British Journal of Pharmacology (1997) 120, 1255–1262

An autoradiographic study of dextromethorphan high-affinity binding sites in rat brain: sodium-dependency and colocalization with paroxetine

*,[1]P. Meoni, †F.C. Tortella & ‡N.G. Bowery

*Department of Pharmacology, The School of Pharmacy, University of London, London WC1N 1AX; †Neuropharmacology Branch, Walter Reed Army Institute of Research, Washington D.C., U.S.A. and ‡Department of Pharmacology, Medical School, University of Birmingham, Birmingham B15 2TT

1 The distribution and some pharmacological properties of centrally located dextromethorphan high-affinity binding sites were investigated by *in vitro* autoradiography.

2 Sodium chloride (50 mM) induced a 7 to 12 fold increase in dextromethorphan binding to rat brain in all areas tested. The effect of sodium was concentration-dependent with a higher dose (120 mM) exerting a smaller effect on binding.

3 [^{3}H]-dextromethorphan binding in the presence of sodium was inhibited in the presence of the anticonvulsant phenytoin at a concentration of 100 μM, while the σ ligand (+)-3-(-3-hydroxyphenyl)-N-(1-propyl)pipendine ((+)-PPP) had no effect on the binding, suggesting an interaction with the DM_2 site.

4 The distribution of the sodium-dependent binding identified in this study correlated significantly with the distribution of the selective 5-HT uptake inhibitor [^{3}H]-paroxetine, and paroxetine and dextromethorphan mutually displaced their binding at concentrations in the low nanomolar range.

5 These data show that dextromethorphan and paroxetine share a sodium-dependent high affinity binding site in rat brain, and suggest that dextromethorphan might interact, in the presence of sodium, with the 5-HT uptake mechanism in rat brain.

Keywords: Dextromethorphan; paroxetine; antitussives; autoradiography; 5-HT uptake; σ receptors

Introduction

Dextromethorphan is a dextrorotatory analogue of morphine that acts centrally to elevate the cough threshold in both man (Eddy *et al*., 1969) and rodents (Benson *et al*., 1953). Besides this well characterized property, it has also been shown to possess an anticonvulsant (Tortella & Musacchio, 1986) and neuroprotectant (Steinberg *et al*., 1991) action in laboratory animals.

Binding sites for dextromethorphan have been described in both the guinea-pig (Craviso & Musacchio, 1980) and rat brain (Klein & Musacchio, 1992), and several centrally acting antitussives such as carbetapentane and caramiphene show high affinity for these sites (Craviso & Musacchio, 1983b). In comparison, dextrorphan, the main active metabolite of dextromethorphan following 0-demethylation by a cytochrome P-450 (Schmid *et al*., 1985), only displaces dextromethorphan binding from these sites with an IC_{50} in the micromolar range (Craviso & Musacchio, 1983b).

The functional role of these dextromethorphan high affinity binding sites has yet to be fully elucidated, but the selective displacement of dextromethorphan from these sites by other centrally acting antitussives such as 3-substituted 17-methyl morphinan analogues, and the efficacy of these substances as neuroprotectants and anticonvulsants (Newman *et al*., 1992) suggest their involvement in the pharmacological actions of dextromethorphan and its derivatives.

Phenytoin and ropizine have been shown to produce a concentration-dependent allosteric increase in the affinity of dextromethorphan to a subpopulation of dextromethorphan high affinity binding sites in guinea-pig (Musacchio *et al*., 1988) and rat brain homogenates (Klein & Musacchio, 1992). The subsequent observation of a differential effect of phenytoin and other anticonvulsants on dextromethorphan binding in different areas of guinea-pig brain (Canoll *et al*., 1990) in an autoradiographical study and the computer-assisted modelling of homologous and heterologous competition studies between dextromethorphan and the σ ligand (+)-3-(-3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-PPP) (Zhou *et al*., 1991; Klein & Musacchio, 1992), has led to a model in which dextromethorphan would be binding in guinea-pig and rat brain to two high affinity and one low affinity site. One of the two high affinity sites, termed DM_1 by Musacchio *et al*. (1989), is allosterically enhanced by phenytoin and ropizine (Musacchio *et al*., 1988; Klein & Musacchio, 1992) and could therefore be involved in the anticonvulsant action of dextromethorphan. The DM_1 site has properties in common with the binding site for (+)-PPP, and should therefore be regarded as a DM_1/σ_1 site (Klein & Musacchio, 1990). By comparison, (+)-PPP only shows a micromolar affinity for the other high affinity binding site (Zhou & Musacchio, 1991; Klein & Musacchio, 1992). DM_2, therefore does not represent a σ site, and binding to this site is inhibited (Canoll *et al*., 1990; Klein & Musacchio, 1992) by phenytoin and ropizine.

SKF 525-A, a wide spectrum inhibitor of cytochrome P-450 (Schenkman *et al*., 1972), and GBR 12909, a dopamine uptake inhibitor with high affinity for cytochrome P450IID1 (Niznik *et al*., 1990), have been shown to displace dextromethorphan binding from the DM_1/σ_1 site (Klein *et al*., 1991), and similar findings have suggested that this site can be identified with a metabolic site.

Antitussive properties have never been described for σ site ligands, thus the DM_2 high affinity site may play a key role in the antitussive action of dextromethorphan. The DM_2 site is relatively less abundant in guinea-pig than in rat brain, where it represents the majority of the binding (Klein & Musacchio, 1992). Most of the behavioural tests that have highlighted the antitussive, anticonvulsant and neuroprotectant properties of dextromethorphan have been conducted in the rat. This prompted us to characterize in more detail dextromethorphan binding in rat brain, in order to elucidate the identity of the DM_2 binding site in this species.

[1]Author for correspondence at present address: Department of Pharmacology, Medical School, University of Birmingham, Vincent Drive, Edgbaston B15 2TT.

As part of the characterization we have considered the possibility that dextromethorphan might interact with the uptake process for 5-hydroxytryptamine (5-HT). α(−)-*Trans*-4-(p-fluorophenyl)-3-[3,4-methylenedioxyphenoxy-methyl]piperidine (paroxetine) is a very potent and selective inhibitor of sodium-dependent 5-HT uptake *in vitro* (Buus-Lassen, 1978; Habert *et al*., 1985) and *in vivo* (Graham & Langer, 1988). We have therefore used it in the present study to examine the possible link between dextromethorphan and the sodium-dependent neuronal 5-HT uptake mechanism.

