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Multiple σ binding sites in guinea-pig and rat brain membranes: G-protein interactions

[1] J.H. Connick, G. Hanlon, J. Roberts, L. France, P.K. Fox & C.D. Nicholson

Department of Pharmacology, Organon Laboratories Limited, Newhouse, Lanarkshire ML1 5SH

1 Evidence is accumulating for multiple sigma (σ) sites in the mammalian CNS.

2 We have addressed this problem and have examined σ site - G-protein coupling in guinea-pig and rat brain membranes.

3 Ditolylorthoguanidine (DTG), (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine (3PPP) and dextromethorphan displaced [^{3}H]-DTG (3.4 nM) with low Hill slopes of 0.5, 0.6 and 0.6, respectively in guinea-pig brain membranes.

4 In the presence of 5'-guanylylimidodiphosphate (Gpp(NH)p; 100 μM), the specific binding of [^{3}H]-DTG was reduced by 36.7%, the Hill slope of 3PPP was increased to near unity, the ability of dextromethorphan to displace DTG was virtually abolished and the Hill slope for DTG remained low (0.7), indicating the presence of at least two binding sites. These data indicate that although Gpp(NH)p removes a dextromethorphan high affinity site, two DTG selective sites remain in the presence of Gpp(NH)p.

5 The present study suggests that DTG binds to at least three sites in guinea-pig brain membranes, at least one of which is G-protein linked.

6 In rat brain membranes, DTG displaced itself (3.4 nM) with a Hill slope near 1. 3PPP displacement of [^{3}H]-DTG was comparable with the guinea-pig (Hill slope 0.5) and displaced from more than 1 site. Dextromethorphan did not displace [^{3}H]-DTG at concentrations below 10 μM.

7 The heterogeneity of σ sites appears to be less in rat than in guinea-pig brain membranes.

Keywords: σ binding sites; ditolyorthoguanidine (DTG); dextromethorphan; guanine nucleotide binding proteins; antipsychotics

Introduction

The sigma (σ) binding site in the mammalian central nervous system has been intensively investigated by use of ligand binding methodology. A unique pattern of sensitivity of the σ site to a wide variety of different compounds, (Walker *et al.*, 1990) together with their anatomical distribution in brain (Gundlach *et al.*, 1986), distinguishes this site from any known extracellular receptor sensitive to neurotransmitters or neuromodulators. However, since it has not proved possible to associate unequivocally modulation of physiological function with the interaction of ligands at the σ site, it remains unknown whether this site is a biologically active receptor. Furthermore, assuming the σ site is a functional receptor it remains unknown whether ligands act as agonists or antagonists.

Amongst the most commonly used and selective ligands used to characterize σ binding are ditolylorthoguanidine (DTG) and (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine (3PPP). DTG was originally reported to bind with high affinity to a single class of binding sites in guinea-pig brain membranes (Weber *et al.*, 1986) and to be a specific σ ligand (Weber *et al.*, 1986). Similarly, 3PPP selectively binds to σ binding sites (Weber *et al.*, 1986). Subsequent investigations of the binding profiles of these ligands have led investigators to suggest that there are two σ sites; σ_1 and σ_2 (Hellewell & Bowen, 1990; Quirion *et al.*, 1992). Other groups have suggested that as many as four binding sites (R_1–R_4) are identifiable (Zhou & Musacchio, 1991).

Multiple affinity states of a single receptor have also been proposed in explanation of modulation of [^{3}H]-3PPP binding in rat brain membranes by GTP binding protein modifying agents (Itzhak, 1989; Beart *et al.*, 1989). These studies, which indicate an interaction with G-proteins, are major pieces of evidence for a receptor role for σ sites.

The aim of the present study was to investigate further the heterogeneity of σ sites and affinity states in guinea-pig brain membranes by use of [^{3}H]-DTG and [^{3}H]-3PPP as ligands. Experiments were also conducted to examine the potential interaction of σ sites with G-proteins. Additional experiments were performed with rat brain membranes to examine the possibility that species differences may exist in the heterogeneity of sites.

