Evidence for sigma-1-like receptors in isolated rat liver mitochondrial membranes

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1 Sigma (σ) receptors have generated a great deal of interest on the basis of their possible roles in various pathologies, including cytoprotection. Although the exact function of sigma-1 (σ_1) receptors is not yet known, their role in the regulation of intracellular Ca^{2+} levels and sterol biosynthesis, functions that could be assigned to mitochondria, are the only mechanisms described.

2 Using preparations of purified rat liver and brain mitochondria we demonstrate herein the presence of σ-like binding sites. $[^3H](+)$-pentazocine, a σ_1 radioligand was used to label these sites.

3 In the liver, $[^3H](+)$-pentazocine labelled one class of binding sites with high affinity ($K_d = 3$ nM), similar to that observed in liver microsomes and synaptic membranes. These sites were located on the outer mitochondrial membranes and displayed high affinity for other σ_1 ligands namely, haloperidol, ifenprodil, carbetapentane or 1,3-di(2-tolyl)guanidine (DTG).

4 The presence of σ_1 receptors on liver mitochondria was confirmed using double fluorescence immunostaining.

5 $[^3H](+)$-pentazocine binding sites were also found on brain mitochondria but they appeared pharmacologically distinct to the liver ones as $[^3H](+)$-pentazocine and typical σ_1 ligands displayed lower affinities for these sites. Nevertheless, $[^3H](+)$-pentazocine binding on both liver and brain mitochondria was modulated by progesterone, a putative endogenous ligand for σ receptors.

6 Our data demonstrates the presence of $[^3H](+)$-pentazocine binding sites with pharmacological characteristics identical to σ_1 receptors on rat liver mitochondrial membranes. The pharmacological significance of these sites and their role on mitochondrial function remain unknown.

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Abbreviations: DHEA, dehydroepiandrosterone; DTG, 1,3-di(2-tolyl)guanidine; EDTA, ethylenediaminetetracetic acid; EGTA, ethylene glycol-bis(b-amino ethyl ether) tetraacetic acid; 3-PPP, 3-(3-hydroxyphenyl)-N-(1-propyl)piperidine; Tris, 2-amino-2-hydroxymethyl-propan-1,3-diol

Introduction

First described by Martin *et al.* (1976), sigma (σ) binding sites were postulated to be new opioid receptor subtypes. Now, it is established that these sites are distinct from any other known receptor. Based on biochemical studies, it is generally accepted that at least two different subtypes of σ receptors exist, termed sigma-1 (σ_1) and sigma-2 (σ_2). The first one shows high affinity for benzomorphans, a stereoselectivity for the (+)-isomers, and is usually labelled with the radioligand $[^3H](+)$-pentazocine (DeHaven-Hudkins *et al.*, 1992; Cagnotto *et al.*, 1994) whereas the second exhibits a lower affinity for (+)-pentazocine and a reverse stereoselectivity. Usually σ_2 binding sites are probed with 1,3-di(2-tolyl)guanidine (DTG) which binds to both subtypes 1 and 2 in roughly equal amounts.

Whatever their subtypes, σ receptors were shown to bind a variety of compounds belonging to different chemical and pharmacological classes including cytochrome P450 inhibitors, Ca^{2+} channel antagonists, antipsychotic and anti-

convulsant agents, cocaine and steroids. Among these hormones, progesterone is considered as a possible, although controversial, endogenous ligand (Su *et al.*, 1988; Schwarz *et al.*, 1989; Bergeron *et al.*, 1999). It appears to be the more potent inhibitor of $[^3H](+)$-pentazocine binding in *in vitro* studies and a close relationship between steroids and the σ_1 receptor subtype was established in different physiological and behavioural tests (Maurice *et al.*, 1999).

σ Receptors are expressed in various areas of the brain but also in peripheral organs including liver, testis, kidney, spleen and intestine. Subcellular distribution studies indicated that σ_1 receptors are highly concentrated and mainly located on microsomal and plasma membranes (McCann & Su, 1990), but some studies also suggest that they could be associated with other organelles of the cell (McCann *et al.*, 1994; Dehaven-Hudkins *et al.*, 1994).

Extensive studies have been performed to elucidate the role of these receptors. The σ_1 subtype has been the most studied and characterized. Although this receptor was recently cloned (Hanner *et al.*, 1996), no clear biological functions have yet been attributed to it. It has been suggested that it modulates

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several neurotransmitter systems (Maurice *et al.*, 1999) and that it is involved both in CNS pathologies such as schizophrenia, dementia, depression and peripheral nervous system diseases (Pascaud *et al.*, 1990; Matsuno & Mita, 1998; Bowen, 2000). σ, especially σ_1, receptor ligands were also shown to possess anti-ischaemic properties (Matsuno & Mita, 1998; Harukuni *et al.*, 2000) and be effective neuroprotective agents in different *in vitro* and *in vivo* models tested (Maurice & Lockhart, 1997).

The reported role of σ_1 binding sites in ischaemia brought our interest to this protein. We recently evidenced anti-ischaemic effects of trimetazidine on various experimental models and observed that these effects were related to a preservation of main mitochondrial functions both in brain and liver of the rat (Elimadi *et al.*, 1998; Zini *et al.*, 1996). It was also shown that [³H]-trimetazidine specifically bound to mitochondrial membranes but these binding sites have not been yet identified (Morin *et al.*, 1998; 2000). Interestingly, among the drugs able to inhibit [³H]-trimetazidine binding, σ ligands competed with high affinity (Morin *et al.*, 2000). Thus the hypothesis was raised that trimetazidine binding sites might be related to σ receptors.

