cord; (Mouse)

1. Introduction

Subtypes of σ sites have been found and characterized in the central nervous system and in a wide variety of peripheral tissues (for review see Su, 1993). Although an array of data are available to indicate their multiple functional role (for review see Su, 1993), no physiological or biochemical function has been incontrovertibly associated with σ receptor(s). The heterogeneity of σ subtypes and the lack of selective σ ligands have largely contributed to the poor consensus on the classification and functional role(s) of σ sites. Based on recent ligand-binding experiments and computer modeling, the possible existence of four pharmacologically distinct σ binding sites has been reported. These are (a) σ_1, (b) σ_2 (for review see Quirion et al., 1992), (c) dextromethorphan selective (Zhou and Musacchio, 1991) and (d) low affinity σ sites (Karbon et al., 1991; Wu et al., 1991; Codd and Shank, 1992; Connick et al., 1992).

Particular efforts have been taken in several laboratories to develop pharmacological tools to distinguish σ_1 from σ_2 sites. They differ from one another primarily in their stereoselectivity for (+)-benzomorphans, tissue distribution and sensitivity to GTP analogs. σ_1 Binding sites show a high (nanomolar) affinity for (+) isomers of benzomorphans such as pentazocine and *N*-allylnormetazocine (SKF 10,047) while only low to moderate affinities were reported for (−) isomers of these compounds (De Costa et al., 1989; Hellewell and Bowen, 1990; DeHaven-Hudkins et al., 1992; Bowen et al., 1993; Cagnotto et al., 1994). σ_1 Sites were found mostly in the central nervous system (CNS). Although

* Corresponding author. University of Minnesota, Department of Veterinary Pathobiology, 1988 Fitch Avenue, 295 Animal Science Bldg., St Paul, MN 55108, USA. Tel. (612) 624-3650, fax (612) 625-0204.

SSDI 0014-2999(95)00383-5

many findings support the possible link between σ_1 sites and GTP-binding regulatory proteins (Itzhak and Stein, 1991; Connick et al., 1992), some data are not compatible with G-protein coupling (DeHaven-Hudkins et al., 1992; Monnet et al, 1994). σ_2 Sites were found in high densities in several peripheral tissues (Dumont and Lemaire, 1991; Hellewell et al., 1994) and cell cultures (Hellewell and Bowen, 1990; Georg and Friedl, 1992). Their stereoselectivity profile for benzomorphanes was opposite to the one for σ_1 sites having a significantly higher affinity for (−) isomers than for (+) isomers of pentazocine and SKF 10,047 (Hellewell and Bowen, 1990; Dumont and Lemaire, 1991; Georg and Friedl, 1992; Hellewell et al., 1994). Other important tools allowing a clear distinction between σ_1 and σ_2 sites include 1,3-di(2-tolyl)guanidine (DTG) and phenytoin. [^{3}H]DTG binds with high, and essentially equal, affinity to both σ_1 and σ_2 sites (Hellewell and Bowen, 1990; Rothman et al., 1991). In the presence of σ_1 blockers, such as (+)-pentazocine, (+)-SKF 10,047 or dextrallorphan, [^{3}H]DTG has been used as a pharmacological tool for characterization of σ_2 sites. The allosteric modulator, phenytoin, also affects σ_1 sites without altering σ_2 binding (Musacchio et al., 1988; McCann and Su, 1991).

Marked species differences have been shown in the pharmacological profile of the σ binding sites in neural tissues of the rat and guinea pig (Klein and Musacchio, 1990; Walker et al., 1990; Rothman et al., 1991) as well as in the proportion of the σ_1 and σ_2 subtypes at the same locations (Leitner et al., 1994). Although the presence of the σ binding sites in spinal cord of the rat has been shown in autoradiographic studies (Gundlach et al., 1986; Aanonsen and Seybold, 1989), no comparative data from quantitative radioligand binding assays have been available characterizing subtypes of σ binding sites in spinal cord. In our previous study, differences in the rank order of potencies for displacers of [^{3}H]DTG binding were found in the mouse brain and spinal cord (Mousseau and Larson, 1994). In the present study, equilibrium bindings of [^{3}H](+)-pentazocine and [^{3}H]DTG to homogenates of mouse spinal cord were compared to those of whole brain, cortex and cerebellum. Subtypes of the σ sites were further characterized in competition experiments using various σ ligands. Some of these findings has been previously reported in abstract form (Kovács et al., 1994).

2. Materials and methods

2.1. Drugs and chemicals

[^{3}H](+)-Pentazocine (35.3 Ci/mmol) and [^{3}H]DTG (39.4 Ci/mmol) were purchased from Dupont/New England Nuclear (Boston, MA, USA). (+)-Pentazocine and (−)-pentazocine were provided by the National Institute on Drug Abuse. Dextromethorphan, 1,3-di(2-tolyl)guanidine (DTG), (+)-*N*-allylnormetazocine and (−)-*N*-allylnormetazocine (SKF 10,047) and (+)-3-(3-hydroxyphenyl)-*N*-1-(propyl)piperidine (3-PPP) were obtained from Research Biochemicals (Natick, MA, USA). Haloperidol was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Membrane preparation

Crude membranes were prepared for [^{3}H](+)-pentazocine and [^{3}H]DTG binding. Male Swiss-Webster mice (20–25 g, Sasco, Omaha, NE, USA) were decapitated and brains, brain sections and spinal cords were rapidly removed and homogenized in 40 volumes of 10 mM Tris-HCl buffer (pH 7.4) at 4°C with a Brinkmann Polytron (setting 8, for 5 s). The homogenate was centrifuged at 30 000 × *g* for 20 min at 4°C. The resulting pellet was resuspended in the same amount of buffer and incubated at 37°C for 30 min. The suspension was then centrifuged (30 000 × *g*, 20 min, 4°C) and the final pellet was resuspended in 15 volumes (350–400 μg/ml protein) of 50 mM Tris-HCl buffer (pH 7.7) at 37°C. The homogenate was used immediately for binding studies.