Preliminary results of this investigation have been presented previously (Connick *et al.*, 1991).

Methods

Brains from male Dunkin-Hartley guinea-pigs (approximately 350 g) or male Wistar rats (approximately 250 g) were homogenized in ice-cold sucrose (0.32 M) with 8 strokes of a Potter-'S' homogenizer at 850 r.p.m. and centrifuged at 900 *g* for 10 min at 4°C. The resultant pellet was resuspended in 10 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4). This was subsequently centrifuged twice (20,000 *g* for 20 min) before final resuspension in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) for experiments with [^{3}H]-DTG or 5 mM Tris-HCl (pH 7.7) for experiments with [^{3}H]-3PPP and 5 ml aliquots frozen at − 70°C.

For radioreceptor assays, aliquots of the frozen membrane suspension were thawed on ice, diluted and 250 μl (i.e. 1 mg of protein) added to either of the following: (i) 3.4 nM [^{3}H]-DTG (53 Ci mmol^{-1}), or (ii) 0.34 nM [^{3}H]-DTG, or (iii) 1.1 nM [^{3}H]-3PPP (115 Ci mmol^{-1}) and unlabelled drugs (up to 22 data points between 10^{-5} M and 10^{-12} M) in a final volume of 1 ml made up with buffer. Non-specific binding was defined

[1] Author for correspondence.

as that remaining in the presence of the drugs used at 10^{-5} M. After 90 min incubation at room temperature, the membrane suspension was rapidly filtered under vacuum through Whatman GF/B filters using a Brandel 24-well cell harvester. The filters were washed 5 times with 1 ml ice-cold buffer, oven dried and placed in 3.5 ml ultima gold scintillation fluid in mini vials and counted.

Data analysis

Results from 3–10 separate experiments were pooled and data were analysed by use of a computerised iterative curve fitting programme (Graphpad Inplot v 3.1). Initially, sigmoidal curves were fitted to the data to obtain estimates of Hill coefficients (nH). Where Hill coefficients were significantly less than one, displacement curves were constructed for both a one and two site model to obtain pIC_{50} values and percentages at each site. Models were compared with respect to the sum of squares value and where applicable an *F*-test (Graphpad Inplot v 4.0) was employed to select the best fit. Where $P<0.05$, results are presented for the more complex model.

Materials

DTG was obtained from Aldrich Chemical Co. (Poole, U.K.) and 3PPP from RBI Inc. (+)- and (−)-SKF 10047 were generous gifts from the NIDA, Bethesda, M.D. Radiochemicals were obtained from NEN du Pont. All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.).

Results

Guinea-pig brain membranes

Displacement curves together with Hill coefficients and pIC_{50} values and percentage occupation estimates from two component curves derived from competition studies, using a range of σ ligands, against [^{3}H]-DTG, binding are presented in Figure 1. Displacement of [^{3}H]-DTG by 3PPP, (+)-SKF 10047 and dextromethorphan gave Hill coefficients of less than 1 and corresponded to displacement of DTG from at least two sites (Figure 1a). No enantiomeric selectivity between (+)- and (−)-SKF 10047 was observed (pIC_{50} values 6.6 ± 0.1 and 6.4 ± 0.1, respectively) but the (−)-enantiomer displaced [^{3}H]-DTG with a Hill slope near 1 (0.93 ± 0.13). DTG and haloperidol also displaced [^{3}H]-DTG with low Hill coefficients suggesting that these ligands also interact with multiple binding sites (Figure 1b). Total specific binding of [^{3}H]-DTG remained unchanged in the presence of either 5′-guanosine monophosphate or adenosine triphosphate but was reduced to $63.3 \pm 3.1\%$ of control ($n = 6$) in the presence of 100 μM Gpp(NH)p. The displacement curves produced by 3PPP (Figure 2a) were also shifted to the right in the presence of Gpp(NH)p and the Hill coefficients were increased to values approaching unity. The ability of dextromethorphan (10^{-12}–10^{-5} M) to displace [^{3}H]-DTG in the presence of Gpp(NH)p was essentially abolished (Figure 2b).