Methods

Dextromethorphan autoradiography

Male Wistar rats weighing 200–300 g were decapitated and the brains were rapidly removed and snap-frozen in liquid nitrogen-cooled isopentane at −70°C. Brains were stored at −80°C before being sectioned. Sagittal sections (10 μm) were cut at −16°C in a cryostat at a level 0.40 mm lateral to the midline and thaw-mounted on gelatine-coated (5% gelatine, 0.05% chromic potassium sulphate) glass slides. Sections were allowed to air-dry before storage at −20°C until use in binding assays.

Before incubation, sections were thawed at room temperature for 15 to 30 min and pre-incubated in ice-cold Tris-HCl solution (50 mM, pH 7.4) for 30 min. Following pre-incubation, sections were dried under a stream of cool air for 15 to 30 min.

For dextromethorphan binding, incubation was initiated by covering each section with a drop of buffer (150 μl) containing an appropriate concentration of [^{3}H]-dextromethorphan (81 Ci mmol^{-1}) in Tris-HCl solution (50 mM, pH 7.4). Non-specific binding was defined as the binding obtained in the presence of unlabelled dextromethorphan 100 μM.

In preliminary studies to assess optimal conditions for autoradiography, the kinetic characteristics of [^{3}H]-dextromethorphan binding were examined to obtain the best ratio of total to non-specific binding, whilst maintaining the highest possible specific binding.

In these experiments, the level of binding was assessed by liquid scintillation spectrometry: the portion of the slide containing the section was cut out with a diamond pencil, placed in a 20 ml plastic scintillation vial with 10 ml of scintillation fluid (Eco-scint) and counted in a liquid scintillation counter (Rackbeta, Packard).

Association of the ligand with its binding site was studied by incubating the sections for various lengths of time (3–96 min) before washing the slides (5 min for three times) in washing buffer at 24 or 4°C after an initial rinse in buffer solution. The dissociation of [^{3}H]-dextromethorphan from the slide-mounted sections was examined after incubation with 20 nM [^{3}H]-dextromethorphan for 60 min at 4°C. The sections were eventually transferred to ice-cooled wash buffer for up to 60 min. These experiments were repeated with three animals, with triplicate determination for each rat.

Saturation experiments were carried out with 5 to 80 nM of [^{3}H]-dextromethorphan while mapping studies were performed with a concentration of radiolabelled ligand of 20 nM. After incubation, slides were washed in fresh Tris-HCl solution (50 mM, pH 7.4) containing 100 mM choline chloride and 0.01% v/v Triton X-100 at 4°C (wash buffer), as described by Craviso and Musacchio (1983a). Preliminary studies (data not shown) demonstrated an increase in specific binding from 50 to 70% of total binding when the sections were washed for 5 min in Tris-HCl solution (50 mM, pH 7.4) without or with choline chloride and Triton, respectively.

In saturation and distribution studies, sections were incubated for 1 h in Tris buffer solution (50 mM, pH 7.4) at 4°C and washed for 2.5 min at the same temperature in wash buffer.

Dried, incubated sections were apposed to ^{3}H-Hyperfilm (Amersham) in slide cassettes with appropriate plastic tritium standards (Amersham) for 7 to 28 days. Films were developed with Kodak D-19 developer for 1 min and fixed for 5 min.

Paroxetine autoradiography

Binding conditions for the study of the distribution of [^{3}H]-paroxetine to rat brain were adapted from the work of Chen *et al*. (1992). Sections were pre-incubated in Tris buffer solution (50 mM at pH 7.4) at room temperature for 20 min. After being dried, the rat tissue was incubated with 1 nM [^{3}H]-paroxetine (15 Ci mmol^{-1}) in Tris HCl solution (containing 300 mM NaCl) for 90 min at room temperature. At the end of the incubation, the sections were quickly dipped in buffer and subsequently washed in the same solution for 30 min. Non-specific binding was defined as binding in the presence of 100 μM fluoxetine.

Dried sections were then processed as for dextromethorphan autoradiography.

Analyses of autoradiograms

The autoradiograms were analysed by a Quantimet 970 (Cambridge Instruments). The density in each region was recorded by positioning a squared cursor on two areas over each region. Mean values for each nucleus were determined from 4 measurements deriving from two adjacent sections. Variability of measurements is expressed as s.e.mean of the values in fmol mg^{-1} wet weight of brain tissue. Individual tables were analysed with a two-way ANOVA to determine a possible effect of treatment and interaction between the effect of treatments and the area measured. Data were subsequently analysed with a one-way ANOVA with Tukey's *post-hoc* test to assess which treatments significantly affected the binding. In the case of a significant interaction between treatments and nuclei, ANOVA was applied to individual nuclei.

Drugs

[^{3}H]-dextromethorphan and [^{3}H]-paroxetine were purchased from New England Nuclear (NEN, Boston, MA, U.S.A.); SKF 525-A (N,N-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was obtained through Research Biochemical International (RBI, Natik, MA, U.S.A.). Paroxetine and fluoxetine were a kind gift of Smithkline Beecham (Harlow, Essex, U.K.). GBR 12909 (1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine dihydrochloride) was obtained from RBI (Natik, MA, U.S.A.). All the other chemicals in this study were obtained from Sigma Chemical Inc. (St. Louis, MO, U.S.A.).

Results

Dextromethorphan binding kinetics

Experiments conducted to assess the kinetics of dextromethorphan binding to rat brain showed the binding to follow a one-site binding isotherm model with the equilibrium reached after 30 min of incubation at 4°C (Figure 1). Preliminary experiments (data not shown) revealed higher levels of specific binding following incubation at 4°C as compared to room temperature. The influence of washing on the level of radioligand bound to the section was best described by a two-site dissociation model, with the fast dissociating site ($t_{1/2}$ = 1.13 min) having a capacity double the slow-dissociating site ($t_{1/2}$ = 5.94 min) (Figure 1). On the basis of these results, binding conditions for successive studies were established as 1 h incubation followed by 2.5 min wash. Under these condi-

tions, specific binding accounted for 70% of total binding when measured with quantitative microdensitometry in distribution studies.

