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Repeated haloperidol treatment decreases σ_1 receptor binding but does not affect its mRNA levels in the guinea pig or rat brain

Atsuko Inoue *, Sayori Sugita, Hideki Shoji, Hisako Ichimoto, Izumi Hide, Yoshihiro Nakata

Department of Pharmacology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

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Abstract

The effects of chronic treatment with haloperidol on sigma (σ) receptors were investigated across brain regions and species. The regional distribution of [^{3}H](+)-pentazocine binding to σ_1 receptor was similar between the guinea pig and rat brains. The highest level of binding was detected in the brain stem and lowest in the striatum and hippocampus. The regional distribution of [^{3}H]1,3-di (2-tolyl) guanidine ([^{3}H]DTG) binding in the presence of 100 nM (+)-pentazocine to σ_2 receptor was similar to that of the [^{3}H](+)-pentazocine binding in the guinea pig brain, while in the rat brain high levels of [^{3}H]DTG binding were detected in the cortex, frontal cortex and cerebellum. The intraperitoneal administration of 2 mg/kg of haloperidol to guinea pig and rats once a day for 21 days produced inhibition of [^{3}H](+)-pentazocine binding but did not affect [^{3}H]DTG binding to σ_2 receptors in any brain region examined. The effects of haloperidol on [^{3}H](+)-penazocine binding in the rat were much weaker than those in the guinea pig. The regional distribution of the level of σ_1 receptor mRNA determined by the ribonuclease protection assay was similar to that of the [^{3}H](+)-pentazocine binding activity, except in the cortex and cerebellum where the levels of σ_1 receptor mRNA were low in guinea pig and rat. Treatment with haloperidol did not affect the levels of σ_1 receptor mRNA in any brain region in either species. These findings suggested that the σ receptors differentially distributed in brain regions are down regulated by treatment with haloperidol across σ receptor subtypes and animal species without changing the transcriptional activity of the σ_1 receptor. The mechanisms by which σ receptors could be differently regulated in vivo by chronic treatment with haloperidol in different species may contribute to the therapeutic efficacy of haloperidol.

Keywords: σ Receptor; Haloperidol; σ_1 Receptor mRNA; [^{3}H]DTG ([^{3}H]1,3-di (2-tolyl) guanidine); [^{3}H](+)-Pentazocine; Species difference

1. Introduction

Sigma (σ) receptors are defined as non-opiate and non-phencyclidine binding sites that mediate the psychomimetic actions of certain opioid derivatives (Martin et al., 1976; Su, 1982; Tam and Cook, 1984). These receptors are widely distributed in the brain as well as in several peripheral tissues of endocrine and immune systems (Su et al., 1988; Itzhak and Stein, 1990; Walker et al., 1990; Ferris et al., 1991). σ receptors can be divided into at least two subtypes, σ_1 and σ_2 receptors (Hellewell and Bowen, 1990; Walker et al., 1990; Quirion et al., 1992). Based on this classification, (+)-pentazocine and (+)-*N*-allylnormetazocine ((+)-SKF10047) are classified as specific σ_1 receptor ligands, and 1, 3-di(2-tolyl)guanidine (DTG), a non-benzomorphan-type σ receptor ligand, has equal high affinities for σ_1 and σ_2 receptors. Thus, each receptor can be identified solely by ligand binding experiments using [^{3}H](+)-pentazocine or [^{3}H]DTG with (+)-pentazocine to mask labeling of σ_1 receptors. The functional significance of these receptors remains to be clarified. σ receptors have been suggested to be involved in the pathogenesis of psychiatric disorders. A wide variety of centrally acting drugs exhibit high to moderate affinities for σ receptors. Anti-psychotic drugs such as haloperidol and rimcazole are high affinity ligands for the σ receptors (Walker et al., 1990; Ferris et al., 1991; Itzhak and Stein, 1991). σ ligands interact with the dopamine neurotransmitter system (Walker et al., 1990; Zhang et al., 1993; Debonnel and de Montigny, 1996). Significant changes have been observed in σ binding activity in discrete brain areas of schizophrenic patients (Weissman et al., 1991;

* Corresponding author. Tel.: +81-82-257-5310; fax: +81-82-257-5314.