Gpp(NH)p had no effect on the Hill slope of the concentration-displacement curve of [^{3}H]-DTG by DTG itself or haloperidol (Figures 2c,d). Indeed, despite a reduction of total binding in the presence of Gpp(NH)p, suggesting a G protein modulation of a proportion of [^{3}H]-DTG binding, the pIC_{50} values for haloperidol were essentially unchanged.

An attempt was also made to reduce the high affinity, Gpp(NH)p-sensitive component of 3.4 nM [^{3}H]-DTG binding by conducting the incubation in the presence of 100 nM dextromethorphan. Under such conditions, [^{3}H]-DTG binding was reduced to $68.2 \pm 2.9\%$ ($n = 4$) of that in the absence of dextromethorphan (i.e. an equivalent reduction to that produced by 100 μM Gpp(NH)p). [^{3}H]-DTG apparently labelled only a single population of sites (Figure 3). 3PPP also appeared to label predominantly a low affinity site ($pIC_{50} = 7.1 \pm 0.01$, 71%) and in addition, occupation of a very low affinity site (pIC_{50} 5.6 ± 0.5, 29%) was revealed (Figure 3). Since no selective agents are so far available which might mask the low affinity component of DTG binding, we attempted to look at the high affinity component by reducing the concentration of [^{3}H]-DTG to 0.34 nM. Under these conditions, DTG displaced [^{3}H]-DTG from essentially one site and yielded an IC_{50} value for complete displacement which was essentially identical to that obtained using 3.4 nM DTG (Table 1). The Hill slopes for haloperidol and 3PPP displacement of [^{3}H]-DTG were less than unity.

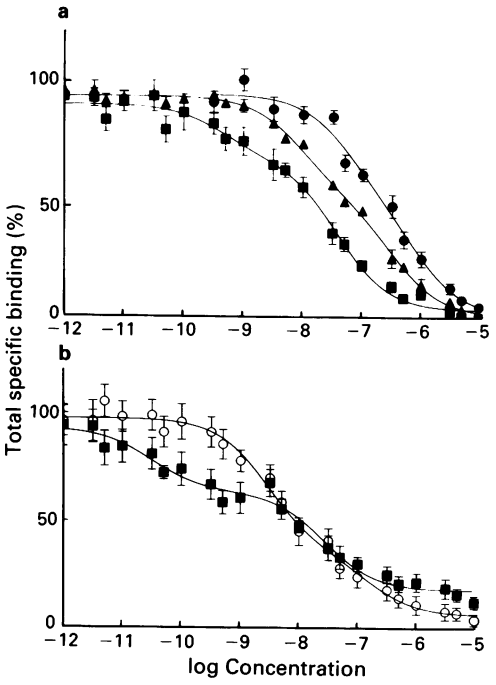

Figure 1 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) binding in guinea-pig brain membranes in the absence of Gpp(NH)p. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain $pIC_{50} \pm$ s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses. (a) (■) (+)-3-(3-hydroxyphenyl)-N-1-(propyl) piperdine (3PPP)-nH = 0.56 ± 0.06, site 1: pIC_{50} 9.3 ± 0.3 (26%); site 2: pIC_{50} 7.4 ± 0.1 (74%). (●) (+)-SKF 10047-nH = 0.75 ± 0.15, site 1: pIC_{50} 7.1 ± 0.5 (54%); site 2: pIC_{50} 6.0 ± 0.8 (46%) or (▲) dextromethorphan-nH = 0.58 ± 0.03, site 1: pIC_{50} 8.0 ± 0.1 (46%); site 2: pIC_{50} 6.4 ± 0.1 (54%). (b) (○) DTG-nH = 0.50 ± 0.07, site 1: pIC_{50} 9.1 ± 0.2 (42%); site 2: pIC_{50} 7.3 ± 0.2 (58%). (■) Haloperidol-nH = 0.36 ± 0.10, site 1: pIC_{50} 10.5 ± 0.4 (33%); site 2: pIC_{50} 7.8 ± 0.2 (67%).

