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Dissociation of the motor effects of (+)-pentazocine from binding to σ_1 sites

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Abstract

Radioligand binding and behavioral studies were conducted to determine whether a relationship existed between the motor effects produced by (+)-pentazocine and its binding to σ sites. Scatchard analyses revealed decreased [^{3}H](+)-pentazocine binding in middle aged rats (5–6 months old) compared to young adult rats (2–3 months old). However, there was no difference between the extent of circling behavior or dystonia produced by microinjection of (+)-pentazocine into the substantia nigra or red nucleus in the older animals compared to the young adult rats. There was also a significant decrease in [^{3}H](+)-pentazocine binding in rats chronically treated with haloperidol. Again, however, despite the reduction in [^{3}H](+)-pentazocine binding, there was no difference between the extent of dystonia produced by unilateral intrarubral microinjection of (+)-pentazocine into animals chronically treated with haloperidol vs. saline. The postural changes produced by (+)-pentazocine could not be attenuated with coadministration of the putative σ receptor antagonist BD1047 (*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine), or the opiate receptor antagonist naloxone. However, the (+)-opiate, (+)-nordihydrocodeinone, partially attenuated the postural effects of (+)-pentazocine, despite its very low affinity for σ_1, σ_2, or opiate receptors. Taken together with previous studies, the results suggest that [^{3}H](+)-pentazocine is a potent and selective probe for σ_1 binding sites, but the in vivo effects of (+)-pentazocine cannot be fully attributed to actions through these sites. Some of the in vivo effects of (+)-pentazocine appear to involve other binding sites that are not detected under the conditions normally used in in vitro assays.

Keywords: Opiate; (+)-Pentazocine; σ Site; Red nucleus; Substantia nigra

1. Introduction

σ Binding sites are non-opiate, non-dopamine, non-phencyclidine sites with a unique drug selectivity pattern and anatomical distribution from other characterized receptors (Quirion et al., 1992). The recent development of novel ligands which exhibit high affinities and/or selectivities for σ binding sites have led to many new insights into the structure and function of these binding sites. One such ligand is [^{3}H](+)-pentazocine which appears to be a potent and selective benzomorphan probe for σ_1 sites (Bowen et al., 1993).

Radioligand binding studies have shown [^{3}H](+)-pentazocine to have high affinity, high selectivity, and low non-specific binding for σ sites. The drug selectivity pattern associated with [^{3}H](+)-pentazocine reflects the typical pharmacological profile for σ sites in guinea-pig brain (Bowen et al., 1993; Hellewell and Bowen, 1990; Tam and Cook, 1984), and is suggestive of a σ_1 site pattern of binding. For example, ligands such as haloperidol, fluphenazine, (+)-3-(2-hydroxyphenyl)-*N*-(1-propyl)-piperidine [(+)-3-PPP], dextrallorphan, (+)-cyclazocine, and (+)-SKF 10,047 all exhibit high affinities for [^{3}H](+)-pentazocine-labelled sites (Bowen et al., 1993).

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In addition, the (+)-enantiomers of benzomorphans and morphinans bind with higher affinity than their corresponding (−)-enantiomers (e.g. Bowen et al., 1993). The ability of both benzomorphan and non-benzomorphan σ site ligands to exhibit high affinity for these binding sites, and for (+)-isomers to have a higher affinity than their corresponding (−)-isomers are hallmarks of interactions with the σ_1 site subtype (Quirion et al., 1992).

However, since the definition of σ binding sites is based largely on the results of radioligand binding assays, the physiological relevance of these sites continues to be debated. In an effort to bridge the gap between receptor binding and functional studies we have, in the past, attempted to correlate the potency of functional effects of σ site ligands to their σ binding affinities (Hemstreet et al., 1993; Matsumoto et al., 1990). Using this approach, we have shown a correlation between the behavioral potency of numerous σ site ligands for producing torsional head postures in rats and their σ binding affinities for [^{3}H]di-*o*-tolylguanidine (DTG) (Matsumoto et al., 1990). We have also demonstrated an association between the efficacy of DTG in producing torsional head postures and circling in rats and its σ binding to [^{3}H]DTG-labelled sites in rats of different ages (Hemstreet et al., 1993; Matsumoto et al., 1989a). However, since DTG acts through both σ_1 and σ_2 sites (Hellewell and Bowen, 1990), we were interested in evaluating the contribution of the various subtypes to motor effects in vivo.

The recent identification of [^{3}H](+)-pentazocine as a selective probe for σ_1 sites and the ability of (+)-pentazocine to produce torsional head postures and circling behavior in rats (Goldstein et al., 1989; Matsumoto et al., 1990), led us to investigate the contribution of σ_1 sites to the motor effects produced by (+)-pentazocine. A portion of this work has been previously published (Walker et al., 1990a).

2. Materials and methods

2.1. [^{3}H](+)-Pentazocine binding

2.1.1. Animals and drug treatments

Male Sprague Dawley rats were purchased from Charles River Laboratories (Boston, MA, USA). [^{3}H](+)-Pentazocine sites were labelled in the following groups of animals: (1) young adult rats (2–3 months old), (2) middle aged rats (5–6 months old), (3) middle aged rats chronically treated with haloperidol or saline for up to 20 days, and (4) young adult rats chronically treated with haloperidol or saline for up to 20 days. Animals that received chronic administration of drugs, including those in the acute group, were injected daily with haloperidol (5 mg/kg, s.c.) or an equal volume of saline between 9:00 a.m. and noon. Three days after the last injection (or single injection in the case of rats in the acute group), the animals were killed by decapitation and the brains removed.