E-mail address: ainoue@hiroshima-u.ac.jp (A. Inoue).

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Shibuya et al., 1992; Helmeste et al., 1996). More recently, a significant association was observed between polymorphisms in the σ_1 receptor gene and schizophrenia (Ishiguro et al., 1998).

Prolonged treatment with haloperidol induces severe extrapyramidal side effects in patients undergoing anti-psychotic therapy. Our previous studies showed that the chronic haloperidol treatment increases dopamine D_2 receptor binding activities as well as its mRNA levels in the rat striatum (Inoue et al., 1997). Itzhak and Alerhand (1989) and Itzhak and Stein (1991) reported that chronic haloperidol treatment induces the opposite effects on dopamine and σ receptors; that is, up-regulation of dopamine D_2 receptor and down-regulation of σ receptor. These findings suggested that σ receptors may play an important role in the psychomotor behavior and the extrapyramidal side effects of neuroleptics. Indeed, σ ligands produce dyskinesia in several animal models. Haloperidol, (+)-SKF10,047 and DTG produce marked dystonia in rats after microinjection into the red nucleus (Walker et al., 1988).

Due to the physiological and pharmacological relevance of σ receptors, it is important to understand the molecular nature of these receptors. Hanner et al. (1996) cloned a gene encoding the σ_1 receptor from guinea pig liver consisting of 223 amino acids that possesses a single putative transmembrane domain. Subsequently, the human (Kekuda et al., 1996; Presad et al., 1998), mouse (Seth et al., 1997; Pan et al., 1998) and rat σ_1 receptors (Seth et al., 1998) have been cloned.

We observed that haloperidol treatment induces down-regulation of σ_1 receptor binding activities but not σ_2 receptor binding activities, and this effect was greater in the guinea pig brain than in rat brain. To understand the molecular nature of these phenomena, we studied the levels of σ_1 receptor mRNA in various areas of the guinea pig and rat brain following treatment with haloperidol.

2. Materials and methods

2.1. Materials

(+)-Pentazocine succinate, DTG, rimcazole and (+)-SKF10047 were synthesized by Otsuka Pharmaceutical (Tokushima, Japan). Drugs were obtained from the following sources: haloperidol (Sigma, St. Louis, MO, USA), [^{3}H](+)-pentazocine (1161.8 GBq/mmol), [^{3}H]DTG (1295.0 GBq/mmol) and [α-^{32}P]UTP (~ 111 TBq/mmol) (DuPont-NEN, Wilmington, DE, USA), T7 polymerase (Promega, Madison, WI, USA) and restriction enzymes (New England Biolabs, Beverly, MA, USA). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan), Katayama Chemical (Osaka, Japan), Kanto Chemical (Tokyo, Japan) and the companies listed above.

2.2. Animals and drug treatment

Male Hartley guinea pigs (200–250 g) and male Wistar rats (160–250 g) were housed under a 12-h light–dark cycle with free access to food and water at 25°C. All procedures were approved by the Animal Care and Use Committee at Hiroshima University School of Medicine. Animals were given intraperitoneal injection of haloperidol (2 mg/kg) or vehicle once a day for 21 days. Haloperidol was dissolved in saline solution containing 6% dimethyl sulfoxide (DMSO) and 0.0075% HCl. For the binding experiments, animals were decapitated 3 days after the last injection and the brains were removed. The brains were dissected into eight regions and stored at −80°C until use for σ receptor binding experiments. For mRNA determination, the brains of the animals were removed 24 h after the last injection, dissected into eight regions, and total RNA was isolated immediately.