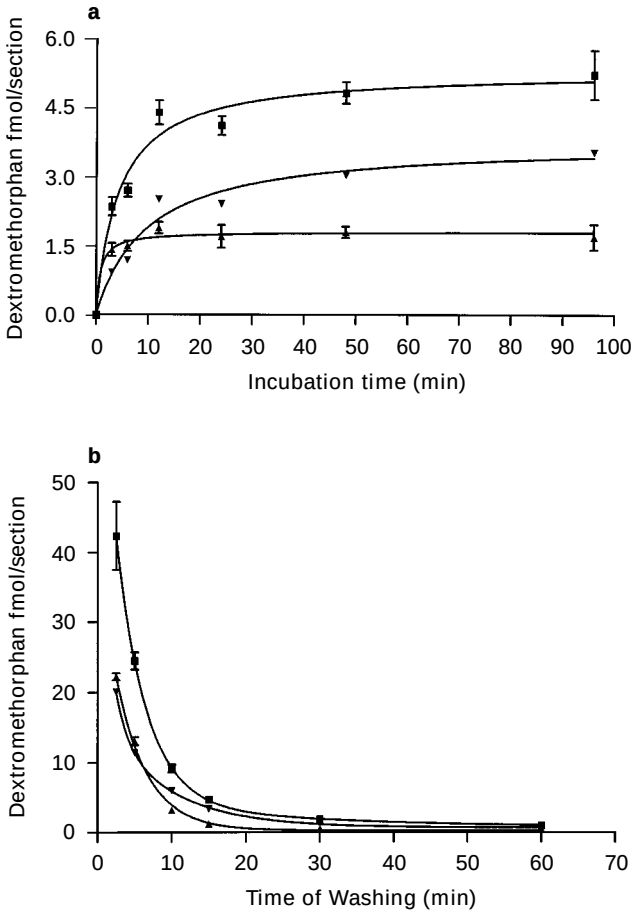

Figure 1 Effect of different incubation (a) and washing times (b) on [^{3}H]-dextromethorphan binding to rat brain. Sections were incubated at 4°C for various lengths of time in the presence of [^{3}H]-dextromethorphan 20 nM in TrisHCl 50 mM, pH 7.4, and subsequently washed for 2.5 min at 4°C in the same buffer containing 100 mM choline chloride and 0.01% Triton X-100. The effect of different washing times was tested after an initial incubation of 60 min at 4°C. (■) Total binding, (▲) non-specific and (▼) specific binding.

Effect of different ions on dextromethorphan binding

Dextromethorphan binding in Tris buffer solution in the absence of any added sodium chloride was very evenly distributed throughout the rat brain. Binding to all nuclei ranged between 10 and 20 fmol mg^{-1} wet weight tissue with less than a two fold ratio between the lowest (frontal cortex) and the highest (superior colliculi) level of binding (Table 1). Addition of sodium chloride (50 mM) to the incubation solution produced on average a seven fold increase in dextromethorphan binding. This increase in dextromethorphan binding with sodium was significant (ANOVA, $P<0.05$), with a significant interaction between treatments and nuclei. One-way ANOVA followed by Tukey's *post-hoc* test applied to individual regions revealed a significant effect of sodium in all areas with the exception of cerebellum, where the effect did not reach statistical significance. The effect of sodium was maximal in the ventral tegmental area, where the binding was increased 12 fold.

Table 2 Autoradiographic saturation analysis of [^{3}H]-dextramethorphan binding in discrete areas of rat brain

Nucleus	K_d	B_{max}
Mammillary bodies	10.5±4.9	286.3±40.1
Ventral tegmental area	8.5±5.2	257.6±43.5
Dorsal raphe	9.7±5.3	228.6±36.3
Superior colliculi	11.5±5.0	204.9±28.0
Central grey area	12.2±5.4	198.7±28.0
Dorsal tegmental area	13.2±5.5	212.6±28.9
Anterior hypothalamus	11.8±6.0	172.9±27.6
Pons	13.8±7.0	87.1±14.8
Hippocampus (CA3 area)	13.9±8.1	77.5±15.1
Dentate gyrus	16.5±9.0	67.2±13.1
Frontal cortex	17.3±6.6	143.9±19.7
Occipital cortex	19.3±9.9	83.9±16.1
Mediodorsal thalamic area	9.3±5.2	94.9±15.4
Medial pretectal nuclei	10.1±5.5	219.5±35.4
Cerebellum	42.4±18.0	42.4±20.3

The tissue sections were incubated with concentrations of [^{3}H]-dextromethorphan ranging fron 5 to 80 nM in buffer containing 120 nM sodium chloride. K_d is expressed as nM and B_{max} as fmol mg^{-1} wet weight tissue. Each set of data are from an average of three animals performed in duplicate± s.e.mean. Data were analysed with GraphPad Prism version 2.01.

Table 1 Effect of sodium on [^{3}H]-dextromethorphan binding in discrete regions of rat brain and of calcium and magnesium on the sodium-dependent binding

	Controls	Na^+ 50 mM[a]	Na^+ 120 mM[a]	Na^+ 50 mM + Ca^{2+} 2.5 mM[a,b]	Na^+ 50 mM + Mg^{2+} 1.2 mM[a,b]
Mammillary bodies	16.3±2.0	132.4±19.4[a]	138.8±7.7[a,b]	127.7±8.5[b]	79.7±10.3[b]
Ventral tegmental area	16.4±3.0	198.1±3.9[a]	157.8±5.7[a,b]	151.8±4.7[b]	114.9±5.5[b]
Dorsal raphe	18.5±2.1	191.5±10.1[a]	142.1±8.4[a]	162.4±11.6[b]	113.9±8.4[b]
Superior colliculi	21.2±3.0	152.7±10.6[a]	114.1±3.6[a,b]	93.7±8.3[b]	58.1±4.7[b]
Central grey area	17.0±2.0	127.8±10.0[a]	106.3±3.0[a]	90.4±5.2[b]	64.2±2.5[b]
Dorsal tegmental area	19.2±2.6	120.1±4.3[a]	102.3±5.4[a,b]	93.3±3.8	60.9±3.4[b]
Anterior hypothalamus	14.5±3.1	109.2±12.4[a]	104.4±4.1[a]	83.2±3.4	45.2±3.1[b]
Pons	11.9±2.3	40.1±6.3[a]	35.1±1.9[a]	25.3±6.1	7.7±3.1[b]
Hippocampus (CA3 area)	15.3±2.6	54.7±6.4[a]	42.1±2.2[a]	19.1±4.1[b]	5.3±1.8[b]
Dentate gyrus	12.3±2.6	42.2±4.6[a]	30.2±1.9[a]	14.9±2.4[b]	1.3±0.7[b]
Frontal cortex	10.9±2.1	75.8±8.4[a]	68.4±2.9[a]	60.5±6.3	27.1±3.6[b]
Occipital cortex	16.2±2.1	62.8±9.4[a]	32.3±2.78[a,b]	12.9±2.9[b]	3.8±1.3[b]
Mediodorsal thalamic area	15.8±2.4	63.0±9.3[a]	59.8±5.1[a]	35.6±3.2	7.1±2.3[b]
Medial pretectal nuclei	11.7±1.5	137.0±7.5[a]	118.0±3.9[a]	97.2±4.1[b]	65.1±4.0[b]
Cerebellum	19.6±3.0	42.3±12.5	19.7±3.2	5.8±1.8[b]	0.8±0.6[b]