[^{3}H]-3PPP binding in guinea-pig brain membranes

3PPP apparently displaced itself from more than one site yielding a Hill coefficient of 0.7 and occupation of primarily a high affinity site pIC_{50} 9.2 (67%) in addition to a lower affinity site ($pIC_{50} = 7.7$; 33%) (Figure 4). DTG also displaced [^{3}H]-3PPP from more than one site (nH = 0.5), again occupying primarily a high affinity site (pIC_{50} 8.8, 60%) in addition to a lower affinity site ($pIC_{50} = 7.0$, 40%) (Figure 4). Dextromethorphan displaced 3PPP from essentially a single site (nH = 0.8 pIC_{50} 7.4) (Figure 4). (+)-SKF 10047 also displaced from more than one site (nH) = 0.34 ± 0.11), occupying some high affinity sites (site 1 $pIC_{50} = 10.4 \pm 0.5$, 27%), although mostly occupying a lower affinity site (site

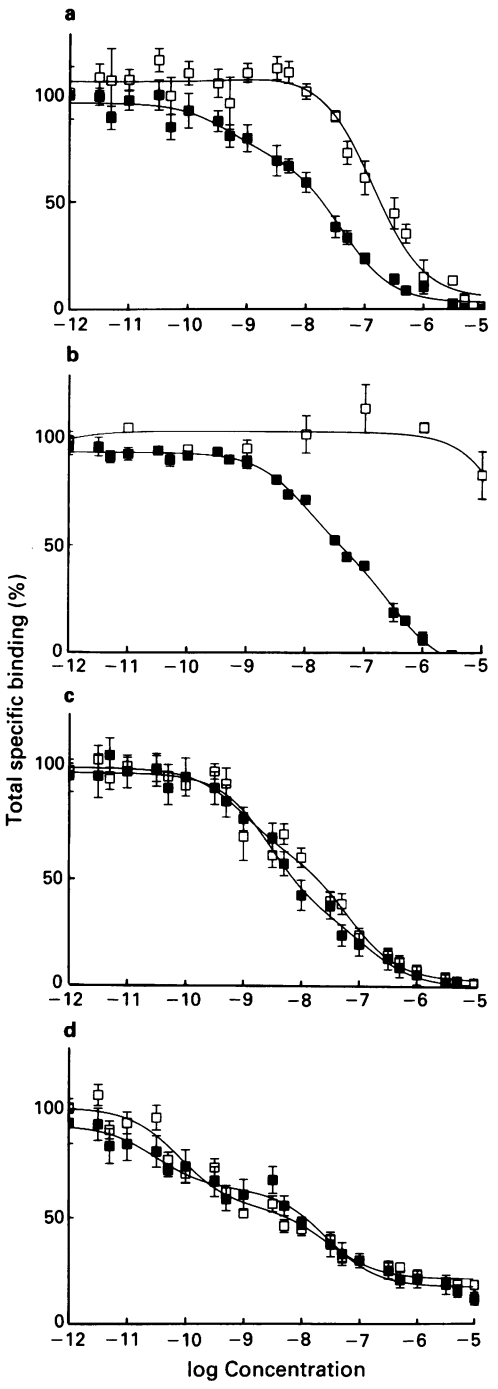

Figure 2 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) binding in guinea-pig brain membranes in the absence of Gpp(NH)p. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses. All pIC_{50} values refer to affinities in the presence of Gpp(NH)p. (a) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine (3PPP) displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 41.0 ± 1.0%; nH = 0.94 ± 0.15, pIC_{50} = 6.5 ± 0.1. (b) Dextromethorphan displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 78.7 ± 5.4%; no effective displacement of [^{3}H]-DTG was observed at concentrations below 10^{-5} M. (c) DTG displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 55.5 ± 1.3%; nH = 0.68 ± 0.06, site 1: pIC_{50} 8.3 ± 0.1 (79%); site 2: pIC_{50} 6.3 ± 0.3 (21%). (d) Haloperidol displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 69.1 ± 3.0% nH = 0.54 ± 0.10, site 1: pIC_{50} 10.3 ± 0.5 (29%); site 2: pIC_{50} 6.3 ± 0.3 (71%).

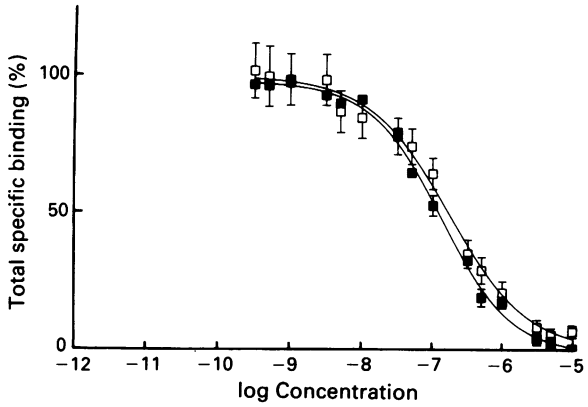