2.3. σ Receptor binding assay

Tissue was homogenized in 10 vol. of ice-cold 50 mM Tris–HCl buffer (pH 7.4) with a Brinkmann Polytron (setting at eight for 15 s) and then centrifuged at 49,000 × *g* for 10 min. The pellet was washed twice with 50 mM Tris–HCl buffer (pH 7.4) and resuspended in 50 vol. of 50 mM Tris–HCl buffer (pH 8.0) to a final protein concentration of 0.6–1 mg/ml. σ_1 receptor was labeled with the σ_1 selective ligand [^{3}H](+)-pentazocine and σ_2 receptor was labeled with the non-selective ligand [^{3}H]DTG in the presence of unlabeled (+)-pentazocine (100 nM). The brain membrane fraction was incubated with each radioligand for 180 min at 37°C, then the reaction mixture was rapidly filtered under vacuum through GF/B glass-fiber filters (Whatman Int., Maidstone, UK) presoaked in 0.3% polyethyleneimine for 30 min prior to use. Filters were washed three times with 3 ml of ice-cold 50 mM Tris–HCl buffer (pH 8.0) and the radioactivities on the filter were measured. In both binding experiments for σ_1 and σ_2 receptors, non-specific binding was determined in the presence of haloperidol (10 μM). The protein concentration was determined according to the method of Lowry et al. (1951).

2.4. Determination of σ_1 receptor mRNA in the guinea pig and rat brain

2.4.1. Preparation of radiolabeled antisense cRNA probe

Total RNA was isolated from each tissue according to the method of Chomczynski and Sacchi (1987). The template cDNAs encoding guinea pig σ_1 receptor and rat σ_1 receptor were prepared by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA isolated from the guinea pig kidney and rat brain, respectively. The primers corresponding to sequences bounding a segment from base 361 to 1052 of guinea pig σ_1 receptor cDNA

(Hanner et al., 1996) defined a fragment of 692 bases, which contained the full coding region and were 5′CCGCTTCACTCGAAGTGATGCAGT3′ (sense) and 5′TGGTCAAGGGTCTTGGCCGAAGAG3′ (antisense). The PCR product was first ligated into the pGEM-T easy vector (Promega), and digested with EcoRI. The fragment of 711 bases was then ligated into pBluescript SK(+) (Strategene, La Jolla, CA, USA) (pg σ_1). The primers corresponding to sequences bounding a segment extending from base 1 to 712 of rat σ_1 receptor cDNA (Seth et al., 1998) defined a fragment of 712 bases, which contained the full coding region and were 5′GACGTTGGTGGTACCAGGCTG3′ (sense) and 5′GCCTGTCTGGTCAGGGGTCTT 3′ (antisense). The PCR product was ligated into the pGEM-T easy vector, and digested with EcoRI and PstI. The fragment of 458 bases was ligated into pBluescript SK(+) (pr σ_1).

The labeled antisense RNA probe was synthesized in vitro using the template cDNA pg σ_1 linearized by digestion with BglII or pr σ_1 with BamHI. After incubation with 10 mM dithiothreitol, 20 units of RNase inhibitor, 1 μg of each linearized DNA, 1.85 MBq [α-^{32}P]UTP with 12 μM UTP, 0.5 mM ATP, GTP and CTP, and 20 units of T7 RNA polymerase at 37°C for 60 min in transcription buffer (Promega), 10 units RNase-free DNase was added to the reaction mixture, and further incubation was carried out for 15 min at 37°C. Labeled RNA was precipitated after extraction with phenol/chloroform/isoamylalcohol (25:24:1). The ^{32}P-UTP-labeled probes were 374 bases in length for the guinea pig and 526 bases in length for the rat (Fig. 1).