In this study, we demonstrate the presence of σ_1 like binding sites on purified mitochondria from rat brain and liver, using [³H](+)-pentazocine as a ligand. This localization was confirmed by means of double fluorescence studies on rat liver sections.

Methods

Preparation of purified rat liver and brain mitochondria

Rat brain mitochondria were isolated by differential centrifugation and purified on a Percoll gradient according to Sims (1990). Male Wistar rats weighing approximately 250 to 300 g were decapitated. Brains were removed, sliced and washed in a buffer containing (mM): sucrose 320, 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) 10 and ethylenediaminetetraacetic acid (EDTA) 1, pH 7.4 at 4°C. The brains were then homogenized in 10 ml per g of tissue of the same buffer and centrifuged 3 min at $1330 \times g$ (Sorvall® RC 28 S). The supernatants (S₁) were kept on ice and the pellets were suspended in 5 ml per *g* of tissue of the same buffer and centrifuged at $1330 \times g$ for 3 min. The pellets were discarded, the supernatants S₂ pooled to S₁ and centrifuged at $21\,200 \times g$ for 10 min. The resulting supernatants (S₃) were kept on ice. The final pellets were suspended in 15% (v v⁻¹) percoll (10 ml g⁻¹ of tissue originally homogenized) and layered on a discontinuous density gradient of 3.5 ml each of 23 and 40% percoll. The tubes were centrifuged at $30\,700 \times g$ for 5 min. The purified mitochondrial rings were recovered at the interface of the 23–40% fractions using a Pasteur pipette and rinsed in the isolation buffer. Microsomal fractions were obtained by centrifuging the supernatants S₃ at $100\,000 \times g$ for 60 min. Protein contents were determined by the method of Lowry *et al.* (1951).

Purified rat liver mitochondria were obtained as previously described (Morin *et al.*, 1998). Briefly, the livers were excised rapidly and placed in a medium containing (mM): sucrose 250, Tris 50 and ethylene glycol-bis(b-amino ethyl ether) tetraacetic acid (EGTA) 5, pH 7.8 at 4°C. Tissue samples (28 g) were minced and homogenized (10 g tissue in 60 ml medium) on ice using a Teflon Potter homogenizer. The homogenates were centrifuged at $600 \times g$ for 10 min (Sorvall® RC 28 S) and the supernatants were centrifuged for 10 min at $3300 \times g$ to obtain mitochondrial pellets. The resulting pellets were washed with medium from which EGTA was omitted and centrifuged for 10 min at $3300 \times g$. The final pellets were suspended in 36 ml of Tris-sucrose buffer (without EGTA) and mitochondria were purified on a discontinuous sucrose gradient according to Morin *et al.* (1998).

The supernatant (from 10 min at $3300 \times g$) was centrifuged at $15\,000 \times g$ for 10 min and then at $170\,000 \times g$ for 1 h (Sorvall® A841 rotor) to obtain a pellet corresponding to the 'microsomal fraction'.

All animal procedures used in this study are in strict accordance with the European Community Council Directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

Preparation of synaptosomal membranes

Male Wistar rats were killed by decapitation, their brains were removed and rapidly dissected. The cerebral cortex was homogenized in 20 volumes of ice-cold sucrose buffer (Tris-HCl 50 mM, sucrose 0.32 M, pH = 7.4 at 4°C) using a Teflon Potter homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min and the pellet was discarded. The supernatant was centrifuged at $50,000 \times g$ for 20 min and the supernatant was discarded. This procedure was repeated two additional times. The final pellet was resuspended in 50 mM Tris-HCl buffer (pH = 8 at 37°C).

Binding assays

Binding of [³H](+)-pentazocine to rat brain and liver mitochondria was measured as follows: Mitochondria (1 mg ml⁻¹) were incubated with [³H](+)-pentazocine in 400 μl of 50 mM Tris-HCl buffer (pH = 8) for 90 min at 37°C. Binding was stopped by the addition of ice-cold binding buffer, and bound and free ligands were separated by rapid filtration through Whatman GF/B glass fibre filters (presoaked in 0.1% polyethylenimine). Each filter was washed twice with an additional 5 ml of ice-cold Tris-buffer (50 mM) and counted in a liquid scintillation counter Packard 1600 TR with an efficiency of 45%. Non-specific binding was defined using 1 μM haloperidol in the liver and 10 μM (±)-pentazocine in the brain. For saturation experiments the range of concentrations of [³H](+)-pentazocine was 0.2–20 nM. For inhibition experiments 2–3 nM [³H](+)-pentazocine was incubated in the absence or in the presence of increasing concentrations (15) of the competing drug.

Enzyme assays

All enzymatic activities were determined by spectrophotometric methods using an Hitachi® UV-3000 spectrophotometer and were shown to be linear in the tissue and protein concentration range used. The determination of monoamine oxidase (EC 1.4.3.4) activity was performed according to the method of Bembenek *et al.* (1990) using kynuramine as a substrate and monitoring the formation of 4-hydroxyquinoline at 316 nm. Cytochrome *c* oxidase (EC 1.9.3.1) activity

was assayed at 37°C according to the method of Rustin *et al*. (1994) by monitoring the oxidation of ferrocytochrome *c* (prepared from type III horse heart cytochrome *c* (Sigma) at 550 nm and NADPH cytochrome *c* reductase (EC 1.6.2.5) was assayed at room temperature as described by Graham (1993).