2.1.2. Membrane preparation

P_2 membrane fractions of pooled brain were prepared as follows. Tissues were homogenized using a Potter-Elvehjem homogenizer and six strokes of a motor-driven pestle in 10 ml ice-cold Tris-sucrose buffer (0.32 M sucrose in 10 mM Tris-HCl, pH 7.4) per gram wet tissue weight. The homogenates were centrifuged at 4°C at 1000 $\times g$ for 10 min and the supernatants saved. The supernatants were then centrifuged at 4°C at 31 000 $\times g$ for 15 min. The pellets were resuspended in 3 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue and allowed to incubate for 30 min at 25°C. Following the incubation, the suspensions were centrifuged at 4°C at 31 000 $\times g$ for 15 min and the pellets resuspended in 1.53 ml of 10 mM Tris-HCl, pH 7.4 per gram tissue. The suspensions were hand homogenized with 5 strokes in a Potter-Elvehjem homogenizer and the aliquots stored at −80°C until use. Protein content was determined by the method of Lowry et al. (1951).

2.1.3. Binding assays

σ Binding sites were labelled with [^{3}H](+)-pentazocine (synthesized as previously described; De Costa et al., 1989; 26.6 Ci/mmol). Each tube contained 500 μg membrane protein, 50 mM Tris-HCl, pH 8.0, and 1 of 14 concentrations of [^{3}H](+)-pentazocine (concentration range 0.3–100 nM). Non-specific binding was determined in the presence of 5 μM (+)-pentazocine. The total reaction volume in each tube was 500 μl and the tubes were run in duplicate. After a 60 min incubation at 25°C, the assays were terminated with 5 ml ice-cold 10 mM Tris-HCl, pH 7.4 or 8.0. The samples were vacuum filtered using a Brandel Cell Harvester through glass fiber filters (Schleicher and Schuell, Keene, NH, USA) that were pre-soaked for at least 30 min in 0.5% polyethyleneimine (Sigma, St. Louis, MO, USA). The filters were then washed twice with 5 ml ice-cold buffer. Filters were placed in Ecoscint cocktail (National Diagnostics, Manville, NJ, USA) and counts were extracted for at least 8 h. A Packard Model 4450 scintillation spectrometer was used for scintillation counting.

2.1.4. Data analysis

Scatchard analysis was conducted with the curve fitting program BDATA (EMF Software, Baltimore, MD, USA). Hill coefficients were calculated from averages of the specific binding at various ligand concentrations using the GraphPad InPlot program (GraphPad Software, San Diego, CA, USA). In all cases where the Hill coefficients were less than 0.80, the data were fit for both one- and two-sites. One-way analyses of variance (ANOVA) were used to evaluate whether changes in B_{max} or K_d were statistically significant (BMDP Statistical Software, Los Angeles, CA, USA).

2.2. Behavioral studies

2.2.1. Animals

Young adult (2–3 months) and middle aged (5–6 months) male Sprague Dawley rats were purchased from either Charles River Laboratories (Boston, MA, USA) or Zivic Miller (Zelienople, PA, USA). The animals were group housed prior to surgery and individually thereafter. All animal care procedures followed those approved by the Brown University and University of California Irvine Animal Care and Use Committees.

Some of the young adult rats were chronically treated with saline or haloperidol prior to behavioral testing. These animals received daily subcutaneous injections of haloperidol (5 mg/kg) or an equal volume of saline as described for the binding studies. The animals were assigned to the following groups and underwent behavioral testing after a 3-day wash-out period: 10 days chronic saline ($n = 17$), 10 days chronic haloperidol ($n = 13$), 20 days chronic saline ($n = 12$), and 20 days chronic haloperidol ($n = 14$). The remaining animals did not undergo a chronic drug treatment regimen prior to behavioral testing and were assigned to the following groups: young adult rat/substantia nigra ($n = 13$), young adult rat/red nucleus ($n = 34$), middle aged rat/substantia nigra ($n = 6$), middle aged rat/red nucleus ($n = 8$).

2.2.2. Surgery

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic apparatus. Guide cannulae, constructed from 24 gauge stainless steel tubing, were implanted with their tips 4.0 mm above the red nucleus or substantia nigra pars reticulata of each animal. The coordinates with reference to lambda and the skull surface were: substantia nigra: 3.2 mm anterior, 2.2 mm lateral, 4.7 mm ventral; red nucleus (young adult rat): 2.5 mm anterior, 0.8 mm lateral, 4.0 mm ventral; red nucleus (middle aged rat): 2.8 mm anterior, 0.8 mm lateral, 4.5 mm ventral (Paxinos and Watson, 1986). Cannulas were fixed in place with stainless steel screws and dental acrylic. Stainless steel stylets kept the cannulas sealed except during drug infusion. A recovery period of at least 3 days was allowed before behavioral testing.

For rats that were chronically treated with haloperidol or saline, surgery was performed either 2 days prior to beginning the drug regimen or on the last day of drug treatment. Preliminary studies in rats chronically treated for 20 days showed that there was no significant difference between the behavioral responses elicited by animals that underwent surgery before the drug treatments and those operated on the last day of treatment ($F(1,12) = 0.60$; n.s.). Therefore, differences between the treatment groups were not due to an interaction between the drug treatments and surgical procedures. The actual procedure for the surgery was identical to that described above. Behavioral testing of chronically treated rats occurred 3 days after the last injection (to match the wash-out period used for the binding experiments).

2.2.3. Behavioral testing

On the day of behavioral testing, 9.3 nmol of (+)-pentazocine (National Institute on Drug Abuse, Rockville, MD, USA) or DTG acetate was administered unilaterally through a 31 gauge microneedle that extended 4.0 mm beyond the tips of the guide cannula into either the red nucleus or substantia nigra as previously described (Goldstein et al., 1989; Matsumoto et al., 1989a, Matsumoto et al., 1990). The microinjections were made in a volume of 0.5 μl over 60 or 72 s using a motor driven infusion pump.