The tissue sections were incubated with 10 nM [^{3}H]-dextromethorphan, as described in Methods, in buffer containing the ions indicated above each column. Binding capacity is expressed as fmol mg^{-1} wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean±s.e.mean of 3–11 rats. [a]$P<0.05$ versus binding in the absence of sodium, [b]$P<0.05$ versus binding in the presence of sodium 50 mM (ANOVA and Tukey's *posthoc* test).

A rise in the concentration of sodium in the incubation buffer did not induce a further increase in dextromethorphan binding. In fact, binding to the medial mammillary bodies, ventral tegmental area, dorsal tegmental area, superior colliculi and occipital cortex was significantly reduced when the sodium concentration was increased to 120 mM as compared to binding in the presence of 50 mM sodium. Ca^{2+} and Mg^{2+}, at the physiological concentration of 2.5 and 1.2 mM, respectively, reduced [^{3}H]-dextromethorphan binding in the presence of 50 mM sodium chloride to enhance maximally the sodium-dependent binding. The reduction following Mg^{2+} was significant in all areas measured, while the same effect after the addition of Ca^{2+} in the incubation buffer did not reach statistical significance in the dorsal tegmental area, anterior hypothalamus, pons, frontal cortex and mediodorsal thalamic area (Table 1).

Saturation studies showed that the sodium-dependent

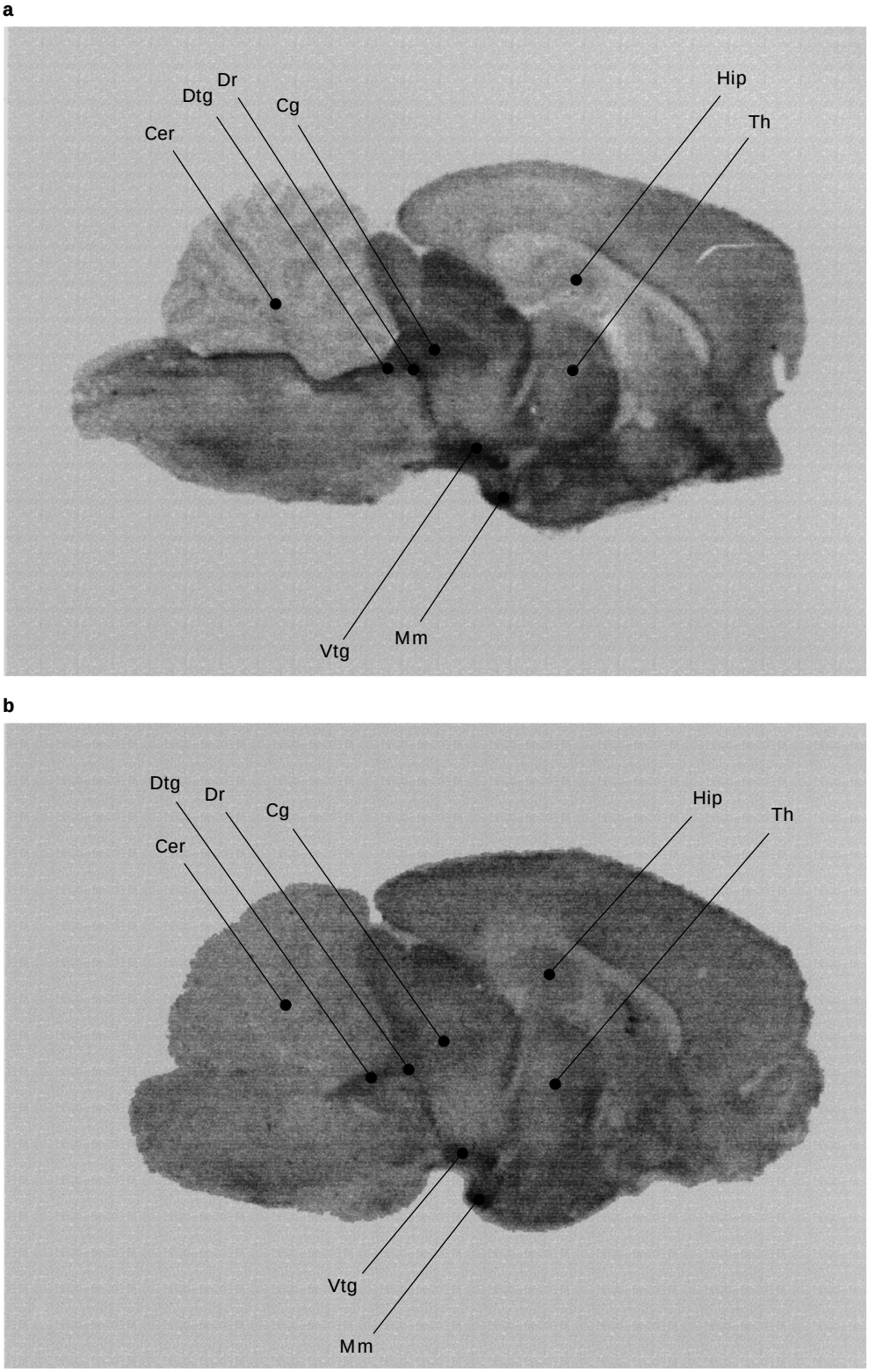

Figure 2 Receptor autoradiographs of [^{3}H]-dextromethorphan (a) and [^{3}H]-paroxetine binding (b) to parasagittal rat sections. Sections were incubated for 1 h at 4°C with [^{3}H]-dextromethorphan or 90 min at 23°C with [^{3}H]-paroxetine. Abbreviations: cerebellum (Cer), dorsal raphe (Dr), central gray (Cg), hippocampus (Hip), thalamus (Th), dorsal tegmental area (Dtg), ventral tegmental area (Vtg), medial mammilllary bodies (Mm).

binding of dextromethorphan to rat brain was saturable with an average K_d value of 14.66 nM (K_d of individual brain areas are presented in Table 2).