Figure 3 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG) in the presence of 100 nM dextromorphan in guinea-pig brain membranes. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses: (■) DTG nH = 0.82 ± 0.06, pIC_{50} = 7.3 ± 0.1; or (▲) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine nH = 0.72 ± 0.10, site 1: pIC_{50} 7.1 ± 0.01 (71%); site 2: pIC_{50} 5.6 ± 0.5 (29%).

2 = 7.1 ± 0.2, 73%). (−)-SKF 10047 displaced 3PPP from essentially a single site (nH) = 0.92 ± 0.08) of low affinity (pIC_{50} 6.3 ± 0.2).

Rat brain membranes

In rat brain membranes DTG has apparently equal affinity for all site(s) present since the Hill coefficient increased (nH = 0.8 ± 0.1; pIC_{50} 7.0) in comparison with results from guinea-pig brain membranes (Figure 5a). 3PPP displacement of [^{3}H]-DTG is essentially comparable with the guinea-pig, revealing displacement from more than 1 site (Figure 5b). In the presence of Gpp(NH)p, the Hill slope for 3PPP displacement increased to near 1 (nH = 0.87 ± 0.18). No decrease in total specific binding was observed (97.3 ± 3.4%) in the presence of Gpp(NH)p. Haloperidol also appears to displace from predominantly 1 site (nH = 0.8, pIC_{50} 8.1) (Figure 5a). Dextromethorphan did not displace [^{3}H]-DTG in rat brain at concentrations below 10 μM, suggesting that the dextromethorphan site observed in guinea-pig brain membranes is either absent or present in very low density in rat brain membranes. No enantiomeric selectivity was observed

Table 1 Displacement of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 0.34 nM) in guinea-pig brain membranes

	pIC_{50}[1]	nH
3PPP	7.2 ± 0.2	0.66 ± 0.10
DTG	7.7 ± 0.2	0.90 ± 0.10
Haloperidol	8.6 ± 0.3	0.75 ± 0.03

Values are mean ± s.e.mean. $n = 4$. 3PPP = (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine.
[1]Data are derived from 5 point displacement curves and no attempt has therefore been made to impose one or two site models on these results.