2.3. Receptor binding assays

Binding of [^{3}H](+)-pentazocine and [^{3}H]DTG to crude membranes of mouse whole brain, cortex, cerebellum and spinal cord tissues was performed in duplicate in 50 mM Tris-HCl buffer (pH 7.7) at 37°C for 210 min and 60 min respectively. For determination of equilibrium dissociation values (K_d) and the number of binding sites (B_{max}) saturation experiments were conducted over a concentration range of 0.05–24 nM [^{3}H](+)-pentazocine and 0.75–72 nM [^{3}H]DTG in the absence and presence of 100 nM (+)-pentazocine. To detect the low affinity [^{3}H]DTG labeled site, 3 nM [^{3}H]DTG was incubated in the presence of unlabeled DTG ranging in concentration from 0.1 nM to 1 μM. For inhibition assays, 0.75 nM [^{3}H](+)-pentazocine or 3 nM [^{3}H]DTG was incubated with 11 concentrations (0.1 nM–100 μM) of the unlabeled test ligands. Nonspecific binding was defined by addition of a final concentration of 10 μM haloperidol. The assays were terminated by rapid filtration through Whatman GF/C glass fiber filter on a Brandel cell harvester using 3 × 4 ml ice-cold 10 mM Tris-HCl buffer (pH 8.0) at 4°C. Filters were pre-soaked in 0.1% polyethylenimine for 2 h at 4°C prior to use. The filter-bound radioactivity was determined by liquid scintillation spectrometry at a 50% efficiency. Membrane protein concentrations were measured using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

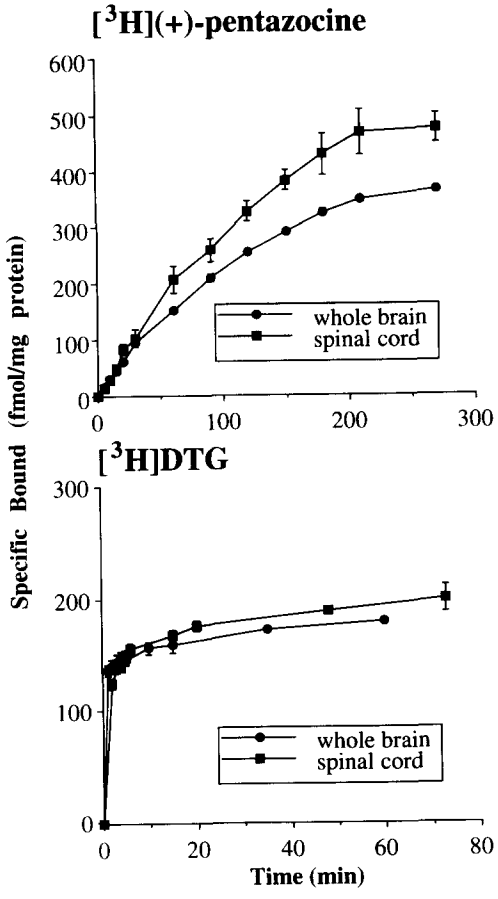

Fig. 1. Time course of binding of [^{3}H](+)-pentazocine (1 nM) and [^{3}H]DTG (3 nM) to mouse whole brain and spinal cord membranes. Experiments were performed in 50 mM Tris-HCl buffer (pH 7.7) at 37°C. The final assay volume was 1 ml with a tissue concentration of approximately 250 μg protein/ml. Non-specific binding was defined by addition of 10 μM haloperidol. The levels of non-specific binding for [^{3}H](+)-pentazocine were 7 fmol/mg protein and 6 fmol/mg protein, for [^{3}H]DTG were 17 fmol/mg protein and 50 fmol/mg protein in the whole brain and spinal cord homogenates, respectively. Each point represents the mean ± S.E.M. of 3 independent determinations performed in triplicate.

Equilibrium-saturation and inhibition binding data were analyzed with the EBDA (Munson and Rodbard, 1980) and LIGAND (McPherson, 1983) computer programs. One- and two-site models were fit to the data and an F-test was used to determine which model afforded the best fit to the data. K_i values, the affinity constants for inhibitors were calculated from IC_{50} values based on the Cheng-Prusoff equation (Cheng and Prusoff, 1973), assuming simple competitive interaction between radioligand and displacer. $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration and K_d is the equilibrium dissociation constants of the radioligand.

2.4. Statistical analysis

Statistical comparison of the K_d and B_{max} mean values was performed using one-way analysis of variance (ANOVA) followed by Scheffe's F-test with the level of significance set at $P < 0.05$. Student's two-tailed, unpaired t-test was used to determine the level of statistical difference between K_i mean values calculated from whole brain versus spinal cord membranes.

3. Results

Specific binding of [^{3}H](+)-pentazocine was monophasic, saturable and reached equilibrium slowly. At 37°C, maximal binding was not observed until 180–210 min of incubation (Fig. 1). After reaching equilibrium, the binding remained constant for at least an additional 4 h time period. Computer-assisted Scatchard analysis resulted in the selection of a one-site model as the best fit for [^{3}H](+)-pentazocine with K_d values that did not significantly vary between regions of the CNS (Table 1). The density of [^{3}H](+)-pentazocine binding sites (B_{max}), however, increased along the neuraxis from 543 ± 34 fmol/mg protein in the cortex to 886 ± 57 fmol/mg protein in the spinal cord (Table 1).