Immunocytochemistry

Cryosections from adult rat liver were fixed in 100% methanol for 10 min. Sections were incubated for 48 h at 4°C with two primary antibodies: σ_1 receptor rabbit polyclonal antibody (diluted 1:200) (Alonso *et al*., 2000) and a mouse monoclonal antibody against the mitochondrial market protein Ab-1 (clone 113-1) (from Lab Vision Corporation, www.labvision.com, Cat No.: MS-627-p1). The Ab-1 antibody was used at a 1:100 dilution. Sections were rinsed in PBS three times and incubated for 2 h with two secondary antibodies, FITC-goat anti-rabbit IgG (Zymed, 1:150) and rhodamine Red-X-conjugated Affinipure goat anti-mouse IgG (Jackson Labs, 1:150). Antibodies were diluted in PBS containing 0.1% gelatin, 0.1% Triton X-100 and 1% normal goat serum. The slides were then mounted with the Prolong Antifade Kit (Molecular Probes Inc.) and observed under Olympus Fluoview laser scanning microscope.

Drugs

[³H](+)-pentazocine was obtained from New England Nuclear (Paris, France). Other drugs and chemicals were obtained from Sigma (St Quentin Fallavier, France) or Merck (Nogent-sur-Marne, France) and were of the highest purity available.

Data analysis

Equilibrium, namely K_d (dissociation constant) and B_{max} (maximal density of binding sites), and inhibition (IC_{50}) binding parameters were calculated by means of a non-linear regression method using a commercially available software (Micropharm, INSERM 1990; Urien, 1995) by modelling the data to one or two component models according to Hill equations as described previously (Morin *et al*., 1998).

A Hill coefficient (nH) value equal one corresponds to a competitive interaction and thus indicates the presence of one class of binding sites. In this case, inhibition constants (K_i) were calculated from the IC_{50} values according to the method of Cheng & Prusoff (1973). All data are presented as the mean±s.e.mean of three or more individual experiments.

Results

Characterization of purified mitochondria

Given that σ binding sites were described to be highly enriched in liver and brain microsomes (McCann *et al*., 1994; Dehaven-Hudkins *et al*., 1994), we evaluated the possible contamination of our mitochondrial preparations by microsomal membranes. Thus, liver and brain mitochondria were characterized for purity for their content in monoamine

oxidase (an outer mitochondrial membrane marker), cytochrome *c* oxidase (an inner mitochondrial membrane marker) and the microsomal marker, NADPH cytochrome *c* reductase and compared to the respective microsomal fractions. A high activity of the two mitochondrial markers was found in the mitochondrial fractions whereas NADPH cytochrome *c* reductase activity was very low and not detectable in purified liver and brain mitochondria, respectively (Table 1).

These results indicate that our purified mitochondrial fractions were devoid of microsomes and thus were appropriate to use in search of mitochondrial σ binding sites. These preparations corresponded to those previously described using the same mitochondrial purification procedures (Sims, 1990; Morin *et al*., 1998).

Characterization of [³H](+)-pentazocine binding sites on rat liver mitochondria

To identify σ like binding sites in mitochondria we used [³H](+)-pentazocine as a ligand. [³H](+)-pentazocine is a ligand of choice to label σ_1 binding sites because it displays high affinity and good selectivity for this subtype (Quirion *et al*., 1992; Hellewell *et al*., 1994). Figure 1A shows that [³H](+)-pentazocine binds to liver mitochondria and reached equilibrium after 90 min incubation at 37°C. When intact mitochondria were used a non-saturable binding component was observed (Figure 1B). It was eliminated when mitochondria were subjected to hypo-osmotic shock and sonication (Figure 1A). This component probably corresponds to an uptake mechanism, which accumulates the ligand within the mitochondria without binding. Therefore all the following experiments were performed on broken mitochondria. Dilution of the suspension (1/100) induced a slow dissociation of the ligand from its binding sites demonstrating that the binding was reversible (Figure 1C).

Saturation binding experiments indicated that [³H](+)-pentazocine recognized a single population of binding sites (nH = 1) over the range of concentration studies with mean K_d and B_{max} values of 2.42±0.50 nM and 1.05±0.08 pmol mg⁻¹ protein, respectively. The linear Scatchard plot confirmed this analysis of the data. A representative binding curve is shown on Figure 2.

In order to confirm the nature of the sites labelled by [³H](+)-pentazocine, several σ reference ligands were examined for their potencies to inhibit its binding (Table 2, Figure

Table 1 Characterization of purified mitochondria by means of marker enzymes

| | *Enzyme activities* | | |
	Cytochrome c oxidase	Monoamine oxidase	Cytochrome c reductase
Liver mitochondria	0.99±0.28	1.68±0.23	ND
Microsomes	0.19±0.06	0.13±0.01	4.98±0.18
Brain mitochondria	0.97±0.33	0.94±0.15	ND
Microsomes	0.24±0.10	0.20±0.02	6.20±0.30