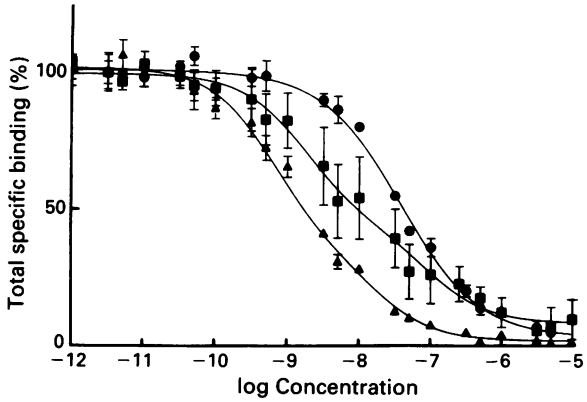

Figure 4 Displacement curves of [^{3}H]-(+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine ([^{3}H]-3PPP) binding in guinea-pig brain membranes. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2, together with percentages at each site given in parentheses: (▲) 3PPP n(H) = 0.70 ± 0.10 site 1: pIC_{50} 9.2 ± 0.1 (67%); site 2: pIC_{50} 7.88 ± 0.2 (33%), (■) DTG nH = 0.53 ± 0.04, site 1: pIC_{50} 8.8 ± 0.2 (60%); site 2: pIC_{50} 7.0 ± 0.2 (40%) or (●) dextromethorphan nH = 0.79 ± 0.07 pIC_{50} = 7.4 ± 0.1.

between (+)- or (−)-SKF 10047, although the Hill slope of (+)-SKF 10047 was lower than the (−)-enantiomer.

Discussion

Although σ binding sites were originally thought to be a single entity, the current consensus is that two subtypes exist, σ_1 and σ_2 (Bowen & Hellewell, 1988; Hellewell & Bowen, 1990; Knight *et al.*, 1991a,b; Rothman *et al.*, 1991; Quirion *et al.*, 1992). Indeed, sophisticated computer assisted models of self and cross-displacement σ ligand binding experiments have identified up to four binding sites (R_1–R_4) for σ ligands (Zhou & Musacchio, 1991; Klein & Musacchio, 1992). Computerized analysis of the binding data from the present study has been restricted to determining whether one or more sites best fits the data. Discussion of data is restricted to an indication of the number of sites and whether ligands have relatively higher or lower affinity. The initial observation which suggested the feasibility of such an approach was that under the conditions used in our experiments, and in contrast to the literature at the time (Weber *et al.*, 1986), DTG and haloperidol displaced [^{3}H]-DTG (3.4 nM) from guinea-pig brain membranes with a Hill-slope considerably less than 1. This is in accord with recent reports demonstrating [^{3}H]-DTG to label at least two binding sites in guinea-pig brain membranes (Karbon *et al.*, 1991; Rothman *et al.*, 1991; De-Haven-Hudkins & Fleissner, 1992). A further study showed two high affinity sites and at least one low affinity site to be labelled by [^{3}H]-DTG in guinea-pig brain membranes (Knight *et al.*, 1991a). However, no investigation has been made of the sensitivity of these sites to guanine nucleotide binding protein modifying agents.

The complexity of [^{3}H]-DTG binding observed in guinea-pig brain membranes, in the present study, most closely parallels the four site model (R_1–R_4) of σ ligand interaction (Zhou & Musacchio, 1991; Klein & Musacchio, 1992). According to this classification, R_1 and R_3 are sites at which σ ligands have a high affinity, R_2 is a dextromethorphan selective site and R_4 is a low affinity site for all examined σ ligands. R_2 would not have been examined in the present study as the radiolabelled ligands used, [^{3}H]-DTG and [^{3}H]-3PPP, have a very low affinity for this site. DTG does not discriminate between R_1 and R_3, whilst haloperidol, 3PPP and particularly dextromethorphan have a higher affinity for