K_d values obtained in different brain areas did not differ significantly (ANOVA test) from each other, and a two-site binding model could not significantly improve the fit to the experimental data.

In the presence of 120 mM NaCl, [^{3}H]-dextromethorphan binding followed a distinctive pattern of distribution in rat brain (Figure 2, Table 1). Binding to mammillary nuclei, ventral tegmental area, dorsal raphe, superior colliculi, central grey, anterior hypothalamus and medial pretectal nuclei was higher than 100 fmol mg^{-1} wet weight of tissue, while the dorsal tegmental area, mediodorsal thalamic nuclei and frontal cortex showed medium levels of binding (50–100 fmol mg^{-1} wet weight of tissue). Low levels of binding (0–50 fmol mg^{-1} wet weight of tissue) were detected in the hippocampus, occipital cortex, pons and cerebellum.

Competition at dextromethorphan binding sites

The σ ligand (+)-PPP, at a concentration of 88 nM, had no effect on dextromethorphan binding (Table 3) in the presence of sodium.

In comparison, Na^+-dependent dextromethorphan binding was clearly inhibited by the anticonvulsant phenytoin (100 μM). On average, phenytoin reduced dextromethorphan binding to 58.4% of control. This effect was statistically significant with no interaction between the treatments and nuclei (ANOVA). Binding of [^{3}H]-dextromethorphan was also reduced by the 5-HT uptake inhibitor paroxetine at a concentration of 20 nM by about 40% (Table 3). Again, the effect was significant with no interaction between treatments and nuclei.

When tested against the sodium-dependent binding of dextromethorphan to rat brain at concentrations of 7.5 and 20.5 nM, respectively, SKF 525-A and GBR 12909 caused a small but statistically significant decrease in dextromethorphan binding to rat brain (Table 4).

Paroxetine binding to rat brain

The binding of [^{3}H]-paroxetine to brain sections had a similar pattern of distribution to that of dextromethorphan. [^{3}H]-paroxetine binding was high (>90 fmol mg^{-1} wet weight of tissue) in the mammillary bodies and ventral tegmental area, medium (45–90 fmol mg^{-1} wet weight of tissue) in the dorsal raphe, superior colliculi and medial pretectal nuclei, and low

Table 3 Effect of (+)-PPP, phenytoin and paroxetine on sodium-dependent [^{3}H]-dextromethorphan binding in discrete regions of rat brain

	Controls	*(+)-PPP 88 nM*	*Phenytoin 100 μM*[a]	*Paroxetine 20 nM*[a]
Mammillary bodies	136.9±7.9	136.4±7.5	89.9±17.5	75.8±4.7
Ventral tegmental area	154.8±6.4	144.3±7.0	94.7±7.9	81.9±2.9
Dorsal raphe	140.7±8.5	126.6±8.1	112.1±21.2	99.6±8.7
Superior colliculi	110.8±3.8	101.2±5.1	71.5±4.1	85.0±3.6
Central grey area	103.5±3.2	100.6±4.7	64.1±2.4	72.0±3.1
Dorsal tegmental area	99.9±5.1	94.4±5.6	52.5±8.6	63.3±4.8
Anterior hypothalamus	100.2±4.1	103.0±4.5	61.9±10.6	46.7±4.7
Pons	36.4±1.9	33.2±3.4	25.4±3.7	29.3±5.3
Hippocampus (CA3 area)	43.6±2.2	35.7±3.5	23.2±1.9	27.1±3.2
Dentate gyrus	32.1±2.0	22.4±3.0	14.7±1.8	21.5±2.2
Frontal cortex	69.0±2.8	61.2±4.8	30.9±3.9	42.6±4.9
Occipital cortex	34.2±2.8	25.8±3.8	17.2±2.6	26.8±3.2
Mediodorsal thalamic area	60.7±5.0	50.3±3.2	48.8±4.1	35.3±2.5
Medial pretectal nuclei	114.0±4.2	110.9±4.0	80.3±3.9	65.2±5.9
Cerebellum	20.3±3.1	9.8±1.9	11.9±1.8	24.8±2.7

The tissue sections were incubated with 10 nM [^{3}H]-dextromethorphan in the presence of sodium 120 mM, as described in Methods, in buffer containing the displacer indicated above each column. Binding capacity is expressed as fmol mg^{-1} wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean±s.e.mean of 3–11 rats. [a]$P<0.05$ versus controls (ANOVA).

Table 4 Effect of cytochrome P-450 inhibitors on [^{3}H]-dextromethorphan binding in discrete areas of rat brain

	Controls	*SKF 525-A 7.5 nM*[a]	*GBR 12909 20.5 nM*[a]
Mammillary bodies	132.8±8.2	129.4±8.3	130.6±7.6
Ventral tegmental area	150.7±6.5	145.1±7.1	137.4±5.2
Dorsal raphe	146.7±6.8	131.2±5.2	133.6±4.4
Superior colliculi	107.1±5.7	91.1±3.9	88.3±4.1
Central grey area	97.2±3.2	91.4±5.3	87.1±2.9
Dorsal tegmental area	82.1±8.7	75.6±4.7	76.8±5.0
Anterior hypothalamus	100.3±7.9	92.0±2.6	89.0±3.2
Pons	33.4±2.6	36.4±2.4	38.2±5.5
Hippocampus (CA3 area)	41.4±5.3	37.3±2.3	28.5±3.0
Dentate gyrus	29.3±4.9	26.6±2.5	29.1±5.7
Frontal cortex	79.4±5.1	76.9±2.8	65.7±5.8
Occipital cortex	36.3±5.4	23.8±3.0	26.3±3.1
Mediodorsal thalamic area	56.2±5.0	52.8±2.9	47.1±2.8
Medial pretectal nuclei	99.8±5.6	99.8±3.7	84.9±6.2
Cerebellum	16.9±3.4	10.9±1.7	11.0±3.4

Tissue sections were incubated with 10 nM [^{3}H]-dextromethorphan in the presence of sodium chloride (120 mM), as described in Methods, in buffer containing the displacer indicated above each column. Binding capacity is expressed as fmol mg^{-1} wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean±s.e.mean of 3–11 rats. [a]$P<0.05$ versus controls (ANOVA).