2.4.2. Ribonuclease protection assay

Approximately, 300,000 and 10,000 cpm of the radiolabeled antisense RNA probes for guinea pig σ_1 receptor and β-actin, respectively, were added to 40 μg of total RNA in hybridization buffer composed of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 mM NaCl and 80% formamide in a total volume of 30 μl. For analysis of rat σ_1 receptor mRNA, 200,000 and 3000 cpm of the radiolabeled antisense RNA probe for rat σ_1 receptor and β-actin, respectively, were added to 40 μg of total RNA. Hybridization was performed at 45°C for 12 h. After incubation, 40 μg/ml of RNase A in 350 μl of RNase digestion buffer, composed of 10 mM Tris–HCl (pH 7.4), 5 mM EDTA and 300 mM NaCl, was added to the mixture. Digestion was carried out for 60 min at 30°C. Twenty microliters of 10% sodium lauryl sulfate (SDS) and 5 μl of 10 mg/ml of proteinase K were added, and further incubation was carried out for 15 min at 37°C. After extraction with phenol/chloroform/isoamyl alcohol (25:24:1), hybridized RNA was precipitated with ethanol and analyzed by electrophoresis on a 4% polyacrylamide (PAGE)/7 M urea gel. After electrophoresis, the gels were dried and exposed to an Imaging Plate to analyze the intensity of each band using a BAS2000 Bioimaging analyzer (Fuji Film, Tokyo, Japan).