Specific binding of [^{3}H]DTG was markedly faster than that of [^{3}H](+)-pentazocine reaching its equilibrium after only a 10 min incubation period at 37°C (Fig. 1) and remained stable for at least 2 h. Saturation binding of [^{3}H]DTG was performed at increasing concentrations of the radioligand between 0.75 and 72 nM. A single high affinity binding site was observed. The best fit estimates of the K_d (16.6–22.3 nM) and B_{max} (1075–1264 fmol/mg protein) values were not significantly different in the whole brain, cortex, cerebellum or spinal cord (Table 2).

σ Binding sites in mouse whole brain and spinal cord were further characterized with competition studies displacing [^{3}H](+)-pentazocine or [^{3}H]DTG with

Table 1
Hot saturation binding parameters of [^{3}H](+)-pentazocine in mouse whole brain, cortex, cerebellum and spinal cord

Tissue	[^{3}H](+)-Pentazocine binding		
	n	K_d (nM)	B_{max} (fmol/mg protein)
Whole brain	8	1.3 ± 0.1	640 ± 37
Cortex	3	1.3 ± 0.6	543 ± 34
Cerebellum	3	1.6 ± 0.2	725 ± 32
Spinal cord	5	1.2 ± 0.1	886 ± 57 [a]

Binding was performed in 50 mM Tris-HCl buffer (pH 7.7) at 37°C. Experiments were conducted over a concentration range of 0.05–24 nM [^{3}H](+)-pentazocine for 210 min. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. The values were determined using the iterative curve-fitting program LIGAND and are the means ± S.E.M. of 3–8 independent determinations performed in duplicate.

[a] Significantly different from corresponding value obtained from whole brain or cortex ($P < 0.05$, ANOVA followed by Scheffe's F test).

Table 2
Hot saturation binding parameters for [^{3}H]DTG in the absence and the presence of 100 nM (+)-pentazocine in mouse whole brain, cortex, cerebellum and spinal cord

Tissue	[^{3}H]DTG binding					
	No (+)-pentazocine added			+100 nM (+)-pentazocine [a]		
	n	K_d (nM)	B_{max} (fmol/mg protein)	n	K_d (nM)	B_{max} (fmol/mg protein)
Whole brain	3	16.6 ± 0.7	1,108 ± 54	3	17.8 ± 0.8	823 ± 30 [b]
Cortex	4	18.5 ± 1.6	1,075 ± 65	4	21.3 ± 1.8	851 ± 41 [b]
Cerebellum	3	19.0 ± 1.8	1,120 ± 88	4	23.1 ± 0.8	795 ± 48 [b]
Spinal cord	4	22.3 ± 0.9	1,264 ± 116	4	30.0 ± 2.3 [c,d]	1148 ± 78

Binding was performed in 50 mM Tris-HCl buffer (pH 7.7) at 37°C. Experiments were conducted over a concentration range of 0.75–72 nM [^{3}H]DTG in the absence and presence of 100 nM (+)-pentazocine for 60 min. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. The values were determined using the iterative curve-fitting program LIGAND and are the means ± S.E.M. of 3–4 independent determinations performed in duplicate.

[a] Note, B_{max} in the presence of 100 nM (+)-pentazocine represents σ_2 binding. Estimated B_{max} of σ_1 site = B_{max} in the absence of (+)-pentazocine − B_{max} in the presence of 100 nM (+)-pentazocine. [b,c] Significantly different from value measured in the same tissue in the absence of (+)-pentazocine ([b]$P < 0.05$, [c]$P < 0.01$; unpaired Student's t-test). [d] Significantly different from values measured in the presence of (+)-pentazocine in the whole brain or the cortex ($P < 0.05$, ANOVA followed by Scheffe's F-test)

various σ ligands. The results of these studies are summarized in Table 3. All σ ligands tested inhibited [^{3}H](+)-pentazocine binding at a single site with high affinity. The calculated K_i values were not significantly different in homogenates of whole brain and spinal cord. Suggesting the involvement of σ_1 sites, the rank order of potency of σ ligands in displacing [^{3}H](+)-pentazocine was established as follows: haloperidol > (+)-pentazocine > (+)-SKF 10,047 > (−)-pentazocine > (+)-3-(3-hydroxyphenyl)-*N*-1-(propyl)piperidine ((+)-3-PPP) > dextromethorphan > DTG > (−)-SKF 10,047. Our finding indicates that

Table 3
Potencies (K_i (nM)) of various σ compounds to inhibit [^{3}H](+)-pentazocine and [^{3}H]DTG binding in mouse whole brain and spinal cord

	[^{3}H](+)-Pentazocine binding	[^{3}H]DTG binding			
		No (+)-pentazocine added			+100 nM (+)-pentazocine
		High affinity [a]	Low affinity	Not distinguished as high or low	
Brain					
(+)-Pentazocine	1.3 ± 0.1	1.3 ± 0.6	1212 ± 77		
DTG	44.9 ± 6.4	9.6 ± 1.2	468 ± 41		12.7 ± 1.6
(+)-SKF 10,047	5.1 ± 1.1	1.5 ± 0.1	11385 ± 970		9771 ± 225
(−)-Pentazocine	15.0 ± 2.0			33.7 ± 3.9	63.6 ± 1.1
(−)-SKF 10,047	935 ± 28			1284 ± 108	2159 ± 155
(+)-3-PPP	22.7 ± 2.7			85.1 ± 2.8	302 ± 38
Haloperidol	0.9 ± 0.1			28.2 ± 0.8	53.6 ± 1.3
Dextromethorphan	27.5 ± 0.6			547 ± 29	1740 ± 122
Spinal cord					
(+)-Pentazocine	1.2 ± 0.1	1.5 ± 0.2	1842 ± 49 [b]		
DTG	44.1 ± 2.2	8.1 ± 0.7	934 ± 69 [c]		83.6 ± 2.4 [c]
(+)-SKF 10,047	4.1 ± 0.8	2.4 ± 0.6	12100 ± 107		9685 ± 103
(−)-Pentazocine	14.1 ± 1.7			31.9 ± 4.3	67.8 ± 3.8
(−)-SKF 10,047	740 ± 34			1212 ± 83	2544 ± 258
(+)-3-PPP	24.2 ± 0.6			71.5 ± 7.4	267 ± 50
Haloperidol	1.0 ± 0.1			23.9 ± 4.2	93.4 ± 9.7 [b]
Dextromethorphan	32.1 ± 1.6			585 ± 99	2015 ± 213