Animals that received intranigral injections were placed into round, plastic containers, 25 cm in diameter. An adjustable elastic harness was fitted around each animal and connected by a metal cable to an optical transducer which encoded the position of the rat as binary signals. A computer, interfaced to the transducer, calculated the number and direction of half turns (180°) per minute for each animal during the 30-min testing session.

Torsional movements of the head were quantified in animals receiving intrarubral injections by measuring the angle of deviation of the head from the horizontal plane, using the eyes of the animals as a reference. Measurements were taken 1, 5, 15, and 30 min after the injection and the peak angle of deviation for each animal was used in the data analysis.

To further evaluate the mechanism through which (+)-pentazocine produced its motor effects, attempts were made to antagonize the head angles produced by (+)-pentazocine using BD1047 (synthesized as described in De Costa et al., 1992), naloxone (Sigma, St. Louis, MO, USA), or (+)-nordihydrocodeinone (synthesized as described in Rice, 1985). BD1047 is a putative σ receptor antagonist with subnanomolar affinity for σ_1 sites (0.93 ± 0.14 nM) and a reasonable affinity for σ_2 sites (47 ± 0.60 nM; Matsumoto et al., 1995). Naloxone is a well established opiate receptor antagonist, and (+)-nordihydrocodeinone is a compound that is structurally related to pentazocine, but has a very low affinity for σ or opiate receptors (> 10000 nM vs. [^{3}H]DTG in rat brain; Matsumoto et al., 1990). The antagonism experiments were conducted in the red nucleus to facilitate interpretation of the data because unlike the substantia nigra which contains a plethora of receptor types, the red nucleus is enriched in σ binding sites, but virtually devoid of other receptors with which non-selective σ site ligands interact (Boyson et al., 1986; Gundlach et al., 1986; Jones and Palacios, 1991; Mansour et al., 1987; Pazos and Palacios, 1985; Quirion et al., 1981). For the antagonism experiments, each animal received two microinjections: 9.3 nmol (+)-pentazocine in the presence and absence of 9.3 nmol of a competing drug. The rats had to meet a 10° criterion upon injection of (+)-pentazocine alone to be further tested in this portion

of the study. This criterion has previously been used for the calculation of ED_{50} values with this paradigm (Matsumoto et al., 1990) and was implemented to reliably assess statistically significant attenuation of torticollis by the competing drugs. A minimum of 24 h intervened between the two injections, and the total drug volume remained 0.5 μl for all injections.

2.2.4. Histology and data analysis

Rats were killed with a lethal dose of sodium pentobarbital and perfused intracardially with 10% formalin. Brains were fixed in a 30% sucrose-formalin solution and coronal sections (40 μm) were taken throughout the extent of the injection site. The sections were stained with cresyl violet and examined under a microscope to localize injection sites. Only those subjects with histologically confirmed injection sites in the red nucleus or substantia nigra were used in the data analysis. Student's *t*-tests or a repeated measures analysis of variance (BMDP Statistical Software; Los Angeles, CA, USA) were used to evaluate the data. However, non-parametric statistics were used to analyze the antagonism data because the rats had to meet a 10° criterion following microinjection of (+)-pentazocine alone to be included in this portion of the study; Wilcoxon signed rank tests were used for this comparison. $P < 0.05$ was considered statistically significant.

2.3. Binding of (+)-nordihydrocodeinone to σ site subtypes

To determine the affinities of (+)-nordihydrocodeinone for σ_1 and σ_2 sites, P_2 membrane fractions were prepared from frozen guinea-pig brains (Pel-Freeze, Rogers, AK) and rat livers as described above for rat brains. The competition binding assays were conducted as previously published, using conditions which selectively label each of the putative σ site subtypes (Bowen et al., 1993; Hellewell et al., 1994; Matsumoto et al., 1995). Briefly, the affinity of (+)-nordihydrocodeinone for σ_1 sites was determined in guinea-pig brain using [^{3}H](+)-pentazocine as the radioligand. The affinity of (+)-nordihydrocodeinone for σ_2 sites was determined in rat liver in the presence of [^{3}H]DTG and a saturating concentration of dextrallorphan to mask σ_1 sites. Non-specific binding was determined in the presence of 10 μM of haloperidol for both assays. The data were analyzed using the curve fitting program GraphPad InPlot (San Diego, CA, USA).

3. Results

3.1. Binding studies

3.1.1. Comparison between young adult and middle aged rats

There was a significant difference between the level of [^{3}H](+)-pentazocine binding in brain membranes prepared from young adult (2–3 months) and middle aged (5–6 months) rats (Fig. 1). The binding parameters (means ± S.E.M.) for the young adult rats were: $K_d = 12 \pm 1$ nM; $B_{max} = 264 \pm 34$ fmol/mg protein. The binding parameters for the middle aged animals were: $K_d = 56 \pm 3$ nM; $B_{max} = 170 \pm 52$ fmol/mg protein. Student's *t*-tests revealed that the difference was significant for K_d ($t = 15.96$, $P < 0.001$) but not for B_{max} ($t = 1.60$, n.s.). The Hill coefficients for the two groups were close to unity (young adult rats = 0.88, middle aged rats = 0.92), suggesting that under the conditions used in our assays, a single population of sites or multiple sites with similar affinities were labelled.