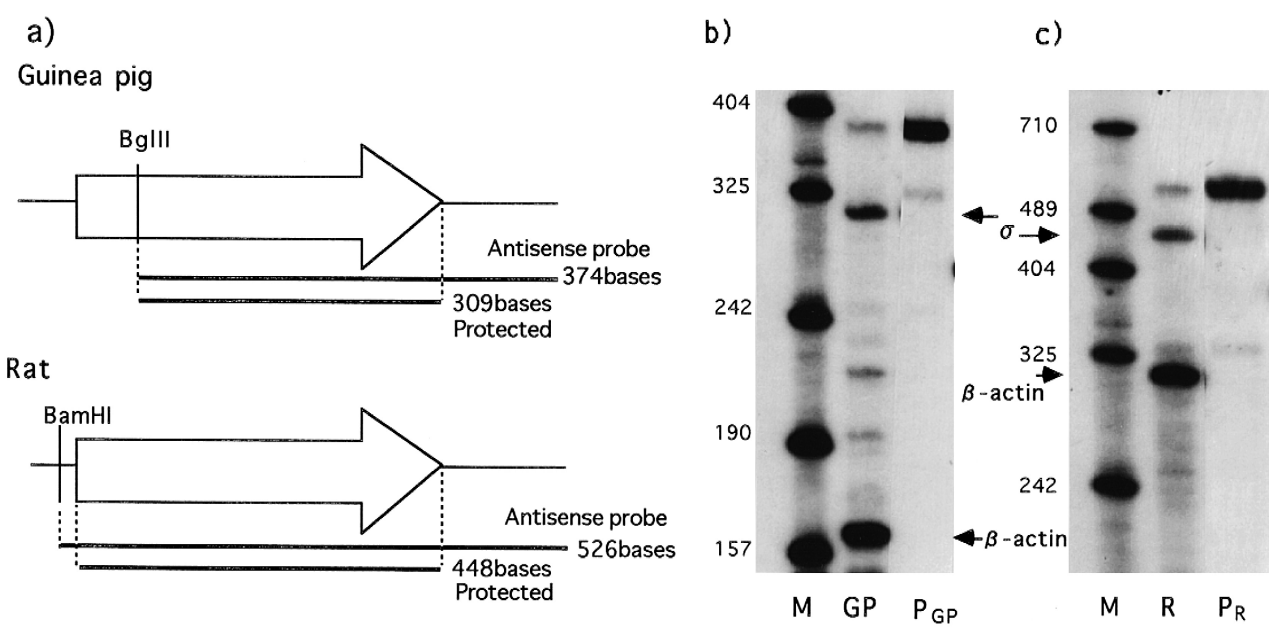

Fig. 1. Ribonuclease protection assay for σ_1 receptor mRNA in guinea pig and rat brain. (a) Illustration of guinea pig and rat σ_1 receptor cDNA and the antisense RNA probes used in this study. The white arrow indicates the partial sequence of σ_1 receptor cDNA of guinea pig or rat amplified by RT-PCR as described in Materials and methods. The lines on both sides of the white arrows represent vector sequences. BglII and BamHI represent the only sites for these restriction enzymes in the cDNA sequence. (b, c) Typical autoradiographs of guinea pig (b) and rat (c) σ_1 receptor mRNA analyzed by ribonuclease protection assay. RNA (40 μg) from the frontal cortex was hybridized with the antisense cRNA probes, digested with RNase A and subjected to 4% PAGE/7 M urea. Lanes show the protected bands for guinea pig (GP) and rat (R) σ_1 receptor and β-actin mRNA, the labeled probes for σ_1 receptor of guinea pig (PGP) and rat (PR), and size marker (M).

2.5. Data analysis

Data are expressed as means ± S.E.M. The ratios of σ_1 binding to total σ binding were compared across brain regions using analysis of variance. Effects of drug treatments in binding studies and in mRNA measurements were evaluated by Student's *t*-test compared to the corresponding control value. Differences were considered to be significant when the P value was less than 0.05.

3. Results

3.1. Regional distribution of σ_1 and σ_2 binding sites in the guinea pig and rat brain

Figs. 2 and 3 show the regional distributions of σ_1 and σ_2 binding sites labeled by [^{3}H](+)-pentazocine and [^{3}H]DTG in the presence of 0.1 μM (+)-pentazocine, respectively, in the guinea pig (Fig. 2) and rat brain (Fig. 3). In both species, σ_1 binding sites were abundant in the brain stem such as the pons and medulla and in the mesencephalon, but low in the striatum and hippocampus. The distribution of σ_2 binding sites in the guinea pig brain was similar to that of σ_1 binding sites, and they were also abundant in the brain stem. In the rat brain, however, the distribution of σ_2 binding sites was not similar to that of σ_1 binding sites, and they were abundant in the cortex, frontal cortex and cerebellum.

The proportions of σ_1 binding sites to total σ binding sites estimated by [^{3}H]DTG binding in the presence and absence of 100 nM (+)-pentazocine are shown in Table 1. Significant variation of the ratio of σ_1 binding was revealed across the rat brain regions. σ_1 binding sites in guinea pig brain regions were predominant over σ_2 binding sites, while in rat brain regions σ_2 binding sites were predominant.

3.2. Effects of haloperidol treatment on σ_1 and σ_2 binding sites

Our previous study indicated that rats exposed to chronic treatment with haloperidol, should be decapitated and analyzed 3 days after the last administration otherwise the residual haloperidol in the brain tissue influenced the results of binding assays (Inoue et al., 1997). In the present study, we followed this previous protocol. Fig. 2a shows the [^{3}H](+)-pentazocine binding to the σ_1 binding site in various brain regions from guinea pigs treated with 2 mg/kg haloperidol intraperitonially once a day for 21 days. Chronic haloperidol treatment induced a marked decrease in [^{3}H] (+)-pentazocine binding in all brain regions examined in this study. Fig. 2b shows [^{3}H]DTG binding in the presence of 100 nM (+)-pentazoicne to the σ_2 binding sites in the same brain regions as examined in [^{3}H](+)-pentazocine binding assay. Chronic treatment with haloperidol did not affect σ_2 binding sites in the guinea pig brain. Fig. 3a shows that chronic haloperidol treatment (2 mg/kg i.p. once a day for 21 days) also decreased [^{3}H] (+)-pentazocine binding in the rat brain regions. The degree of decrease in each rat brain region was significant but smaller than those in the guinea pig brain. The treat-