Binding was performed in 50 mM Tris-HCl buffer (pH 7.7) at 37°C. 0.75 nM [^{3}H](+)-pentazocine was incubated with 11 concentrations (0.1 nM–100 μM) of the unlabeled test ligands for 210 min. For [^{3}H]DTG inhibition assays 3 nM radioligand was incubated with various concentrations of unlabeled test ligands ranging from 0.1 nM to 100 μM (to 1 μM for DTG) in the absence or in the presence of 100 nM (+)-pentazocine. Non-specific binding was defined using 10 μM haloperidol. The K_i values were determined using the iterative curve-fitting program LIGAND and are the means ± S.E.M. of 3–4 independent determinations performed in duplicate.

[a] Note, because DTG labels both σ_1 and σ_2 sites with equally high affinity, 'high affinity' [^{3}H]DTG binding means σ_1 and σ_2 sites together for DTG. As (+)-pentazocine and (+)-SKF 10,047 have a markedly higher affinity for σ_1 than σ_2 sites, the term 'high affinity' is associated only with σ_1 binding for those ligands. [b,c] Significantly different from corresponding value obtained from brain ([b]$P < 0.05$; [c]$P < 0.01$; unpaired Student's t-test).

(+)-pentazocine and (+)-SKF 10,047 were 10 to 200 times more potent displacers of [^{3}H](+)-pentazocine than their (−) isomers providing further evidence for stereospecific σ_1 binding in mouse brain and spinal cord.

Of several σ ligands tested in competition experiments, (+)-pentazocine and (+)-SKF 10,047 revealed two distinct, high affinity [^{3}H]DTG binding sites in either tissues (Fig. 2; Table 2). As both (+)-pentazocine and (+)-SKF 10,047 compete primarily for σ_1 sites (Quirion et al., 1992) and DTG binds to both σ_1 and σ_2 sites with essentially equal affinities, the two high affinity [^{3}H]DTG labeled sites in our study presumably corresponded to the σ_1 and σ_2 population of σ sites. Competition experiments revealed that 100 nM (+)-pentazocine was sufficient to inhibit approximately 30% of the high affinity [^{3}H]DTG binding (Fig. 3) yielding a K_i of 1.3–1.5 nM (Table 2). This portion of the high affinity [^{3}H]DTG binding was considered as

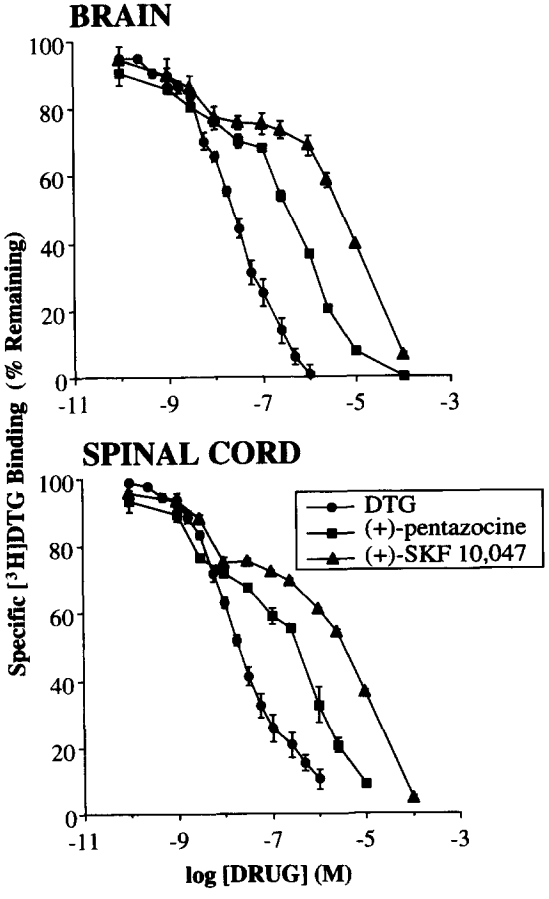

Fig. 2. Inhibition of [^{3}H]DTG binding by DTG, (+)-pentazocine and (+)-SKF 10,047 in mouse whole brain and spinal cord. [^{3}H]DTG (3 nM) was incubated in 50 mM Tris-HCl buffer (pH 7.7) at 37°C for 60 min in the presence of unlabeled ligands ranging in concentration from 0.1 nM to 100 μM. For DTG a concentration range from 0.1 nM to 1 μM was used to calculate the equilibrium binding data. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. Each point represents the mean ± S.E.M. of 3–4 independent determinations performed in duplicate.