Enzymes activities are expressed in nmol min⁻¹ mg⁻¹ protein. Data shown are the mean±s.e.mean of 3–4 experiments. ND, not detectable.
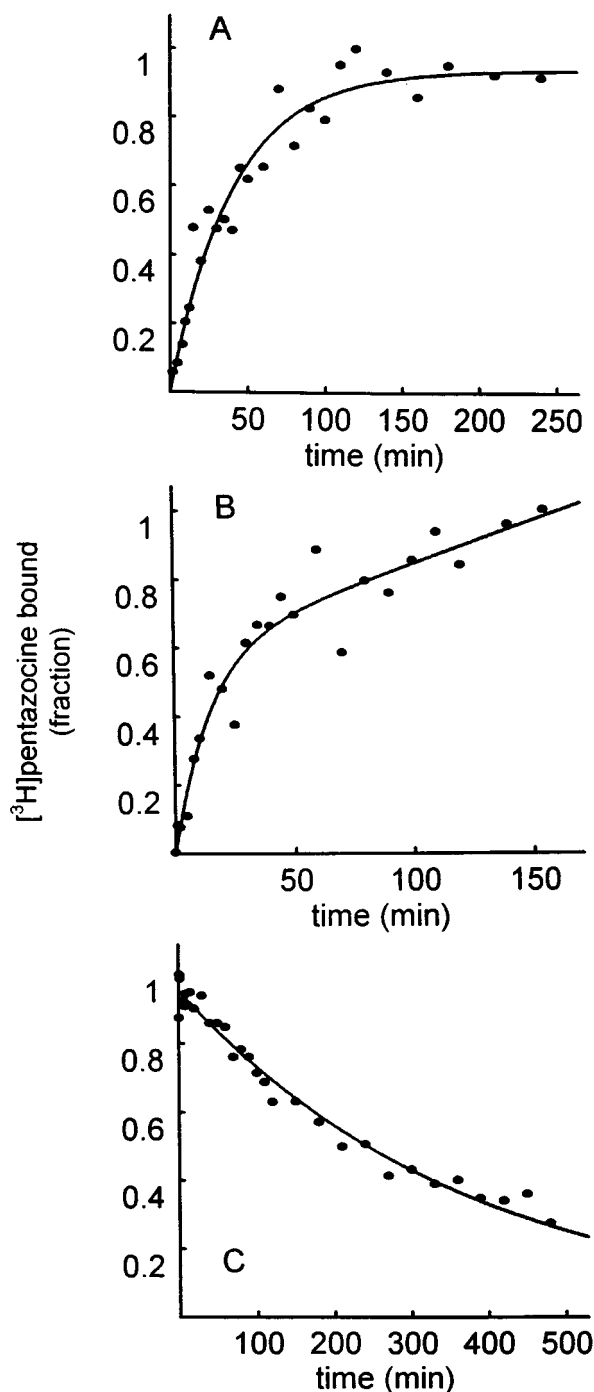
Figure 1 Association and dissociation kinetics of [³H](+)-pentazocine to rat liver mitochondria. Association experiment: Liver mitochondria (1 mg ml⁻¹) were incubated with 2 nM [³H](+)-pentazocine at 37°C. Specific binding in intact (B) and broken mitochondria (A). Dissociation experiment (C): after equilibrium was reached, dissociation was induced by dilution and specific binding monitored for 500 min (C). Data shown are plotted as fractions of the maximal binding value ([³H](+)-pentazocine bound = 1) and are from typical experiments representing the means of duplicate determinations.

3). Among the drugs tested haloperidol and (±)-pentazocine were the most potent with K_i values of 1.93 ± 0.76 and 5.27 ± 0.62 nM, respectively.
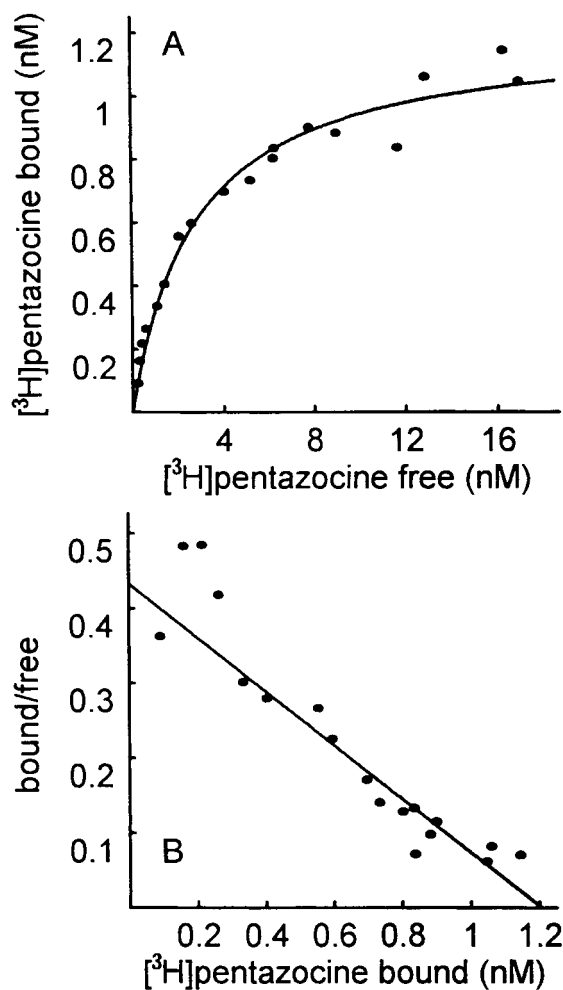

Figure 2 Representative equilibrium binding curve of [³H](+)-pentazocine binding to liver mitochondria. Concentration of [³H](+)-pentazocine (0.2–2 nM) was incubated with sonicated mitochondria (1 mg ml⁻¹) for 90 min at 37°C and non-specific binding was defined using 1 μM haloperidol. Equilibrium parameters were estimated by a non-linear regression analysis. In this particular experiment, K_d and B_{max} values were 2.78 nM and 1.20 pmol mg⁻¹ protein, respectively. (A) Direct plot. (B) Scatchard plot.