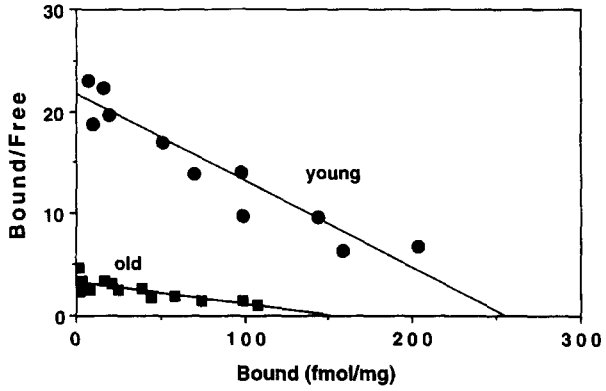

Fig. 1. Scatchard analysis of [^{3}H](+)-pentazocine binding in young adult rats (young, 2–3 months old) and middle aged rats (old, 5–6 months old). Various concentrations of [^{3}H](+)-pentazocine (concentration range 0.3–100 nM) were incubated with pooled brain membranes from rats of the two age groups; non-specific binding was determined in the presence of 5 μM (+)-pentazocine. The points in each plot represent the average values from 3–4 replications. The binding parameters (means ± S.E.M.) for the young adult animals were: $K_d = 12 \pm 1$ nM, $B_{max} = 264 \pm 34$ fmol/mg protein; the values from the middle aged rats were: $K_d = 56 \pm 3$ nM, $B_{max} = 170 \pm 52$ fmol/mg protein. The Hill coefficients were close to unity: young adult rats = 0.88, middle aged rats = 0.92.

3.1.2. Comparison between middle aged rats chronically treated with haloperidol vs. saline

[^{3}H](+)-Pentazocine binding was rapidly and profoundly reduced by chronic haloperidol treatment in middle aged rats ($F(3,126) = 11.39$, $P < 0.0001$; Fig. 2). It was impossible to determine K_d and B_{max} values because the level of binding was so low in the haloperidol-treated groups. Therefore, it is unknown whether the haloperidol-induced changes in the middle aged rats resulted from a decreased affinity and/or number of sites.

3.1.3. Comparison between young adult rats chronically treated with haloperidol vs. saline

Chronic haloperidol treatment also decreased binding to brain σ sites labelled with [^{3}H](+)-pentazocine in young adult rats (Fig. 3). A summary of the binding parameters is shown in Table 1. Analyses of variance revealed that the changes were significant for B_{max} ($F(3,7) = 4.74$; $P < 0.05$) but not K_d ($F(3,7) = 1.82$; n.s.). Under the condi-

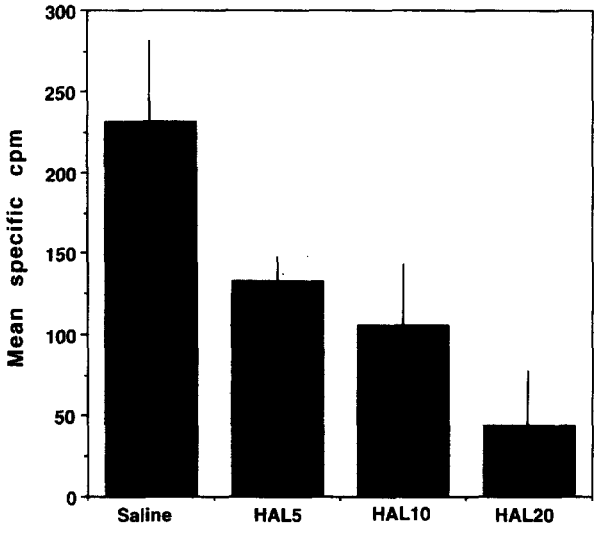

Fig. 2. Reduction in [^{3}H](+)-pentazocine binding in brain membranes from middle aged rats (5–6 months old) chronically treated with haloperidol. Rats were treated with haloperiol (5 mg/kg, s.c.) or an equivalent volume of saline. Saline-treated controls were injected for 20 days, while haloperidol-treated rats were injected for 5, 10, or 20 days (HAL5, HAL10, HAL20). The mean ± S.E.M. specific cpm for 5 nM [^{3}H](+)-pentazocine is summarized; non-specific binding was determined in the presence of 5 μM (+)-pentazocine. The data are corrected for protein. It is impossible to determine whether the observed changes are due to a decreased affinity or number of receptors because the level of binding was so low. However, the reduction was statistically significant ($P < 0.0001$).

tions used in our study, it was difficult to ascertain whether [^{3}H](+)-pentazocine labelled a single or multiple binding sites. Analysis of the data using Hill coefficients and the residuals from one- and two-site fits of both the summary and individual Scatchards favored a one-site model. However, there were hints of the existence of multiple sites (e.g. borderline unitary Hill coefficients and the apparent presence of two sites in some Scatchards), although they could not be reliably resolved under the conditions used in our assays. The only condition where a two-site model better fit the data was the group acutely treated with haloperidol. In this case, a one-site fit yielded a sum of squares of 2545 (df = 13) while a two-site fit yielded a sum of squares of 859 (df = 10). The binding parameters for a two-site fit for this group were as follows: $K_{d1} = 2.45$ nM, $B_{max1} = 44.4$ fmol/mg protein; $K_{d2} = 1001$ nM, $B_{max2} = 5104$ fmol/mg protein.

3.2. Behavioral studies

3.2.1. Comparison between young adult and middle aged rats

Despite the significant difference in [^{3}H](+)-pentazocine binding between young adult and middle aged rats, there was no difference between the behavioral responses produced by (+)-pentazocine in the two groups of animals. Unilateral microinjection of (+)-pentazocine into the substantia nigra pars reticulata produced a comparable amount of circling behavior in young adult and middle aged rats ($t = 0.20$, n.s.; Fig. 4A). Similarly, there was no significant difference between the degree of head angle produced by unilateral intrarubral microinjection of (+)-pentazocine into the two groups of rats ($t = 0.20$, n.s.; Fig. 4B).