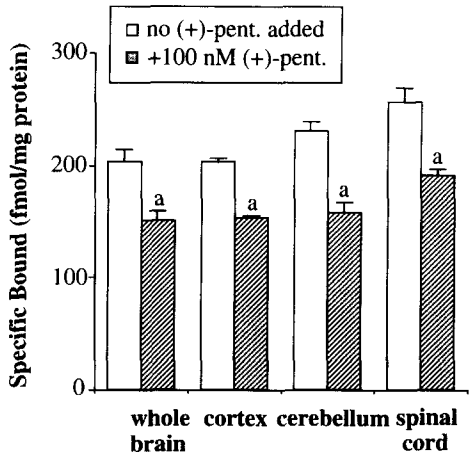

Fig. 3. Specific binding of [^{3}H]DTG to mouse whole brain, cortex, cerebellum and spinal cord homogenates. Membranes were incubated with 3 nM [^{3}H]DTG in 50 mM Tris-HCl buffer (pH 7.7) at 37°C for 60 min, in the absence and the presence of 100 nM (+)-pentazocine. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. Each value represents the mean ± S.E.M. of 3 independent determinations performed in triplicate. [a] Statistically different from bound [^{3}H]DTG values calculated in the absence of (+)-pentazocine, $P < 0.05$, paired Student's t-test.

σ_1 binding. Hence, the presence of 100 nM (+)-pentazocine could in fact mask σ_1 sites providing an effective pharmacological tool to study heterogeneity of the σ sites. In the presence of 100 nM (+)-pentazocine, competition between [^{3}H]DTG and a variety of σ ligands produced a rank order of potency of ligands that closely resembled that of the σ_2 site. The absence of stereoselectivity of [^{3}H]DTG binding for (−)-pentazocine and (−)-SKF 10,047 also indicated that the mouse whole brain and spinal cord homogenates contain binding sites with characteristics of the σ_2 site. At σ_2 sites, studied in the presence of 100 nM (+)-pentazocine, inhibition of [^{3}H]DTG binding by DTG, (+)-pentazocine or haloperidol was found to have significantly higher potencies in the brain than the spinal cord membranes (Table 3). Also, significant regional differences were found in the rank order of potencies for σ ligands in displacing [^{3}H]DTG in the presence of 100 nM (+)-pentazocine in the spinal cord versus whole brain. The rank order of potency was in whole brain: DTG > haloperidol > (−)-pentazocine > (+)-3-PPP > (+)-pentazocine > dextromethorphan > (−)-SKF 10,047 > (+)-SKF 10,047, whereas in spinal cord: (−)-pentazocine > DTG > haloperidol > (+)-3-PPP > (+)-pentazocine > dextromethorphan > (−)-SKF 10,047 > (+)-SKF 10,047.

After establishing the presence of σ_1 and σ_2 sites in tissues of the mouse CNS, equilibrium binding parameters for these two high affinity [^{3}H]DTG labeled σ sites were assessed. When the σ_1 site was covered by 100 nM (+)-pentazocine, equal densities and same affinities for the σ_2 sites were found in the whole

Table 4
Cold saturation binding parameters for [^{3}H]DTG in mouse whole brain, cortex, cerebellum and spinal cord

Tissue	[^{3}H]DTG binding				
	High affinity			Low affinity	
	n	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
Whole brain	8	9.6 ± 1.2	637 ± 95	468 ± 41	5590 ± 281
Cortex	3	8.7 ± 1.2	640 ± 124	242 ± 35	1973 ± 78
Cerebellum	3	9.4 ± 1.1	687 ± 54	667 ± 109	3485 ± 206
Spinal cord	5	8.1 ± 0.7	617 ± 53	943 ± 69 [a]	11369 ± 1345 [a,b]

Binding was performed in 50 mM Tris-HCl buffer (pH 7.7) at 37°C. For determination of equilibrium dissociation values (K_d) and the number of binding sites (B_{max}), 3 nM [^{3}H]DTG was incubated for 60 min in the presence of unlabeled ligand ranging in concentration from 0.1 nM to 1 μM. Non-specific binding was defined by addition of a final concentration of 10 μM haloperidol. The values were determined using the iterative curve-fitting program LIGAND and are the means ± S.E.M. of 3–8 independent determinations performed in duplicate.
[a] Significantly different from corresponding value obtained from whole brain or cortex ($P < 0.05$, ANOVA followed by Scheffe's F-test).
[b] Significantly different from corresponding value obtained from cerebellum ($P < 0.05$, ANOVA followed by Scheffe's F-test).

brain, cortex and cerebellum membranes (Table 2). The density of σ_1 sites was calculated by subtracting the number of [^{3}H]DTG labeled sites (B_{max}) detected in the presence of 100 nM (+)-pentazocine from that of measured in the absence of (+)-pentazocine. For whole brain, cortex and cerebellum we found σ_1 sites densities of 285, 224 and 325 fmol/mg protein, respectively. In spinal cord, however, hot saturation experiments revealed an increased K_d of DTG which was accompanied by an unchanged B_{max} in the presence of 100 nM (+)-pentazocine (Table 2). This suggests a very low density of σ_1 sites in the spinal cord of the mouse.

To detect the low affinity [^{3}H]DTG labeled site, cold saturation experiments were performed. Scatchard analysis of these data indicated the existence of two classes of binding sites as the two-site model yielded a significantly better fit (F-test, $P < 0.001$) than fitting experimental data to a one-site model. The calculated numerical values are reported in Table 4. The best fit estimates of the K_{dH} (8.1–9.6 nM) and B_{maxH} (617–687 fmol/mg protein) values for the high affinity [^{3}H]DTG labeled binding were not significantly different in the whole brain, cortex, cerebellum or spinal cord. [^{3}H]DTG also labeled another, bigger population of binding sites with lower affinity. The affinity of DTG to the low affinity σ site was significantly lower in the spinal cord than in the whole brain or the cortex. The density of [^{3}H]DTG labeled low affinity binding site in mouse spinal cord was approximately 6 times greater than the amount observed in cortex and approximately 3 times greater compared to the cerebellum.