The K_i value for (±)-pentazocine was slightly higher than the K_d value determined from equilibrium experiments (2.42 ± 0.50 nM) but it is probably due to the use of the (+)-pentazocine radioligand which has higher affinity than the racemic compound (Bowen *et al.*, 1989; Quirion *et al.*, 1992). Opipramol, ifenprodil, carbetapentane, DTG and amiodarone were found to be very potent inhibitors ($K_i < 100$ nM) of [³H](+)-pentazocine binding whereas progesterone and testosterone showed moderate inhibition with K_i values around 1 μM. The other steroids hormones were less active with K_i values higher than 4 μM.

The rank order of potency at the [³H](+)-pentazocine binding sites is similar between the liver mitochondria and the well-characterized brain membranes (Cagnotto *et al.*, 1994; Dehaven-Hudkins *et al.*, 1994; McCann *et al.*, 1994; Table 2) showing a strong correlation ($r = 0.975$, $P < 0.0001$; Figure 4). A similar relation was found between liver mitochondria and liver microsomes ($r = 0.966$; $P < 0.0001$;

Figure 4). These observations provided strong evidence that [³H](+)-pentazocine binding sites located on mitochondria are similar to σ_1 receptors.

Modulation of progesterone on [³H](+)-pentazocine binding to liver mitochondria

Progesterone has been proposed as a putative endogenous ligand for σ binding sites. We investigated the interaction of progesterone with the liver mitochondrial [³H](+)-pentazocine binding sites. Inhibition experiments (Table 2) showed that progesterone competed with [³H](+)-pentazocine binding sites (nH = 1). The results displayed on Table 3 reinforced the significance of these data. Increasing concentrations of progesterone increased the K_d for [³H](+)-pentazocine binding without altering its B_{max} value in a significant manner.

Sub-mitochondrial location of [³H](+)-pentazocine binding sites

The sub-mitochondrial localization of [³H](+)-pentazocine binding sites was determined using a digitonin solubilization protocol to separate the outer from the inner mitochondrial membrane as described by Anholt *et al.* (1986). Intact liver mitochondria were incubated with a low digitonin concentration (0.6 mg digitonin mg^{-1} protein), which resulted in the extraction of monoamine oxidase (an outer membrane marker) without affecting cytochrome *c* oxidase activity (an inner membrane marker, Figure 5), confirming previous results (Anholt *et al.*, 1986; Morin *et al.*, 1998). Under these conditions, [³H](+)-pentazocine binding to mitochondria was present in the digitonin extract enriched in outer membranes only (Figure 5), suggesting that [³H](+)-pentazocine binding sites are located on the outer mitochondrial membrane.

Immunostaining for σ_1 receptors of liver mitochondria

In order to confirm the presence of σ receptors on mitochondria, liver sections were double immunostained for σ_1 receptors and a mitochondria marker (mitochondria Ab-1 primary antibody). Using conventional immunofluorescence the presence of σ_1 receptor (green) and mitochondria Ab-1 (red) in rat liver was clear (Figure 6). When confocal images of σ_1 receptor staining and mitochondria Ab-1 staining were merged, a colocalization (yellow) of their respective antigens was observed.

Study of [³H](+)-pentazocine binding on rat brain mitochondria

To investigate whether the mitochondrial location of [³H](+)-pentazocine binding sites is exclusive to liver, we examined whether [³H](+)-pentazocine binding was present in rat brain mitochondria. Indeed, [³H](+)-pentazocine binding was found in rat brain mitochondria. However, these sites differ from those observed on brain membrane fraction and on liver mitochondria. Although the number of sites was similar in
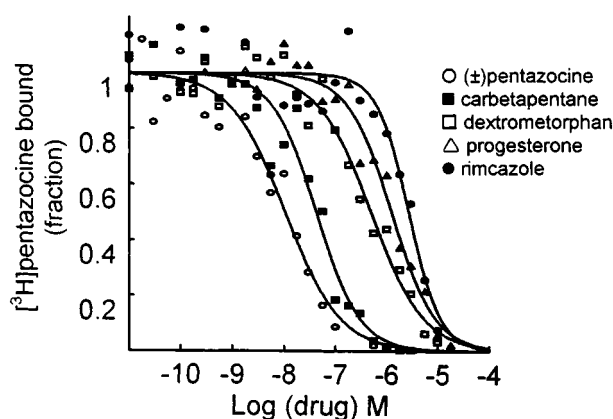

Figure 3 Effect of several σ receptor ligands on [³H](+)-pentazocine binding to rat liver mitochondria. Incubations were carried out with 2–3 nM [³H](+)-pentazocine at 37°C for 90 min. Data shown are from typical experiments and are plotted as fractions of the control binding value (in the absence of inhibitor: [³H](+)-pentazocine bound = 1).