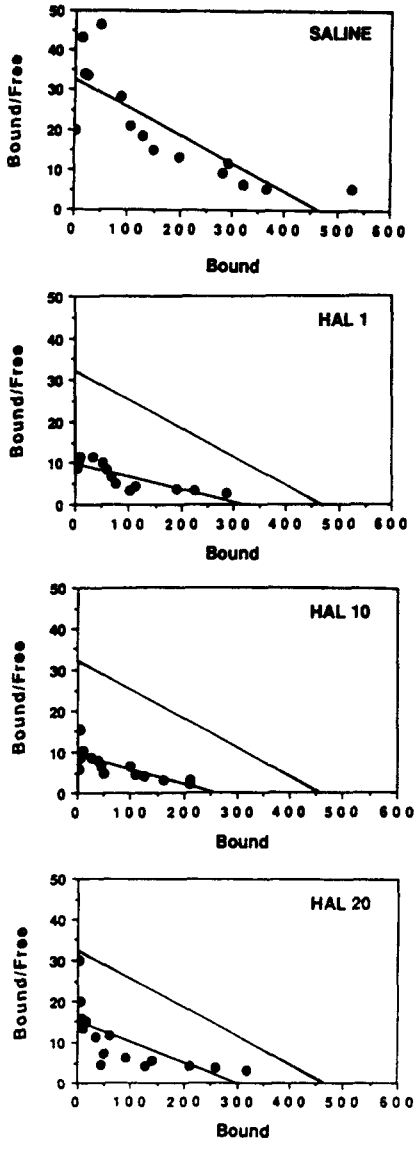

Fig. 3. [^{3}H](+)-Pentazocine binding in young adult rats (2–3 months old) chronically treated with haloperidol. Various concentrations of [^{3}H](+)-pentazocine (concentration range 0.3–100 nM) were incubated with pooled brain membranes from rats treated with haloperidol (5 mg/kg, s.c.) for 1, 10 or 20 days (HAL1, HAL10, HAL20) or an equal volume of saline for 20 days. Non-specific binding was determined in the presence of 5 μM (+)-pentazocine. Data from three replications were averaged to generate the summary plots. The plain line in each panel replicates the Scatchard for the control group while the line fitting the data points shows the plot for the drug condition. There was a significant decrease in B_{max} ($P < 0.05$), but not K_d.

Table 1
Summary of [^{3}H](+)-pentazocine Scatchards in chronically treated rats

Treatment	K_d (nM)	B_{max} (fmol/mg)	Hill coefficient
Saline (20 days)	17±3	506±42	0.91
Haloperidol (1 day)	44±9	393±58	0.75
Haloperidol (10 days)	38±5	299±18	0.84
Haloperidol (20 days)	36±5	395±47	0.77

Various concentrations of [^{3}H](+)-pentazocine (concentration range 0.3–100 nM) were incubated with pooled brain membranes from rats treated with haloperidol (5 mg/kg, s.c.) or an equal volume of saline for up to 20 days. Non-specific binding was determined in the presence of 5 μM (+)-pentazocine. The values represent means±S.E.M. from 3 replications, each run in duplicate.

3.2.2. Comparison between young adult rats chronically treated with haloperidol vs. saline

Although there was a reduction in [^{3}H](+)-pentazocine binding following chronic haloperidol treatment, there was no difference between the degree of head angle produced by intrarubral microinjections of (+)-pentazocine in rats chronically treated with haloperidol vs. saline for 10 or 20 days ($F(1,16) = 0.06$; n.s.; Fig. 5A). This contrasts with the pattern seen with DTG where there was a significant decrease in the head angles produced by intrarubral microinjections of DTG in rats chronically treated with haloperidol vs. saline ($F(1,32) = 4.60$; $P < 0.04$; Fig. 5B) in addition to the previously reported reduction in binding to σ sites labelled with [^{3}H]DTG (Matsumoto et al., 1989b).

3.2.3. Lack of effect of σ and opiate receptor antagonists

The head angles produced by microinjection of (+)-pentazocine into the red nucleus could not be attenuated by the putative σ receptor antagonist BD1047 (Wicoxon stat = 9.00, n.s.) or the opiate receptor antagonist naloxone (Wilcoxon stat = 3.00, n.s.). However, there was a statistically significant attenuation of the head angles produced by (+)-pentazocine when (+)-nordihydrocodeinone was coadministered (Wilcoxon stat = 1.50, $P < 0.03$; Fig. 6). When microinjected into the red nucleus, (+)-nordihydrocodeinone, BD1047, and naloxone had no significant effects on their own as compared to a control injection of saline (naloxone $t = 0.48$, n.s.; (+)-nordihydrocodeinone previously reported in Matsumoto et al., 1990; BD1047 previously reported in Matsumoto et al., 1995).

3.3. Binding affinities of (+)-nordihydrocodeinone for σ site subtypes

(+)-Nordihydrocodeinone had only a low affinity for σ_1 sites (16.7 μM) and was inactive at σ_2 sites (less than 30% displacement at 100 μM).