(<45 fmol mg^{-1} wet weight of tissue) in all other areas tested (Figure 2 and Table 5).

When dextromethorphan was present in the incubation buffer, it reduced the binding of [^{3}H]-paroxetine to 64.9% of controls. This effect was statistically significant at 40 nM with no interaction between treatment and nuclei.

The correlation plot between the distributions of dextromethorphan and paroxetine in rat brain is shown in Figure 3. Data points are best described by a straight line (correlation was significant, $r=0.922$). The slope of the line differed significantly from zero ($P<0.0001$), revealing a non-casual relationship between the distributions. The intercept of the line on the abscissae may suggest the presence of a component of dextromethorphan binding that is not shared by paroxetine.

Discussion

The present work provides autoradiographical evidence for the existence of a sodium-dependent fraction of dextromethorphan binding to rat brain. This sodium-dependent binding, characterized for the first time in this study, constitutes the majority of dextromethorphan binding to rat brain, and shares most of the pharmacological properties with the DM_2 site identified in previous studies (Zhou *et al*., 1991; Klein & Musacchio, 1992).

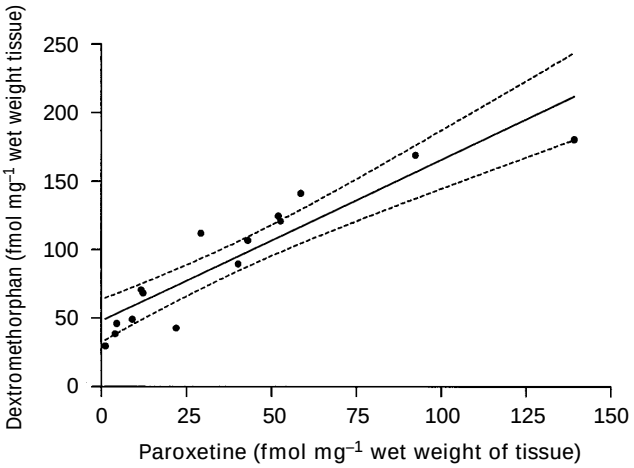

Figure 3 Correlation between dextromethorphan and paroxetine binding in rat brain. Corresponding values of [^{3}H]-dextromethorphan and [^{3}H]-paroxetine binding measured in different brain areas were plotted on the X and Y axis, respectively. Linear regression analysis gave a straight line ($r=0.992$, $P<0.0001$) whose slope was significantly different from zero. Dashed lines represents 95% confidence intervals.

In particular, the sodium-dependent binding was unaffected by a concentration of (+)-PPP equal to 4 times the K_d value for the common DM_1/σ_1 site in the rat (Klein & Musacchio, 1992), and it was only minimally affected (8 and 11% reduction respectively) by concentrations of SKF 525-A and GBR 12909 equal to three times their K_d for the DM_1/σ_1 site (Klein *et al*., 1991). Dextromethorphan binding in guinea-pig brain has been shown to be reduced by about 50% by these two cytochrome P450 inhibitors (Klein *et al*., 1991).

The present study showed for the sodium-dependent binding of dextromethorphan an average K_d of 14.7 nM; that is appreciably lower than the K_d for the DM_1/σ_1 site in rat brain (47 nM, Klein & Musacchio, 1992). This difference in K_d between the DM_1/σ_1 and the DM_2 site might also be reflected in the higher value of K_d in the cerebellum. The cerebellum was the only area in which dextromethorphan binding was not affected by sodium, and could therefore present relatively high levels of DM_1/σ_1 binding.

Phenytoin significantly decreased the sodium-dependent binding of dextromethorphan. This anticonvulsant was shown to interact with the voltage-dependent sodium channel in rat brain (Francis & McIntyre-Burnahm, 1992), and its effect on sodium-dependent binding might be either the result of a direct competition with dextromethorphan or an indirect effect following the action on the sodium channel.

The proposed identity of the sodium-dependent dextromethorphan binding with 5-HT uptake and the requirement of external sodium for this process seem to suggest a direct interaction of phenytoin with the dextromethorphan binding site or to a connected allosteric site.

Previous studies on the distribution and characteristics of dextromethorphan binding to guinea-pig and rat brain have all been conducted in sodium phosphate 50 mM (Musacchio *et al*., 1988; Klein & Musacchio, 1992), and therefore reflect the properties of both the sodium-dependent and non-dependent binding site.

A specific effect of sodium following the addition of sodium chloride to the incubation buffer is supported by the analogous effect on binding exerted by sodium phosphate (data not shown).

This effect of sodium led us to investigate the possible interaction of dextromethorphan with a sodium-dependent uptake mechanism. The particularly high levels of sodium-dependent dextromethorphan binding in the medial mammillary, ventral tegmental area and dorsal raphe prompted us to examine the relationship between dextromethorphan binding and the 5-HT uptake mechanism.

Table 5 Effect of dextromethorphan on [^{3}H]-paroxetine binding in discrete areas of rat brain

Nucleus	*Controls*	*Dextromethorphan 20 nM*	*Dextromethorphan 40 nM*[a]
Mammillary bodies	139.2±12.6	132.5±15.1	81.8±8.8
Ventral tegmental area	92.5±12.9	80.0±10.6	56.0±8.2
Dorsal raphe	58.6±8.1	65.2±8.0	43.8±6.0
Superior colliculi	52.7±5.5	64.9±5.7	35.8±4.3
Central grey area	29.3±3.3	43.7±5.7	27.4±3.6
Dorsal tegmental area	43.0±5.7	40.5±4.5	28.0±4.53
Anterior hypothalamus	40.2±7.3	28.2±4.4	18.4±4.1
Pons	21.9±4.3	17.7±3.3	8.1±2.3
Hippocampus (CA3 area)	9.1±2.4	9.0±2.6	6.2±2.6
Dentate gyrus	4.0±1.5	3.0±0.9	3.4±0.6
Frontal cortex	12.2±2.1	10.3±1.6	16.1±4.4
Occipital cortex	4.5±3.1	7.0±3.1	3.4±1.1
Mediodorsal thalamic area	11.7±3.9	8.5±2.2	4.1±1.0
Medial pretectal nuclei	52.0±8.5	46.3±6.2	39.8±4.4
Cerebellum	1.2±0.8	8.2±3.6	1.1±0.8

The tissue sections were incubated with 1 nM [^{3}H]-paroxetine, as described in Methods, in buffer containing dextromethorphan at the concentration indicated above each column. Binding capacity is expressed as fmol mg^{-1} wet weight of tissue. Each value is the mean of triplicate determinations and represents the mean±s.e.mean of 3 rats. [a]$P<0.05$ versus controls (ANOVA).