4. Discussion

The present study provides the first equilibrium binding data and pharmacological characterization of σ binding sites in mouse whole brain, cortex, cerebellum and spinal cord labeled by [^{3}H](+)-pentazocine and [^{3}H]DTG.

[^{3}H](+)-Pentazocine has been proposed to bind to a single, high affinity site in the CNS (De Costa et al., 1989; DeHaven-Hudkins et al., 1992; Bowen et al., 1993; Cagnotto et al., 1994). The presence of another σ site in the human cerebellum recognized by [^{3}H](+)-pentazocine with affinity in the nanomolar range has been recently reported (Zabetian et al., 1994). In the present study [^{3}H](+)-pentazocine labeled a single class of σ sites with similar K_d values throughout the CNS. Regional differences in the density of σ_1 receptor subtype in rat (Leitner et al., 1994; McCann et al., 1994) or guinea pig brain (Walker et al., 1992) have previously been reported. In our present experiments, a significantly larger population of [^{3}H](+)-pentazocine labeled sites was found in the spinal cord as compared to the cortex or the whole brain. This suggested a differential distribution of [^{3}H](+)-pentazocine labeled σ_1 sites. K_i data from competition studies using various σ ligands revealed that [^{3}H](+)-pentazocine labeled binding sites in the whole brain and spinal cord were identical.

The present data confirm that [^{3}H]DTG interacts with more than one class of binding sites (Hellewell and Bowen, 1990; Karbon et al., 1991; Rothman et al., 1991; Codd and Shank, 1992; Connick et al., 1992). It labeled two sites, σ_1 and σ_2 sites, with high affinity, and an additional, large population of binding sites with low affinity. The presence of the σ_2 receptor subtype in mouse brain and spinal cord was confirmed by the rank order of potency and lack of selectivity of σ ligands when the σ_1 site was masked with 100 nM (+)-pentazocine. Similar densities and affinities for σ_2 sites were detected in whole brain, cortex and cerebellum. However, we found several discrepancies in the characterization of spinal σ_2 binding sites: (a) In the spinal cord, [^{3}H]DTG labeled a larger population of the σ_2 sites with lower affinity. (b) Potency of (+)-pentazocine, haloperidol and DTG displacing [^{3}H]DTG was 1.5-, 1.7-, and 6.6-fold higher in the brain than in the spinal cord. (c) The rank order of potency for (−)-pentazocine, haloperidol and DTG was different

in spinal cord from that found in the brain. These discrepancies raise the possibility that the σ_2 sites located in the spinal cord may not be identical with those found in various brain structures.

A large population of [^{3}H]DTG labeled low affinity binding has been described in the guinea pig (Karbon et al., 1991; Zhou and Musacchio, 1991) and rat brains (Codd and Shank, 1992). The existence of the low affinity [^{3}H]DTG binding site in the mouse CNS was confirmed in our cold saturation experiments using 3 nM [^{3}H]DTG. The density of low affinity σ sites was increased while their affinity was decreased along the neuraxis from cortex to spinal cord. The pharmacological characteristics and function of the low affinity site are presently unclear. The possibility that a substantial component of [^{3}H]DTG binding described as binding to low affinity sites may represent nonspecific binding has also been raised (Basile et al., 1994).

Variation in the ratio of σ_1 and σ_2 binding across brain regions is subject of intense research. Survey of autoradiographic distribution of [^{3}H](+)-pentazocine and [^{3}H]DTG binding in various brain regions of the guinea pig showed significant correlations, but in certain nuclei different ratios were found (Walker et al. 1992). The different distribution of σ_1 and σ_2 binding among brain regions is consistent with receptor binding assays using rat brain (Leitner et al., 1994; McCann et al., 1994). Enrichment of σ_2 sites in rat cortex, and a ratio of σ_1 and σ_2 sites close to 1.0 in rat cerebellum were reported by McCann and coworkers (1994). However, the amount of ligand bound to σ_2 sites exceeded the amount bound to σ_1 sites by 300% in both cortex and cerebellum in an other study (Leitner et al., 1994). The presence of larger densities of [^{3}H]DTG labeled σ_2 sites compared to [^{3}H](+)-pentazocine labeled σ_1 sites in the cortex and cerebellum was also confirmed by the present data. The preponderance of σ_2 sites was revealed in the mouse spinal cord as well. However, the density of the [^{3}H](+)-pentazocine labeled σ_1 population was found not to match that of the [^{3}H]DTG labeled σ_1 sites throughout the mouse CNS. Proportions of (+)-pentazocine labeled σ_1 to [^{3}H]DTG labeled σ_1 site varied by anatomical regions producing ratios ranging from 2.2, 2.4, 2.2 to 7.6 in the whole brain, cortex, cerebellum and spinal cord, respectively. This is in agreement with the proposal that in rat brain [^{3}H]DTG labels primarily the σ_2 site under physiological conditions (Codd and Shank, 1992; Connor and Chavkin, 1992).