4. Discussion

There appears to be a dissociation between the motor effects produced by (+)-pentazocine and its binding to σ_1 sites. Despite a significant reduction in [^{3}H](+)-pentazocine binding in the brains of middle aged rats compared to young adult rats, there was no difference between the motor effects elicited by (+)-pentazocine after microinjection into the brains of rats in the two groups. Similarly, chronic haloperidol treatment dramatically attenuated [^{3}H](+)-pentazocine binding, but there was no difference in the extent of head angle produced by (+)-pentazocine when microinjected into the red nucleus of rats chronically treated with haloperidol vs. saline. It therefore appears that unlike the pattern seen with the σ site ligand, DTG, where

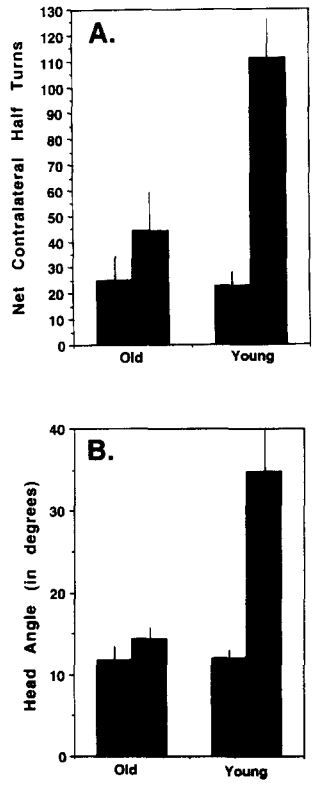

Fig. 4. Effects of (+)-pentazocine (mean±S.E.M.), as compared to DTG, in the red nucleus and substantia nigra of middle aged (old, 5–6 months old) and young adult (young, 2–3 months old) animals. Panel A: Unilateral microinjection of 9.3 nmol (+)-pentazocine (solid bars) into the substantia nigra pars reticulata produced a comparable number of mean net contraleteral half circles in middle aged and young adult rats during the 30 min testing session. Panel B: Unilateral microinjection of 9.3 nmol (+)-pentazocine (solid bars) into the red nucleus produced a comparable change in head angle in young adult and middle aged animals. For comparison, previously reported data for the σ site ligand, DTG (hatched bars), under conditions identical to the ones reported for (+)-pentazocine, are also shown (Matsumoto et al., 1989a). In contrast to the pattern seen with (+)-pentazocine, DTG elicited weaker effects in the middle aged animals, and also had poorer σ binding parameters in these older rats (Matsumoto et al., 1989a).

both drug-induced and naturally occurring changes in the number and affinity of [^{3}H]DTG-labelled σ sites have functional ramifications for the control of movement and posture (Hemstreet et al., 1993; Matsumoto et al., 1989a, 1990), this does not appear to be the case for [^{3}H](+)-pentazocine-labelled sites (see Author's note).

Under the conditions used in this study, [^{3}H](+)-pentazocine would be expected to selectively label σ_1 sites (Bowen et al., 1993). Therefore, the lack of relationship between binding to [^{3}H](+)-pentazocine-labelled sites and motor effects under a number of other conditions, suggests that classical σ_1 sites were not associated with the motor effects observed in this report.

Since the rat brain has been shown to contain σ_1 and σ_2 sites in similar proportions (60%:40%; Bowen et al., 1993; Vilner et al., 1992), a logical explanation would be that σ_2 sites mediated the motor effects induced by (+)-pentazocine. This would be consistent with previous observations that: (1) the drug selectivity pattern associated with σ-induced postural changes after intrarubral microinjection is characterized by relatively low affinity for (+)-opiates and a high affinity for compounds such as DTG and haloperidol, which is suggestive of a σ_2 site pattern (Matsumoto et al., 1990; Quirion et al., 1992); and (2) the potencies of benzomorphan σ site ligands in producing circling behavior are significantly correlated with their affinities for σ_2 sites (Walker et al., 1993), emphasizing the potential importance of σ_2 sites in motor function. However, the present data suggest that an involvement of σ_2 sites alone cannot fully explain the motor effects produced by (+)-pentazocine. If the motor effects of (+)-pentazocine were mediated through σ_2 sites, then the σ receptor antagonist, BD1047, which has a 47 nM K_i for σ_2 sites, should have antagonized the head angles produced by (+)-pentazocine as it does for DTG-induced dystonia (Matsumoto et al., 1995). However, this was not observed. Furthermore, (+)-nordihydrocodeinone, which lacks or has only very low affinity for σ_1 and σ_2 sites was able to partially attenuate the head angles produced by microinjection of (+)-pentazocine into the rat red nucleus, suggesting that at least a portion of the dystonia produced by (+)-pentazocine was not mediated through σ_1 or σ_2 sites.