The significant colocalization between dextromethorphan binding and the selective 5-HT uptake inhibitor paroxetine, and their mutual displacement strongly suggest an interaction of dextromethorphan at this site. Competition of these two ligands at a metabolic site is very unlikely, since the two cytochrome P450 inhibitors did not affect the majority of binding, and this enzyme is implicated in the main metabolic pathway of both drugs.

Analysis of the dextromethorphan saturation curve suggests binding to one site only. This conclusion is contradicted by evidence emerging from the present study such as the residual binding in the absence of sodium, that follows a different pattern of distribution, and the heterogeneous effect of different pharmacological agents in different brain areas.

Binding to the cerebellum for example, was not potentiated by sodium ions, was reduced by (+)-PPP and paroxetine had no effect on the levels of binding. The cerebellum could therefore express particularly high levels of sodium-independent binding, that appears to have the same pharmacological profile as the DM_1/σ_1 site.

This heterogeneity in the modulation of dextromethorphan binding to rat brain revealed in this study suggests an unequal distribution of the sodium-dependent and non-dependent binding sites, as confirmed by the two different patterns of distribution for [^{3}H]-dextromethorphan obtained in the absence and presence of sodium.

The binding properties in different brain areas therefore appeared to be determined by the relative proportion of sodium-dependent binding, which was influenced by paroxetine, and sodium-independent binding which corresponded to the previously identified DM_1/σ_1 site.

Since σ ligands have never been shown to possess any antitussive activity, the sodium-dependent site identified in this study as the DM_2 site may represent a good candidate for a role in cough modulation, and its possible identity with 5-HT uptake may provide an important clue to the mechanism of action of the antitussive effect of dextromethorphan.

5-HT has been demonstrated to play a key role in the antitussive mechanism of morphine, dihydrocodeine and dextromethorphan (Kamei *et al*., 1987; 1988), and a further study showed an increase in the release of 5-HT from the nucleus of the solitary tract in the rat following dextromethorphan administration (Kamei *et al*., 1992). Since this nucleus represents an important relay in the modulation of cough, a local effect of dextromethorphan through DM_2 sites could be implicated.

Other evidence for the possible functional effects of the interaction of dextromethorphan with 5-HT uptake have appeared in the literature: Ahtee (1975) demonstrated inhibition of 5-HT uptake in human platelets by nanomolar concentrations of the antitussive agent, and Finnegan *et al*. (1991) demonstrated a protective effect of dextromethorphan on the acute depletion of 5-HT produced by the administration of *p*-chloroamphetamine in rats.

An action of dextromethorphan at 5-HT uptake sites in man is also supported by evidence of adverse reactions witnessed by clinicians following the coadministration of dextromethorphan and monoamine oxidase (MAO) inhibitors (Sovner & Wolfe, 1988; Nierenberg & Semprebon, 1993). The symptoms described in these reports correspond to the central nervous system 5-HT syndrome, which is a condition resulting from the interaction of drugs which enhance 5-hydroxytryptaminergic tone at receptors in the central nervous system. The same effect following the administration of MAO inhibitors and dextromethorphan has been observed in rabbits (Sinclair, 1976) and ascribed to the inhibition of 5-HT uptake (Sinclair & Lo, 1976).

Further studies are required to establish unequivocally the identity of the sodium-dependent dextromethorphan high affinity binding site with the 5-HT uptake mechanism, but the commonality between a subpopulation of dextromethorphan binding sites and paroxetine binding might lead the way to new possible applications for 5-HT uptake inhibitors and dextromethorphan.

References

AHTEE, L. (1975). Dextromethorphan inhibits 5-hydroxytryptamine uptake by human blood platelets and decreases 5-hydroxyindoleacetic acid content in rat brain. *J. Pharm. Pharmacol*., **27,** 107–180.

BENSON, W.M., STEFKO, P.L. & RANDALL, L.O. (1953). Comparative pharmacology of lervorphan, racemorphan and dextrorphan and related methyl ethters. *J. Pharmacol. Exp. Ther*., **109,** 189–200.

BUUS-LASSEN, J. (1978). Potent and long-lasting potentiation of two 5-hydroxytriptophan-induced effects in mice by three selective 5HT uptake inhibitors. *Eur. J. Pharmacol*., **47,** 351–358.

CANOLL, P.D., SMITH, P.R. & MUSACCHIO, J.M. (1990). Ropizine concurrently enhances and inhibits [^{3}H]-dextromethorphan binding to different structures of the guinea-pig brain: autoradiographic evidence for multiple binding sites. *Life Sci*., PL9–PL16.

CHEN, H., CLARK, D. & GOLDMAN, D. (1992). Quantitative autoradiography of [^{3}H]-paroxetine binding sites in rat brain. *J. Pharmacol. Toxicol. Meth*., **4,** 209–216.

CRAVISO, G.L. & MUSACCHIO, J.M. (1980). High affinity binding of the antitussive dextromethorphan to guinea-pig brain. *Eur. J. Pharmacol*., **65,** 451–453.

CRAVISO, G.L. & MUSACCHIO, J.M. (1983a). High affinity dextromethorphan binding sites in guinea-pig brain. I. Intitial characterization. *Mol. Pharmacol*., **23,** 619–628.

CRAVISO, G.L. & MUSACCHIO, J.M. (1983b). High affinity dextromethorphan binding sites in guinea-pig brain. II. Competition experiments. *Mol. Pharmacol*., **23,** 629–640.