Marked species differences have been described among the characteristics of σ binding sites (Klein and Musacchio, 1990; Walker et al., 1990; Rothman et al., 1991; Cagnotto et al., 1994; Leitner et al., 1994). In our study, the potency of (+)-SKF 10,047 relative to (+)-pentazocine, in displacing bound [^{3}H](+)-pentazocine was 5–22 times higher in mouse brain as compared to guinea pig brain (De Costa et al., 1989; DeHaven-Hudkins et al., 1992). Also, DTG has been characterized as a good inhibitor of [^{3}H](+)-pentazocine binding in rat (Cagnotto et al., 1994) or guinea pig brain (De Costa et al., 1989; DeHaven-Hudkins et al., 1992). But we found it to be second to the last in the rank order of potency, followed only by (−)-SKF 10,047. Recent findings suggest a significant species difference in the proportions of the σ receptor subtypes as well. Varying ratios of the [^{3}H]DTG labeled σ_2 to [^{3}H](+)-pentazocine labeled σ_1 binding have been reported; 1.63–3.51 for rat brain regions and 0.67 for guinea pig whole brain (Leitner et al., 1994). Here we report ratios of 1.29, 1.57, 1.01 and 1.30 for whole brain, cortex, cerebellum and spinal cord of the mouse, respectively. Our findings confirmed the presence of species differences in the properties of σ receptor subtypes.

In conclusion, the present study illustrates the complexity of the use of [^{3}H]DTG as a ligand for σ receptors (Connick et al., 1992) as the following discrepancies were found comparing the binding of [^{3}H](+)-pentazocine and [^{3}H]DTG. (a) The density and distribution of σ_1 sites labeled by [^{3}H]DTG and calculated either from hot or cold saturation experiments, were not paralleled by those of labeled by [^{3}H](+)-pentazocine. (b) The brain and spinal [^{3}H](+)-pentazocine labeled σ_1 binding sites exhibited similar pharmacological properties whereas different pharmacological profiles were found for the σ_2 sites in these two tissues. Introduction of ligands showing selectivity for σ_2 receptor subtypes (Bonhaus et al., 1993; Bertha et al., 1994; De Costa et al., 1994) can provide additional evidence for the distinct nature of the spinal σ_2 site which we observed when we compared it to the σ_2 site of the brain.

Acknowledgements

This work was supported by U.S. Public Health Service Grant DA 04090. We thank Dr. Dénes Budai for his careful review of the manuscript and Drs. Virginia M. Goettl and Julie S. Kreeger for their helpful editorial suggestions.

References

Aanonsen, L.M. and V.S. Seybold, 1989, Phencyclidine and sigma receptors in rat spinal cord: binding characterization and quantitative autoradiography, Synapse 4, 1.

Basile, A.S., B.R. De Costa and I.A. Paul, 1994, Multiple [^{3}H]DTG binding sites in guinea pig cerebellum: evidence for the presence of non-specific binding, Eur. J. Pharmacol. 252, 139.

Bertha, C.M., B.J. Vilner, W. Williams, K.C. Rice and W.D. Bowen, 1994, E-8-benzylidene-2-methyl-5-phenylmorphans: a novel class of high affinity ligands which exhibit sigma 1 or sigma 2 subtype selectivity, Soc. Neurosci. Abstr. 314.2.

Bonhaus, D.W., D.N. Loury, L.B. Jakeman, Z. To, A. DeSouza, R.M. Eglen and E.H.F. Wong, 1993, [^{3}H]BIMU-1, a 5-hydroxytryptamine$_3$ receptor ligand in NG-108 cells, selectively labels sigma-2 binding sites in guinea pig hippocampus, J. Pharmacol. Exp. Ther. 267, 961.

Bowen, W.D., B.R. De Costa, S.B. Hellewell, J.M. Walker and K.C. Rice, 1993, [^{3}H](+)-Pentazocine: a potent and highly selective benzomorphan-based probe for sigma-1 receptors, Mol. Neuropharmacol. 3, 117.

Cagnotto, A., A. Bastone and T. Mennini, 1994, [^{3}H](+)-Pentazocine binding to rat brain σ_1 receptors, Eur. J. Pharmacol. Mol. Pharmacol. 266, 131.

Cheng, Y.C. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3090.

Codd, E.E. and R.P. Shank, 1992, 1,3-Di(2-[5-^{3}H]tolyl)guanidine labels more than one site in rat forebrain, Eur. J. Pharmacol. 217, 149.

Connick, J.H., G. Hanlon, J. Roberts, L. France, P.K. Fox and C.D. Nicholson, 1992, Multiple σ binding sites in guinea-pig and rat brain membranes: G-protein interactions, Br. J. Pharmacol. 107, 726.

Connor, M.A. and C. Chavkin, 1992, Ionic zinc may function as an endogenous ligand for the haloperidol-sensitive σ_2 receptor in rat brain, Mol. Pharmacol. 42, 471.

De Costa, B.R., W.D. Bowen, S.B. Hellewell, J.M. Walker, A. Thurkauf, A.E. Jacobson and K.C. Rice, 1989, Synthesis and evaluation of optically pure [^{3}H]-(+)-pentazocine, a highly potent and selective radioligand for σ receptors, FEBS Lett. 251, 53.

De Costa, B.R., X. He, C. Dominguez, J. Cutts, W. Williams and W.D. Bowen, 1994, A new approach to the design of sigma-2-selective ligands: synthesis and evaluation of *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)ethylamine-related polyamines at σ-1 and σ-2 receptor subtypes, J. Med. Chem. 37, 314.

DeHaven-Hudkins, D.L., L.C. Fleissner and F.Y. Ford-Rice, 1992, Characterization of the binding of [^{3}H](+)-pentazocine to σ recognition sites in guinea pig brain, Eur. J. Pharmacol. Mol. Pharmacol. 227, 371.

Dumont, M. and S. Lemaire, 1991, Interaction of 1,3-di(2-[5-^{3}H]tolyl) guanidine with σ_2 binding sites in rat heart membrane preparations, Eur. J. Pharmacol. 209, 245.