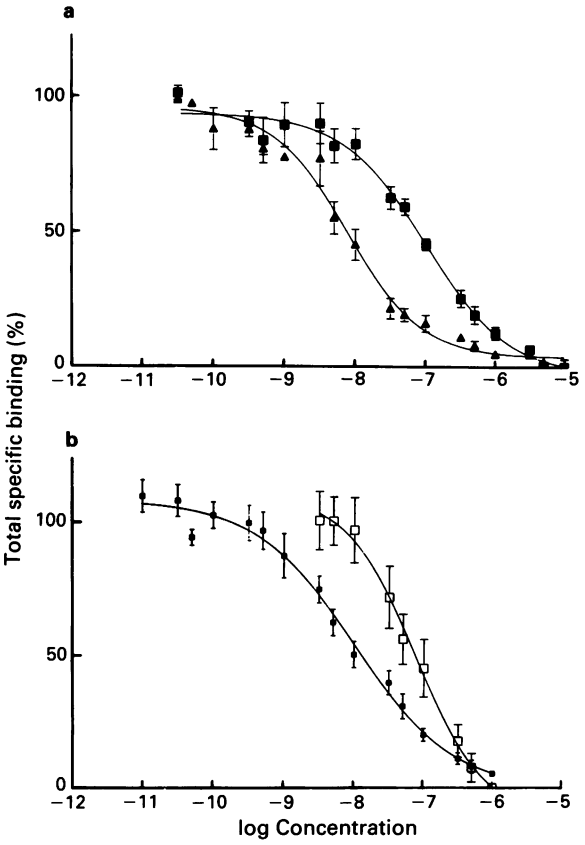

Figure 5 Displacement of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) in rat brain membranes. Data (± s.e.mean, vertical bars) from 3–10 experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 or at one site where nH = 1, together with percentages at each site given in parentheses. (a) (■) DTG nH = 0.79 ± 0.11 pIC_{50} = 7.0 ± 0.1 or (▲) haloperidol nH = 0.84 ± 0.09 pIC_{50} = 8.1 ± 0.1 and (b) (■) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine in the absence (nH = 0.51 ± 0.06, site 1: 8.3 ± 0.1 (68%), site 2: pIC_{50} 6.4 ± 0.3 (32%)) or (□) presence (nH = 0.87 ± 0.18 pIC_{50} = 7.1 ± 0.1) of Gpp(NH)p. No change in specific binding was observed.

R_1 (Zhou & Musacchio, 1991).

According to this classification, [^{3}H]-DTG can be anticipated to bind to R_1, R_3 and R_4 sites in guinea-pig brain membranes. The reduction in the total specific binding of [^{3}H]-DTG, which was induced by Gpp(NH)p in the present study, without a change in the Hill slope of the DTG displacement curve, is explicable if one of the high affinity sites (R_1 and R_3) is G-protein linked. The reduction in total [^{3}H]-DTG binding representing a dramatic reduction in DTG binding to this site. The inability of dextromethorphan to displace [^{3}H]-DTG in the presence of Gpp(NH)p, indicates that it is the dextromethorphan high affinity site R_1 that is no longer labelled. The Gpp(NH)p-induced reduction in [^{3}H]-DTG binding suggests that DTG is an agonist at this G-protein linked site, as has previously been suggested for 3PPP, SKF 10047 and dextromethorphan in rat brain (Itzhak, 1989; Beart *et al.*, 1989). Reducing the concentration of [^{3}H]-DTG from 3.4 nM to 0.34 nM resulted in a reduction in labelling of the low affinity site, R_4. DTG, which does not distinguish between R_1 or R_3 (Zhou & Musacchio, 1991), has a Hill slope close to one. Haloperidol and 3PPP which have higher affinities for R_1 (Zhou & Musacchio, 1991) have lower Hill slopes. To avoid labelling the low affinity site it may be advisable to use low concentrations of [^{3}H]-DTG, at least in assays with guinea-pig brain membranes.