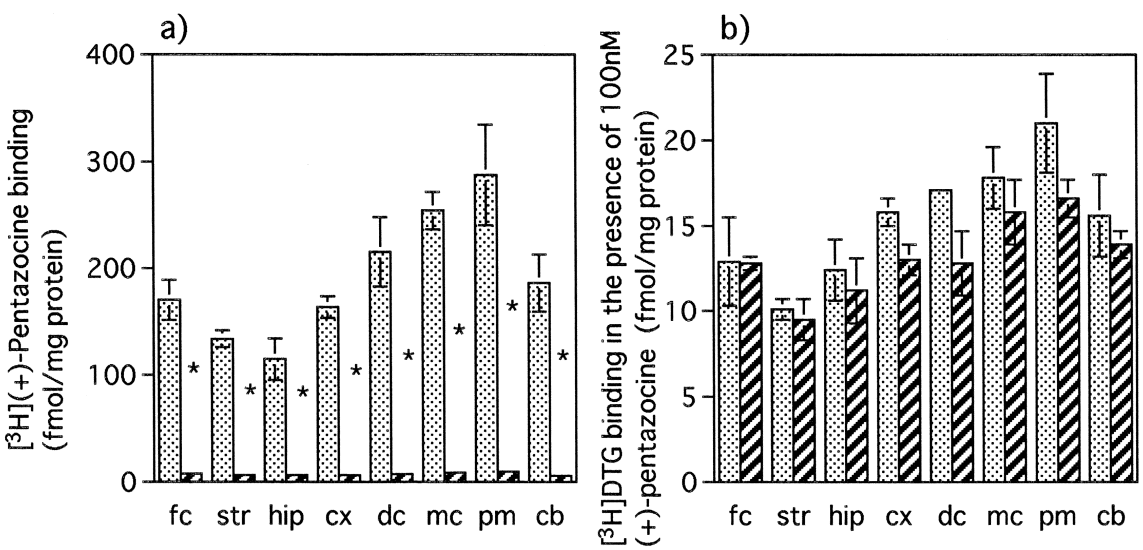

Fig. 2. Distributions of σ_1 and σ_2 binding sites in the guinea pig brain and effects of chronic treatment with haloperidol. (a) σ_1 binding sites are expressed as [^{3}H](+)-pentazocine binding in the brain regions of guinea pigs treated with vehicle (square with vertically dotted lines) or 2 mg/kg (i.p. 21 days) haloperidol (▨). Data are means ± S.E.M. (bars) from three to seven separate experiments. $^*P < 0.05$, significantly different from the value in the vehicle-treated animals. (b) σ_2 binding sites are expressed as [^{3}H]DTG binding in the presence of 100 nM (+)-pentazocine in the brain regions of guinea pigs treated with vehicle (square with vertically dotted lines) or 2 mg/kg (i.p. 21 days) haloperidol (▨). Data are means ± S.E.M. (bars) from three to seven separate experiments. The brain was dissected into eight regions as follows: fc, frontal cortex; str, striatum; hip, hippocampus; cx, cortex; dc, diencephalon; mc, mesencephalon; pm, pons and medulla; cb, cerebellum.