EDDY, N.B., FRIEBEL, H., HAHN, K.J. & HALBACH, H. (1969). Codeine and its derivatives for pain and cough relief. 4. Potential alternatives for cough relief. *Bull. WHO*, 639–719.

FINNEGAN, K.T., KERR, J.T. & LANGSTON, J.W. (1991). Dextromethorphan protects against the neurotoxic effect of p-chloro amphetamine in rats. *Brain Res*., **558,** 109–111.

FRANCIS, J. & MCINTYRE-BURNHAM, W. (1992). [^{3}H]-phenytoin identifies a novel anticonvulsant-binding domain on voltage-dependent sodium channels. *Mol. Pharmacol*., **42,** 1097–1103.

GRAHAM, D. & LANGER, S.Z. (1988). The neuronal sodium-dependent serotonin transporter: studies with [^{3}H]-imipramine and [^{3}H]-paroxetine. In *Neuronal Serotonin*, ed. Osborne, N.N. & Hamon, H. p. 376, Chichester: John Wiley & Sons.

HABERT, E., GRAHAM, D., TAHRAOUI, L., CLAUSTRE, Y. & LANGER, S.Z. (1985). Characterization of [^{3}H]-paroxetine binding to rat cortical membranes. *Eur. J. Pharmacol*., **118,** 107–114.

KAMEI, J., OGAWA, M. & KASUYA, Y. (1987). Monoamines and the mechanisms of action of antitussive drugs in rats. *Arch. Int. Pharmacodyn. Ther*., **290,** 117–127.

KAMEI, J., OGAWA, M. & KASUYA, Y. (1988). Supersensitivity of 5,7-dihydroxytrytamine treated rats to the respiratory depressant and antitussive effects of dihydrocodeine. *Eur. J. Pharmacol*., **153,** 305–308.

KAMEI, J., MORI, T., HIGARASHI, H. & KASUYA, Y. (1992). Serotonin release in the nucleus of the solitary tract and its modulation by antitussive drugs. *Res. Comm. Pathol. Pharmacol*., **76,** 371–374.

KLEIN, M., CANOLL, P.D. & MUSACCHIO, J.M. (1991). SKF 525A and cytochrome P450 ligands inhibit with high affinity the binding of [^{3}H]-dextromethorphan and sigma ligands to guinea-pig brain. *Life Sci*., **48,** 543–550.

KLEIN, M. & MUSACCHIO, J.M. (1990). Computer-assisted analysis of dextromethorphan and (+)PPP binding sites in the rat brain. Allosteric effects of ropizine. *Life. Sci*., **47,** 1625–1634.

KLEIN, M. & MUSACCHIO, J.M. (1992). High-affinity dextromethorphan and (+)-3-(-3-hydroxyphenyl)-N-(1-propyl)piperidine binding sites in rat brain. Allosteric effect of ropizine. *J. Pharmacol. Exp. Ther*., **260,** 990–999.

MUSACCHIO, J.M., KLEIN, M. & CANOLL, P.D. (1989). Dextromethorphan and sigma ligands: common sites but diverse effects. *Life Sci*., **45,** 1721–1732.

MUSACCHIO, J.M., KLEIN, M. & SANTIAGO, L.J. (1988). High affinity dextromethorphan binding sites in guinea-pig brain: further characterization and allosterical interactions. *J. Pharmacol. Exp. Ther*., **247,** 424–431.

NEWMAN, A.H., BEVAN, K., BOWERY, N.G. & TORTELLA, F. (1992). Synthesis and evaluation of 3-substituted 17-methylmorphinan analogs as potential anticonvulsant agents. *J. Med. Chem*., **35,** 4135–4142.

NIERENBERG, D.W. & SEMPREBON, M. (1993). The central nervous serotonin syndrome. *Clin. Pharmacol. Ther*., **53,** 84–88.

NIZNIK, H.B., TYNDALE, R.F., SALLEE, F.R., GONZALEZ, F.J., HARDWICK, J.P., INABA, T. & KALOW, W. (1990). The dopamine transporter and cytochrome P450IID1 (debrisoline 4-hydroxylase) in brain: resolution and identification of two distinct [^{3}H]-GBR 12935 binding proteins. *Arch. Biochem. Biophys*., **276,** 424–432.

SCHENKMAN, J.B., BEVERLEY, B.J. & CINTI, D.L. (1972). Diethylaminoethyl 2,2-diphenylvalerateHCl (SKF 525A) in vivo and in vitro effects of metabolism by rat liver microsomes – formation of an oxygenated complex. *Biochem. Pharmacol*., **21,** 2373–2383.

SCHMID, B., BIRCHER, R., PREISIG, R. & KUPFER, A. (1985). Polymorphic dextromethorphan metabolism: co-segregation of oxidative o-demethylation with debrisoquine hydroxylation. *Clin. Pharmacol. Ther*., **38,** 618–624.

SINCLAIR, J.G. (1973). Dextromethorphan-monoamine oxidase inhibitor interaction in rabbits. *J. Pharm. Pharmacol*., **25,** 803–808.

SINCLAIR, J.B. & LO, G.F. (1976). The blockade of serotonin uptake into synaptosomes: relationship to an interaction witth monoamine oxidase inhibitors. *Can. J. Physiol. Pharmacol*., **55,** 180–187.

SOVNER, R. & WOLFE, J. (1988). Interaction between dextromethorphan and monoamine oxidase inhibitor therapy with isocarboxazid [letter]. *New Engl. J. Med*., **319,** 1671.

STEINBERG, G.K., ENG, H.L., KUNIS, D.M., GRANT, G.A., POLIJAK & DE LA PAZ R. (1991). Dextromethorphan alters cerebral blood flow and protects against cerebral injury following focal ischemia. *Neurosci Lett*., **133,** 225–228.

TORTELLA, F.C. & MUSACCHIO, J.M. (1986). Dextromethorphan and carbetapentane: centrally acting non-opioid antitussive agents witth novel anticonvulsant properties. *Brain Res*., **383,** 314–319.

ZHOU, G. & MUSACCHIO, J.M. (1991). Computer-assisted modelling of multiple dextromethorphan and sigma binding sites in guinea-pig brain. *Eur. J. Pharmacol. – Mol. Pharmacol. Sect*., **206,** 261–269.

(Received September 23, 1996
Revised December 11, 1996
Accepted January 3, 1997)