The presence of Gpp(NH)p or dextromethorphan (100 nM) dramatically reduces [^{3}H]-DTG binding to R_1; the principle site labelled under these conditions is R_3. The DTG binding revealed by [^{3}H]-DTG in the presence of 100 nM dextromethorphan showed no enantiomeric selectivity for SKF 10047 displacement, suggesting that this site (R_3) may be equivalent to the σ_2 site of Hellewell & Bowen (1990). A concentration of 100 nM dextromethorphan may not be sufficient to occupy all R_1 sites since 3PPP still displaced DTG with a Hill-slope of less than 1.

Results obtained with [^{3}H]-3PPP in guinea-pig brain membranes are consistent with the above discussion. Dextromethorphan completely displaced [^{3}H]-3PPP from essentially one site, indicating that 3PPP primarily labels R_1 under the conditions used in the present study. SKF 10047 showed enantiomeric selectivity for [^{3}H]-3PPP displacement, indicating that R_1 is equivalent to the σ_1 site of Hellewell & Bowen (1990). DTG and 3PPP itself displaced [^{3}H]-3PPP from more than one site or affinity state.

The complexity of the analysis when using guinea-pig brain membranes may be reduced by conducting similar experiments with rat membranes, where interaction with the R_4 binding site is not anticipated (Klein & Musacchio, 1991). It has been suggested that rat brain membranes contain predominantly R_3 (Klein & Musacchio, 1991), R_1, the other affinity site, being found in much lower density (10%) than in guinea-pig brain membranes. Nevertheless, the use of [^{3}H]-(+)-pentazocine has allowed the presence of σ_1 and σ_2 sites in rat brain to be confirmed (Vilner & Bowen, 1992). In contrast to the guinea-pig, no reduction of total specific [^{3}H]-DTG binding was shown in the rat in response to Gpp(NH)p; this can be interpreted as being due to the small amount of the G-protein linked high affinity site (σ_1/R_1) and confirms that the other DTG high affinity site (σ_2/R_3) is unlikely to be G-protein linked. However, Gpp(NH)p was still able to increase the Hill-slope of 3PPP displacement of [^{3}H]-DTG. The results obtained with Gpp(NH)p are comparable with those obtained previously in the rat when [^{3}H]-3PPP was used as the σ ligand (Beart *et al.*, 1989; Itzhak, 1989). Since no change in total specific [^{3}H]-DTG binding was observed in the presence of Gpp(NH)p, this result might not have been expected. However, similar findings have been made previously. Itzhak & Stein (1992) also found [^{3}H]-DTG binding in rat brain membranes not to be modulated by guanine nucleotides, whilst in the same preparation [^{3}H]-3PPP binding demonstrated sensitivity to guanine nucleotides. Several aspects of the inhibition of σ ligand binding by guanine nucleotides are parallelled by the enhancement of σ binding by phenytoin (McCann & Su, 1992). The contribution of interactions at this site have not been addressed by our work and require further study.

In conclusion, the present study illustrates the complexity of the use of DTG as a radiolabel for σ receptors. Clearly this compound labels several affinity states/sites. The problem is compounded if guinea-pig brain membranes are used; more selective labels for the different sites are required to aid our understanding of the affinity of compounds for the individual sites. At least one site appears to be coupled to G-proteins and thus, may be linked to signal transduction mechanisms. No unequivocal evidence exists to identify the specific second messenger pathway but the existence of linkage to a G-protein strongly suggests a functional consequence of σ binding and thus a receptor function. Such evidence is, however, circumstantial and it remains possible, though unlikely, that guanine nucleotides could interact directly with a proportion of σ binding sites. Given the widespread distribution of σ binding sites and their presence in a variety of subcellular fractions, evidence for a potential σ receptor may be strengthened by investigating the guanine nucleotide sensitivity of such fractions. G-protein linkage should be restricted to the plasma membrane fraction and would not be expected in the microsomal fraction (subject to the purity of the respective fractions). To date, no differences in the pharmacology of σ binding has been observed between the various fractions (Knight *et al.*, 1991a).

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