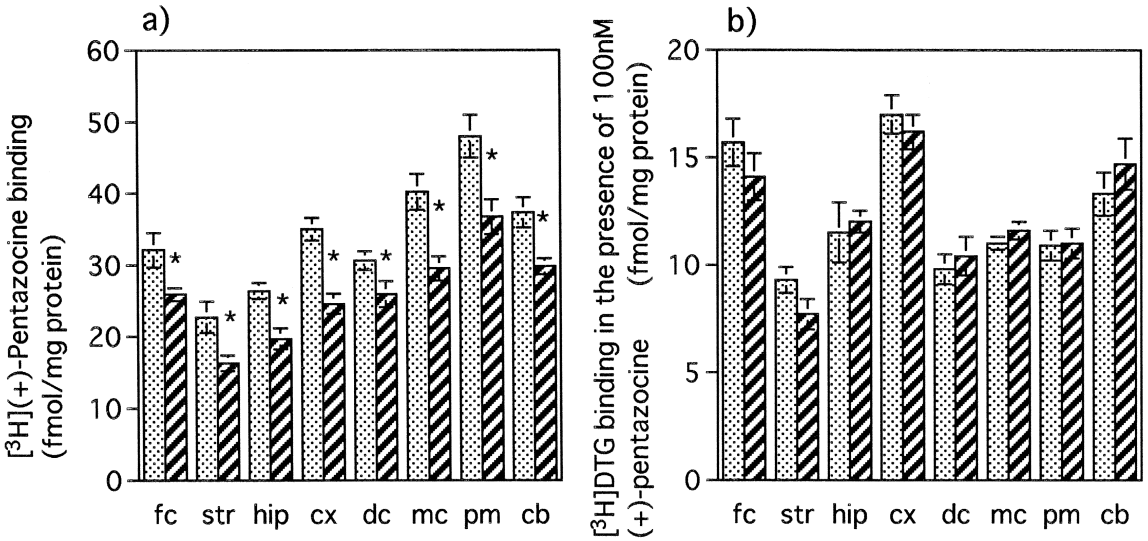

Fig. 3. Distributions of σ_1 and σ_2 binding sites in the rat brain and the effects of chronic treatment with haloperidol. (a) σ_1 binding sites are expressed as [^{3}H](+)-pentazocine binding in the brain regions of rats treated with vehicle (square with vertically dotted lines) or 2 mg/kg (i.p. 21 days) haloperidol (▨). Data are means ± S.E.M. (bars) from three to seven separate experiments. $^*P < 0.05$, significantly different from the values in vehicle-treated animals. (b) σ_2 binding sites are expressed as [^{3}H]DTG binding in the presence of 100 nM (+)-pentazocine in the brain regions of rats treated with vehicle (square with vertically dotted lines) or 2 mg/kg (i.p. for 21 days) haloperidol (▨). Data are means ± S.E.M. (bars) from three to seven separate experiments. The brain was dissected into eight regions as follows: fc, frontal cortex; str, striatum; hip, hippocampus; cx, cortex; dc, diencephalon; mc, mesencephalon; pm, pons and medulla; cb, cerebellum.

ment had no effect on [^{3}H]DTG binding in the presence of 100 nM (+)-pentazocine in guinea pig brain regions similarly to the observations in rats (Fig. 3b).

3.3. Effects of haloperidol on [^{3}H](+)-pentazocine binding in the guinea pig brain

We investigated the effects of chronic and acute treatment on [^{3}H](+)-pentazocine binding in the frontal cortex of guinea pigs treated with various doses of haloperidol (0.01–0.5 mg/kg). Even in a single treatment, as little as 0.05 mg/kg haloperidol induced an approximately 50% decrease in [^{3}H](+)-pentazocine binding in the frontal cortex, and more than 0.1 mg/kg haloperidol treatment prevented [^{3}H](+)-pentazocine binding almost completely. Chronic treatment with more than 0.01 mg/kg haloperidol blocked [^{3}H](+)-pentazocine binding almost completely.

Scatchard analysis indicated that the maximum binding capacity of [^{3}H](+)-pentazocine was decreased markedly in the cortex of guinea pigs treated with 2 mg/kg haloperidol compared to controls (vehicle treatment). The values of B_{max} were 0.043 pmol/mg protein for the haloperidol treatment group and 0.63 pmol/mg protein for the control. The K_d values were 4.4 nM and 2.3 nM, respectively.

The effects of haloperidol on σ_1 receptor were long-lasting and reversible, since the inhibition of [^{3}H](+)-pentazocine binding to the cortex in the guinea pig produced by a single treatment with haloperidol decreased slowly and was still evident 14 days later. These effects of haloperidol on σ_1 receptors in the guinea pig brain were comparable with those reported previously in guinea pigs (Klein et al., 1994) and in rats (Itzhak and Stein, 1991).