Table 2 Inhibition of [³H](+)-pentazocine by various sigma reference ligands

		K_i (nM)		
Sigma ligand	*Liver mitochondria*	*Liver microsomes*	*Brain membrane*	*Brain mitochondria*
Haloperidol	1.93±0.76	14.8±1.44	6.19±2.96	1348±830
(±)-Pentazocine	5.27±0.62	19.0±6.35	14.3±2.28	105±16
Opipramol	9.38±3.26	37.6±2.62	34.2±7.6	>10,000
Ifenprodil	14.6±1.17	152±43.2	19.3±8.21	>10,000
Carbetapentane	23.3±1.54	73.9±14.0	47.6±4.89	>10,000
DTG	31.5±11.6	53.2±13.1	53.5±4.75	669±255
Amiodarone	49.3±7.99	124±28.3	67±38	1806±1140
(+)-3-PPP	75.6±7.55	189±17.6	368±195	7170±3200
Dextrometorphan	271±17	528±6.49	652±33	>10,000
Tamoxifen	495±75	2031±785	1108±262	1911±1779
Progesterone	751±114	1039±42	1339±353	1033±1078
Testosterone	1172±412	1020±196	910±97	2072±911
Rimcazole	1540±10	1221±61	867±185	>10,000
DHEA	4364±400	7286±1807	5081±1172	>10,000

Data shown are the mean±s.e.mean of 3–4 experiments. [³H](+)-pentazocine concentration was 2–3 nM. IC$_{50}$ values were estimated by a non-linear regression analysis by a competitive model since all Hill coefficient (nH) values were equal to 1. All drugs displaced 100% of [³H](+)-pentazocine specific binding. DHEA, dehydroepiandrosterone; DTG, 1,3-dil(2-tolyl)guanidine; 3-PPP, 3-(3-hydroxyphenyl)-N-(1-propyl)piperidine.
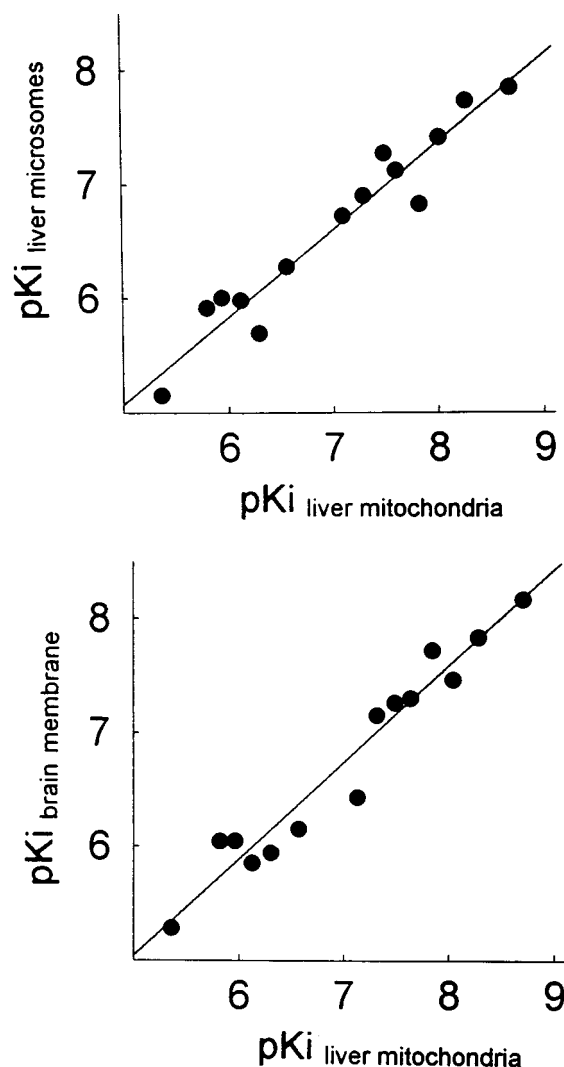
Figure 4 Relationship between the affinity of drugs for [³H](+)-pentazocine binding sites from liver mitochondria, liver microsomes and brain membranes. Plotted are pK_i values listed in Table 2. Regression line was obtained by least squares fit analysis of the data points.

Table 3 Effect of progesterone on [³H](+)-pentazocine to liver mitochondria

	[³H](+)-pentazocine binding	
	K_d (nM)	B_{max} (pmol mg^{-1} protein)
Control	2.57 ± 0.23	1.12 ± 0.17
+0.5 μM progesterone	5.90 ± 0.70	1.51 ± 0.20
+1.5 μM progesterone	8.79 ± 1.01	0.85 ± 0.10
+2 μM progesterone	28.5 ± 8.0	2.45 ± 0.74

[³H](+)-pentazocine binding was measured in the absence (control) or in the presence of increasing concentrations of progesterone. Binding parameters were determined by non-linear regression analysis. Data shown are the mean $\pm$ s.e.mean of three experiments done in duplicate.

brain ($B_{max} = 1.26 \pm 0.09$ pmol mg^{-1} protein) and liver ($B_{max} = 1.05 \pm 0.08$ pmol mg^{-1} protein), [³H](+)-pentazocine had 15-fold lower affinity in brain ($K_d = 31.6 \pm 3.9$ nM)
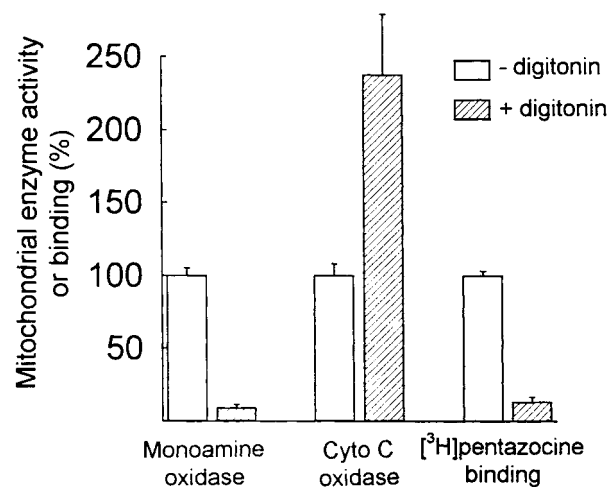
Figure 5 [³H](+)-pentazocine binding sites are located on the outer mitochondrial membranes. Freshly isolated mitochondria (8 mg ml^{-1}) were treated or not with 0.6 mg digitonin mg^{-1} protein in 1 ml Tris-sucrose buffer for 15 min at 4°C. After incubation the mitochondrial suspension was diluted and centrifuged at $15,000 \times g$ for 7 min. Cytochrome *c* oxidase activity, monoamine oxidase activity and [³H](+)-pentazocine (2 nM) binding were then assayed in the pellet. The maximal activities of the markers (100% values) were 1.57 nmol min^{-1} mg^{-1} protein for monoamine oxidase, 0.87 nmol min^{-1} mg^{-1} protein for cytochrome *c* oxidase, and 0.41 pmol mg^{-1} protein for [³H](+)-pentazocine binding. Each data point is the mean $\pm$ s.e.mean of duplicate measurements obtained from 3–4 experiments.