Georg, A. and A. Friedl, 1992, Characterization of specific binding sites for [^{3}H]-1,3-di-*o*-tolyl-guanidine (DTG) in rat glioma cell line C6-BU-1, Glia 6, 258.

Gundlach, A.L., B.L. Largent and S.H. Snyder, 1986, Autoradiographic localization of sigma receptor binding sites in guinea pig and rat central nervous system with (+)^{3}H-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine, J. Neurosci. 6, 1757.

Hellewell, S.B. and W.D. Bowen, 1990, 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.

Hellewell, S.B., A. Bruce, G. Feinstein, J. Orringer, W. Williams and W.D. Bowen, 1994, Rat liver and kidney contain high densities of σ_1 and σ_2 receptors: characterization by ligand binding and photoaffinity labeling, Eur. J. Pharmacol. Mol. Pharmacol. 268, 9.

Itzhak, Y. and I. Stein, 1991, Regulation of sigma receptors and responsiveness to guanine nucleotides following repeated exposure of rats to haloperidol: further evidence for multiple sigma binding sites, Brain Res. 566, 166.

Karbon, E.W., K. Naper and M.J. Pontecorvo, 1991, [^{3}H]DTG and [^{3}H](+)-3-PPP label pharmacologically distinct σ binding sites in guinea pig brain membranes, Eur. J. Pharmacol. 193, 21.

Klein, M. and J.M. Musacchio, 1990, Computer-assisted analysis of dextromethorphan and (+)-3-(-3-hydroxyphenyl)-*N*-(1-propyl)piperidine binding sites in rat brain. Allosteric effects of ropizine, Life Sci. 47, 1625.

Kovács, K.J., D. Budai and A.A. Larson, 1994, Comparison of sigma receptor binding in mouse brain and spinal cord, Soc. Neurosci. Abstr. 478.17.

Leitner, M.L., A.G. Hohmann, S.L. Patrick and J.M. Walker, 1994, Regional variation in the ratio of σ_1 to σ_2 binding in rat brain, Eur. J. Pharmacol. 259, 65.

Lowry, O.H., M.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

McCann, D.J. and T.P. Su, 1991, Solubilization and characterization of haloperidol-sensitive (+)-[^{3}H]SKF-10,047 binding sites (sigma sites) from rat liver membranes, J. Pharmacol. Exp. Ther. 257, 547.

McCann, D.J., A.D. Weissman and T.P. Su, 1994, Sigma-1 and sigma-2 sites in rat brain: comparison of regional, ontogenetic, and subcellular patterns, Synapse 17, 182.

McPherson, G.A., 1983, A practical computer-based approach to the analysis of radioligand binding experiments, Comput. Programs Biomed. 17, 107.

Monnet, F.P., G. Debonnel, R. Bergeron, B. Gronier and C. De Montigny, 1994, The effects of sigma ligands and neuropeptide Y on *N*-methyl-D-aspartate-induced neuronal activation of CA_3 dorsal hippocampus neurones are differentially affected by pertussis toxin, Br. J. Pharmacol. 112, 709.

Mousseau, D.D. and A.A. Larson, 1994, Substance P analogs displace sigma binding differentially in the brain and spinal cord of the adult mouse, Metab. Brain Dis. 9, 249.

Munson, P.J. and D. Rodbard, 1980, LIGAND: a versatile computerized approach for characterization of ligand-binding systems, Anal. Biochem. 107, 220.

Musacchio, J.M., M. Klein and L.J. Santiago, 1988, High affinity dextromethorphan binding sites in guinea pig brain: further characterization and allosteric interactions, J. Pharmacol. Exp. Ther. 247, 424.

Quirion, R., W.D. Bowen, Y. Itzhak, J.L. Junien, J.M. Musacchio, R.B. Rothman, T.P. Su, S.W. Tam and D.P. Taylor, 1992, A proposal for the classification of sigma binding sites, Trends Pharmacol. Sci. 13, 85.

Rothman, R.B., A. Reid, A. Mahboubi, C.H. Kim, C.B. De, A.E. Jacobson and K.C. Rice, 1991, 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, Mol. Pharmacol. 39, 222.

Su, T.P., 1993, Delineating biochemical and functional properties of sigma receptors: emerging concepts, Crit. Rev. Neurobiol. 7, 187.

Walker, J.M., W.D. Bowen, F.O. Walker, R.R. Matsumoto, B.R. De Costa and K.C. Rice, 1990, Sigma receptors: biology and function, Pharmacol. Rev. 42, 355.

Walker, J.M., W.D. Bowen, S.R. Goldstein, A.H. Roberts, S.L. Patrick, A.G. Hohmann and B.R. De Costa, 1992, Autoradiographic distribution of [^{3}H](+)-pentazocine and [^{3}H]1,3-di-*o*-tolylguanidine (DTG) binding sites in guinea pig brain: a comparative study, Brain Res. 581, 33.

Wu, X.Z., J.A. Bell, C.E. Spivak, E.D. London and T.P. Su, 1991, Electrophysiological and binding studies on intact NCB-20 cells suggest presence of a low affinity sigma receptor, J. Pharmacol. Exp. Ther. 257, 351.

Zabetian, C.P., J.K. Staley, D.D. Flynn and D.C. Mash, 1994, [^{3}H](+)-Pentazocine binding to sigma recognition sites in human cerebellum, Life Sci. 55, PL 389.

Zhou, G.Z. and J.M. Musacchio, 1991, Computer-assisted modeling of multiple dextromethorphan and sigma binding sites in guinea pig brain, Eur. J. Pharmacol. Mol. Pharmacol. 206, 261.