(+)-Nordihydrocodeinone has previously been shown

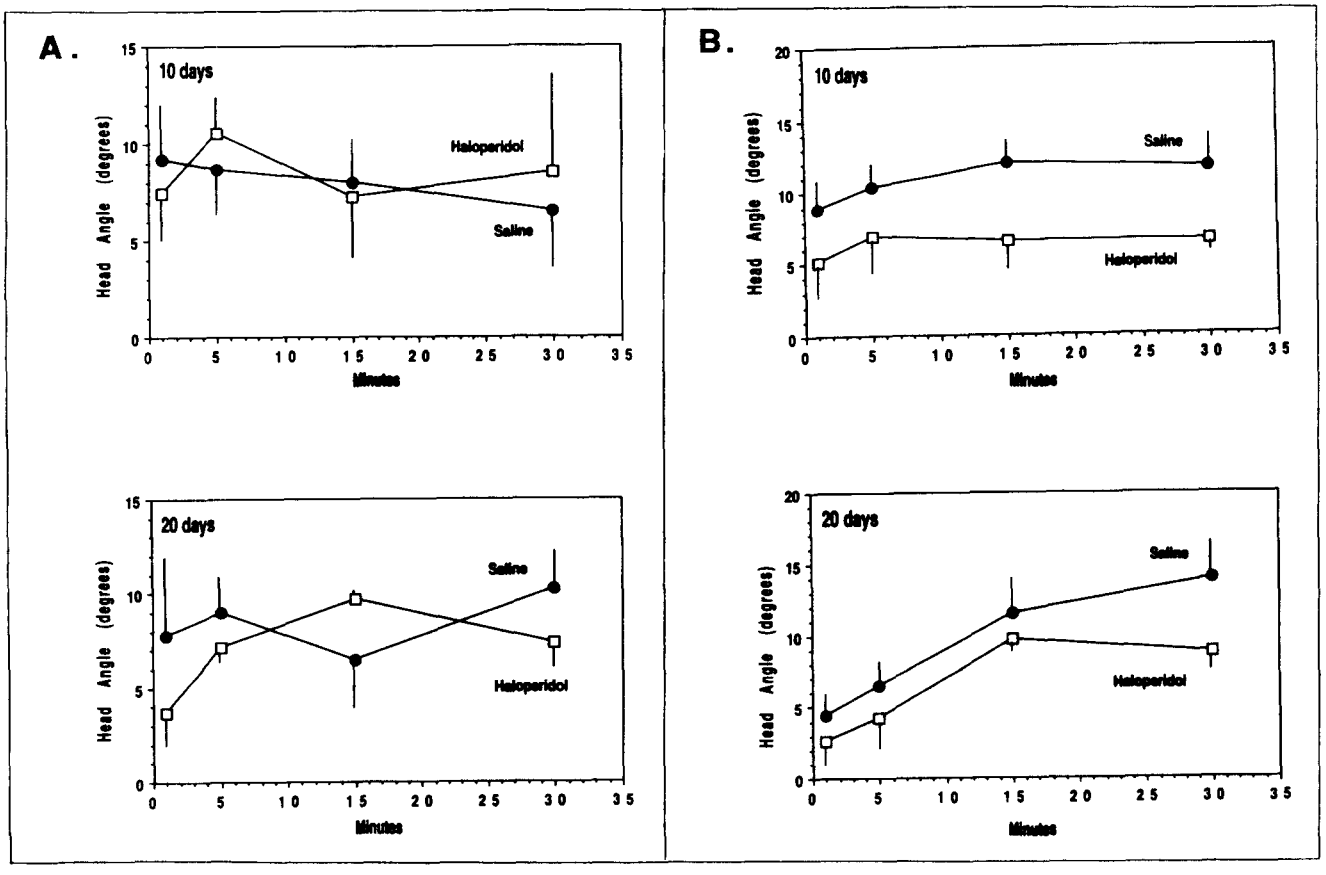

Fig. 5. Panel A: No alteration in the behavior produced by microinjection of (+)-pentazocine into the red nucleus of rats chronically treated with haloperidol. After a 3 day wash out, unilateral microinjection of 9.3 nmol (+)-pentazocine was made into rats chronically treated with haloperidol (5 mg/kg, s.c.) or saline for 10 days (upper panel) or 20 days (lower panel). Panel B: The data for microinjections of 9.3 nmol DTG into the rat red nucleus after chronic haloperidol treatment, under conditions identical to the ones reported for (+)-pentazocine. In contrast to the pattern seen with (+)-pentazocine, DTG elicited weaker effects in rats chronically treated with haloperidol ($P < 0.04$) and also had poorer σ binding parameters in haloperidol-treated rats.

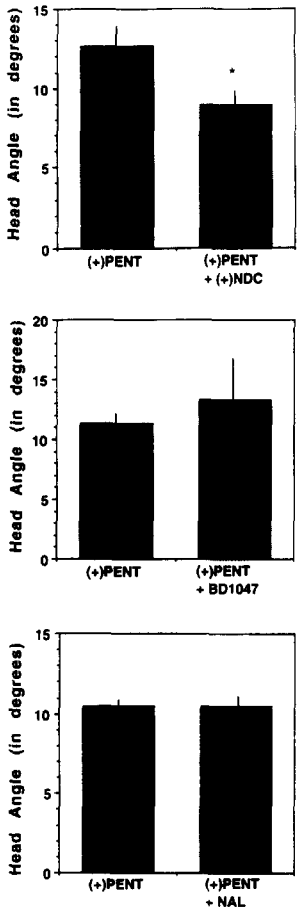

Fig. 6. Reduction in the extent of dystonia produced by (+)-pentazocine in the presence of (+)-nordihydrocodeinone, but not BD1047 or naloxone. Unilateral microinjections of 9.3 nmol (+)-pentazocine were made into the red nucleus of rats. Subsequent intrarubral administrations of 9.3 nmol (+)-pentazocine were made in the presence of 9.3 nmol of either BD1047 (σ receptor antagonist), naloxone (NAL, opiate receptor antagonist), or (+)-nordihydrocodeinone (NDC, structurally related (+)-opiate devoid of σ or opiate activity). The attenuation of the (+)-pentazocine response was significant for only (+)-nordihydrocodeinone ($P < 0.03$), suggesting that at least part of the (+)-pentazocine response was mediated through a mechanism other than that involving typical σ or opiate receptors.

to attenuate oxotremorine-induced analgesia in rats (Walker et al., 1990b). Benzomorphan σ site ligands such as (+)-pentazocine and dextrallorphan also had similar actions (Walker et al., 1990b). However, the ability of these compounds to attenuate oxotremorine-induced analgesia could not be attributed to actions at σ sites because other σ site ligands such as DTG, (+)-SKF 10,047, and (+)-cyclazocine were without effect (Walker et al., 1990b). We have previously demonstrated that unilateral microinjection of (+)-nordihydrocodeinone alone into the rat red nucleus has no effect (Matsumoto et al., 1990), although in the present study, it was able to partially reduce the dystonic postures produced by (+)-pentazocine. Taken together, the data suggest that (+)-nordihydrocodeinone acts as a partial agonist/antagonist at a non-σ_1/non-σ_2 site that is also sensitive to some other (+)-opiates. It is unlikely that this yet unidentified site represents a type of opiate receptor because the opiate receptor antagonist, naloxone, was incapable of attenuating the head angles produced by (+)-pentazocine.