compared to the liver. Inhibition experiments confirmed this difference as all the σ compounds tested were less potent when tested on brain than on liver mitochondria (Table 2). In addition, the selective antagonist rimcazole was without effect (Table 2). The only notable exceptions were steroid hormones, which exhibit the same affinities in both liver and brain mitochondria preparations.

Discussion

During the past few years σ_1 recognition sites have been extensively studied. These studies were almost exclusively carried out on microsomal and plasma membranes because σ_1 recognition sites are more abundant in these cellular fractions whatever the organ studied. However, several observations have suggested that these sites could be also present on other organelles (Cagnotto *et al.*, 1994; Dehaven-Hudkins *et al.*, 1994; Alonso *et al.*, 2000) but no clear demonstration and pharmacological characterization using purified preparations were performed. In the present study, we demonstrate the presence of [³H](+)-pentazocine binding sites on purified preparation of mitochondria and provide the pharmacological profile of these mitochondrial binding sites, which proves their similarity with σ_1 receptors.

In liver mitochondria, [³H](+)-pentazocine labels one class of high affinity ($K_d = 3$ nM) binding sites, similar to that observed in synaptic membranes (Dehaven-Hudkins *et al.*, 1994; Nguyen *et al.*, 1996). The mitochondrial sites also display high affinity for other σ_1 ligands such as haloperidol, opipramol, ifenprodil or carbetapentane. The binding inhibition profile obtained with these reference compounds is
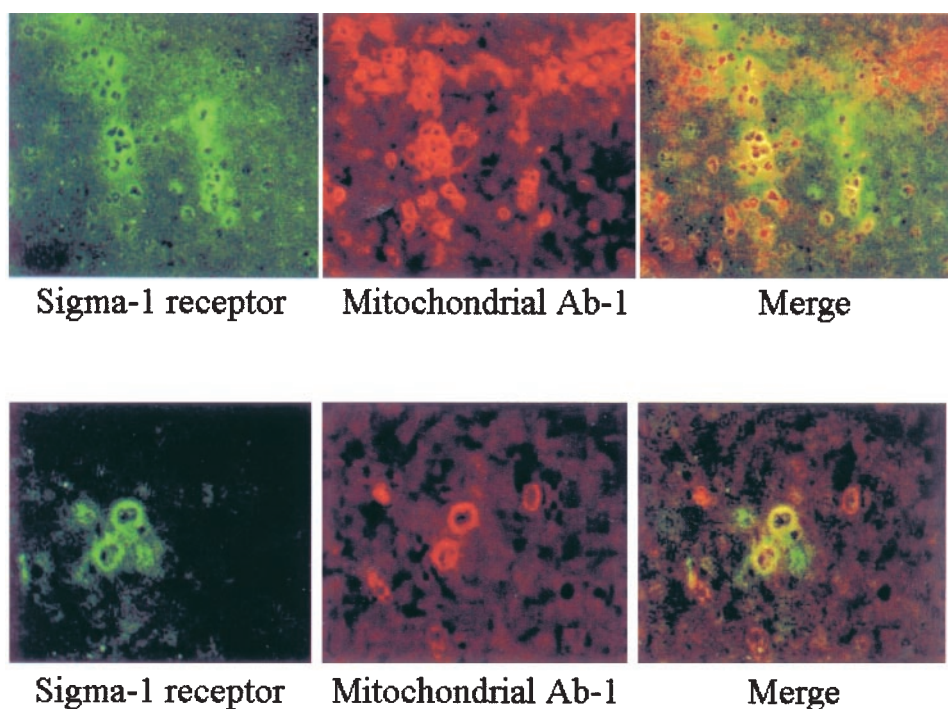
Figure 6   Colocalization of σ_1 receptor and mitochondrial Ab-1 marker in rat liver. As described under Methods rat liver sections were incubated with σ_1 receptor and mitochondria Ab-1 primary antibodies. Using conventional immunofluorescence the presence of σ_1 receptor (green) and mitochondria Ab-1 (red) in rat liver is clear. Confocal images of σ_1 receptor staining and mitochondria Ab-1 staining were merged, showing a colocalization (yellow) of their respective antigens. Magnification: $\times 20$ (top) and $\times 40$ (bottom).

similar to those reported previously for synaptic membranes (McCann *et al*., 1994; Dehaven-Hudkins *et al*., 1994) and a close relationship exists between the K_i values obtained from liver mitochondria and either liver microsomes or synaptic membranes. On the other hand, [³H](+)-pentazocine binding is modulated by progesterone, a putative endogenous ligand for σ_1 receptors, in agreement with previous findings on other biological materials (Su *et al*., 1988; Schwarz *et al*., 1989; Bergeron *et al*., 1999). Taken together, these results indicate that rat liver mitochondrial [³H](+)-pentazocine binding sites present pharmacological characteristics identical to the σ_1 receptors. Interestingly, this is not the case for [³H](+)-pentazocine binding sites found on brain mitochondria. Indeed, [³H](+)-pentazocine and typical σ_1 ligands display lower affinities for these sites. For example, haloperidol is 700-fold less potent on brain than on liver mitochondria and opipramol, ifenprodil and carbetapentane were ineffective on the former. This indicates that mitochondrial brain sites differ from the liver ones and from the typical peripheral σ_1 receptors. In addition, they do not appear to be related to σ_2 receptors because the σ_2 binding ligand DTG showed a moderate affinity for these sites ($K_i = 0.6$ μM). Although these sites are distinct from σ_1, they exhibit common binding recognition properties for steroid hormone ligands and the antisteroid agent tamoxifen, which have similar affinities for liver and brain mitochondrial [³H](+)-pentazocine binding sites. This observation indicates that these sites could be related entities.

In support of our binding data, double fluorescence studies, using both an anti-σ_1 antibody (Alonso *et al*., 2000) and a mitochondrial marker, clearly show the presence of immunoreactive σ_1 protein(s) on liver mitochondrial membranes.

The pharmacological significance of these binding sites is unknown. The same comment can be made for the σ receptors. Indeed, although numerous pharmacological effects have been associated with σ_1 receptors (reviewed by Matsuno & Mita, 1998; Maurice *et al*., 1999; Bowen, 2000), the cellular transduction mechanism and the functional role of these receptors remain unclear. Among the effects described, σ ligands exert cytoprotective and more particularly neuroprotective properties (Maurice & Lockhart, 1997). This function of the ligands relates to their ability to directly inhibit the excitatory neurotransmitter system (Maurice & Lockhart, 1997). More recently, several lines of evidence indicated that σ receptors could mediate some of their effects by altering intracellular effects. For example, they were shown to modulate intracellular calcium and more particularly facilitate the mobilization of intracellular calcium pools (Hayashi *et al*., 2000). This increase, due to the release of calcium from endoplasmic reticulum stores, has been suggested to contribute to the cytoprotective effect, reducing indirectly presynaptic glutamate release (Maurice & Lockhart, 1997). These data indicate that σ ligands might use calcium signal as cellular transduction mechanism and may participate to the control of cellular calcium homeostasis. Since mitochondria are known to store calcium (Gunter *et al*., 1994) and are supposed to play a role in the transduction of the calcium signal (Ichas *et al*., 1997) we can speculate that mitochondrial [³H](+)-pentazocine binding sites might be a pharmacological site regulating calcium stores.

A possible relationship between σ receptors and sterol metabolism has also been evoked. Indeed, the σ_1 receptor has been cloned in several species (Hanner *et al.*, 1996; Kekuda *et al.*, 1996; Seth *et al.*, 1997) and shows a 30% identity and a 60% homology to a yeast sterol C8-C7 isomerase suggesting a role for this drug-binding protein in sterol synthesis (Moebius *et al.*, 1997). In addition, certain neurosteroids have been shown to exhibit modulatory effects *via* σ receptors (Monnet *et al.*, 1995; Bergeron *et al.*, 1996). Interestingly, another drug-receptor, the peripheral-type benzodiazepine receptor, is precisely localized on the outer mitochondrial membrane where it is involved in the transport of cholesterol into mitochondria, the first step in steroid biosynthesis (Papadopoulos *et al.*, 1997; Li *et al.*, 2001). Although the role of peripheral-type benzodiazepine receptors in cholesterol transport into mitochondria of steroidogenic cells has been shown, it is important to note that a more general role for this ubiquitous receptor protein in intracellular cholesterol compartmentalization has been proposed (Li & Papadopoulos, 1998). The recent finding that this receptor is a high-affinity cholesterol binding protein (Li *et al.*, 2001; Lacapere *et al.*, 2001) further supports its function in cholesterol compartmentalization.

Given that our data demonstrate that [³H](+)-pentazocine binding sites are also located on the outer membrane of mitochondria and are modulated by several steroid hor-mones, especially progesterone which is considered to be a putative endogenous ligand (Su *et al.*, 1988; Schwarz *et al.*, 1989; Bergeron *et al.*, 1999), a possible modulation of steroid biosynthesis and/or cholesterol movement across mitochon-drial membrane, initial step in membrane biogenesis and steroid biosynthesis, by σ ligands cannot be excluded. This might also contribute to the cytoprotective effect of these drugs. Although the role of σ_1 receptors has not yet been defined, their involvement in this process seems plausible. The effect of steroids and Ca^{2+} on σ_1 receptors together with their presence on the mitochondrial membranes, presented herein, support this hypothesis. Studies to test these hypotheses are in progress in our laboratory. In conclusion, we report herein the presence and the pharmacological characteristics of mitochondrial σ_1 binding sites. The assignment of these drug-binding sites to mitochondria, considering the vital role of these organelles in respiration, apoptosis, sterol metabo-lism etc, opens a new area for the application of σ_1 drug ligands in search of a role of this drug-binding protein.

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