Additional evidence for the involvement of non-σ_1/non-σ_2 sites in the motor effects of (+)-pentazocine is derived from an examination of the binding associated with [^{3}H](+)-pentazocine in the rat brain. The borderline unitary Hill coefficients for [^{3}H](+)-pentazocine in some Scatchard plots suggests that the radioligand may interact with more than one population of sites. Furthermore, if σ_1 and σ_2 sites were the only binding sites present in the rat brain, [^{3}H](+)-pentazocine, which is σ_1 selective, should have labelled fewer sites than [^{3}H]DTG, which has about equal affinity for both σ_1 and σ_2 sites. Although this pattern of results is observed in rat liver membranes (Hellewell and Bowen, 1990; Hellewell et al., 1994), in rat brain, [^{3}H]DTG and [^{3}H](+)-pentazocine label a similar number of sites, suggesting that [^{3}H](+)-pentazocine interacts with additional sites. Although the possible involvement of a non-σ site mechanism cannot be ruled out, it is possible that another less characterized σ site may be involved (Booth et al., 1993; Bowen et al., 1989, 1995; Codd and Shank, 1992; Enomoto et al., 1993; Matsumoto et al., 1992; Vilner et al., 1992, 1993, 1995a, 1995b; Wu et al., 1991).

The most likely possibility involves the existence of a low affinity (+)-pentazocine binding site in rat brain that is not labelled well by [^{3}H](+)-pentazocine under the conditions used in our assays. In the chronic haloperidol studies, we obtained a K_d value for a low affinity site of 1001 nM in brain membranes from animals acutely treated with haloperidol. In previous studies, we have shown that (+)-pentazocine has an IC_{50} value against [^{3}H]DTG in the rat brain of 1106 nM (Matsumoto et al., 1990). Since these values are consistent with the affinity of (+)-pentazocine for σ_2 sites (Bowen et al., 1993), they have been assumed to represent binding to this σ subtype and until recently, there was no reason to consider other alternatives. However, a low affinity [^{3}H](+)-SKF 10,047 site has been documented in NCB-20 cells that appears to be involved in σ-mediated effects on K^+ currents (Wu et al., 1991). Although this [^{3}H](+)-SKF 10,047 site has low affinity for (+)-benzomorphans, it exhibits only moderate enantioselectivity for their (−)-isomers and has significantly lower affinity for haloperidol and (−)-pentazocine than reported for σ_2 sites (Wu et al., 1991). Therefore, it has been speculated that this low affinity site represents a distinct binding site for (+)-opiates from σ_2 sites (Vilner et al., 1992). Similar low affinity sites for (+)-opiates that are atypical of σ_2 sites have been reported in a number of tumor cell lines (Vilner et al., 1995b). Among these cell lines, three apparent classes of [^{3}H](+)-pentazocine binding sites could be distinguished based on K_d values in the range of 1–7 nM, 30–60 nM, and 125–350 nM. In

addition, low affinity sites for [^{3}H]DTG have been reported in the brains of Wistar rats (Codd and Shank, 1992; Enomoto et al., 1993). Since DTG has a high affinity for both σ_1 and σ_2 sites (Bowen et al., 1993; Hellewell and Bowen, 1990; Quirion et al., 1992), the micromolar affinities associated with these sites cannot be attributed to interactions with either of these two subtypes. Recently, a putative σ_3 site which selectively binds a class of novel phenylaminotetralins, but which has low affinity for prototypic σ site ligands such as haloperidol, (+)-pentazocine, and DTG has been identified (Booth et al., 1993); and Bowen and co-workers have additionally characterized a novel σ-like site which has low affinity for haloperidol, (+)-pentazocine, and DTG, but which has high affinity for σ site-active aryl ethylenediamines such as BD1008 (Bowen et al., 1995). Further studies are needed to fully characterize these low affinity sites because they may be mediating functional effects produced by (+)-pentazocine and other σ site ligands.

Taken together, the data suggest that [^{3}H](+)-pentazocine is a potent and selective probe for σ_1 binding sites in vitro, but the in vivo effects of (+)-pentazocine cannot be fully attributed to actions at this site. One explanation is that there is a low affinity site in rat brain that is not labelled efficiently with [^{3}H](+)-pentazocine, but which may nonetheless mediate some of the physiological actions of the unlabelled drug.

5. Author note

In a previous book chapter, it was stated that repeated administration of haloperidol reduced the motor effects of (+)-pentazocine after intranigral and intrarubral injections in rats (Walker et al., 1994). This statement is incorrect and regrettably, we failed to detect this error before publication. The sentence should read, '...we found that repeated injections of haloperidol led to reduced motor responses to intrarubral microinjections of DTG in rats'. As demonstrated in the current paper, the motor effects of (+)-pentazocine do *not* mirror effects on [^{3}H](+)-pentazocine binding following chronic haloperidol treatment. Chronic administration of haloperidol reduces the motor effects of DTG after intrarubral injections, and age-related decreases in [^{3}H]DTG binding are reflected as decreased efficacy of intrarubral and intranigral injections of DTG.

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