As haloperidol has high affinities for both σ and dopamine D_2 receptors, we investigated whether other σ ligands (5 mg/kg DTG, 11 mg/kg rimcazole and 40 mg/kg (+)-SKF10047) or dopamine antagonists (15 mg/kg chlorpromazine, 100 mg/kg sulpiride and 1 mg/kg spiperone) have similar effects on [^{3}H](+)-pentazocine binding in guinea pigs. Chronic treatment with any of these ligands for 21 days did not decrease [^{3}H](+)-pentazocine binding in the guinea pig frontal cortex. Thus, only

Table 1
Regional variations in the proportions of σ_1 binding in the guinea pig and rat brain

Brain region	Ratio of σ_1 (% of total σ binding activity)	
	Guinea pig	Rat
Frontal cortex	58.7 ± 7.3	26.7 ± 2.3
Striatum	50.6 ± 4.3	35.8 ± 3.4
Hippocampus	45.4 ± 0.8	30.4 ± 2.8
Cortex	48.7 ± 3.9	30.5 ± 1.7
Diencephalon	55.0 ± 8.9	37.9 ± 3.5
Mesencephalon	63.4 ± 1.9	38.4 ± 2.7
Pons and medulla	56.6 ± 2.9	44.3 ± 2.2
Cerebellum	57.6 ± 3.0	33.3 ± 2.1

The ratio of σ_1 sites to total σ sites was derived by dividing the value obtained by subtracting [^{3}H]DTG specific binding activity in the presence of (+)-pentazocine (100 nM) from that in its absence by [^{3}H]DTG specific binding activity in the absence of (+)-pentazocine. Non-specific binding was determined in the presence of 10-μM haloperidol. Data are means ± S.E.M. of three to six independent experiments.

haloperidol induced a decrease in [^{3}H](+)-pentazocine binding.

3.4. Effects of haloperidol treatment on the level of σ_1 receptor mRNA

Fig 1 shows a schematic illustration of σ_1 receptor cDNA and its antisense cRNA probe (Fig. 1a) and the typical electrophoresis pattern in the measurement of σ_1 receptor mRNA by ribonuclease protection assay (Fig. 1b,c). The band of mRNA for guinea pig σ_1 migrated at a position corresponding to 309 bases, while that for β-actin used as an internal standard corresponded to 160 bases (Fig. 1b). The band of mRNA for rat σ_1 migrated at a position corresponding to 448 bases, while that for β-actin used as an internal standard corresponded to 310 bases (Fig. 1c).

Fig. 4a and b shows typical autoradiographic bands of gels, the intensities of which indicate the relative levels of the mRNA of σ_1 receptor and β-actin in different brain regions of guinea pigs treated intraperitoneally with 2 mg/kg haloperidol (Fig. 4b) or vehicle (Fig. 4a) once a day for 21 days. The ratios of each band intensity for σ_1 receptor mRNA relative to that of β-actin mRNA of the same sample were calculated and are shown in Fig. 4c. σ_1 receptor mRNA was relatively highly expressed but was distributed unevenly in the different brain regions. High levels of σ_1 receptor mRNA were observed in brain stem regions such as the pons and medulla, mesencephalon and diencephalon. The distribution of σ_1 receptor mRNA across the brain regions was similar to that of σ_1 binding sites detected by the binding activities of [^{3}H](+)-pentazocine shown in Fig. 2. The levels of σ_1 receptor mRNA were increased slightly by haloperidol treatment but this was not significant.

Fig. 5a and b shows typical autoradiographic bands of gels, the intensities of which indicate the relative levels of the mRNA of σ_1 receptor and β-actin in different brain regions of rats treated intraperitoneally with 2 mg/kg haloperidol (Fig. 5b) or vehicle (Fig. 5a) once a day for 21 days. The ratios of each band intensity for σ_1 receptor mRNA relative to that σ_1-actin mRNA of the same sample were calculated and are shown in Fig. 5c. As in guinea pigs, σ_1 receptor mRNA in the rat brain was distributed unevenly across the different brain regions, and relatively high levels were observed in the brain stem regions such as the pons and medulla, mesencephalon and diencephalon. The distribution of σ_1 receptor mRNA across the brain regions was similar to that of σ_1 binding sites detected by the binding activities of [^{3}H](+)-pentazocine

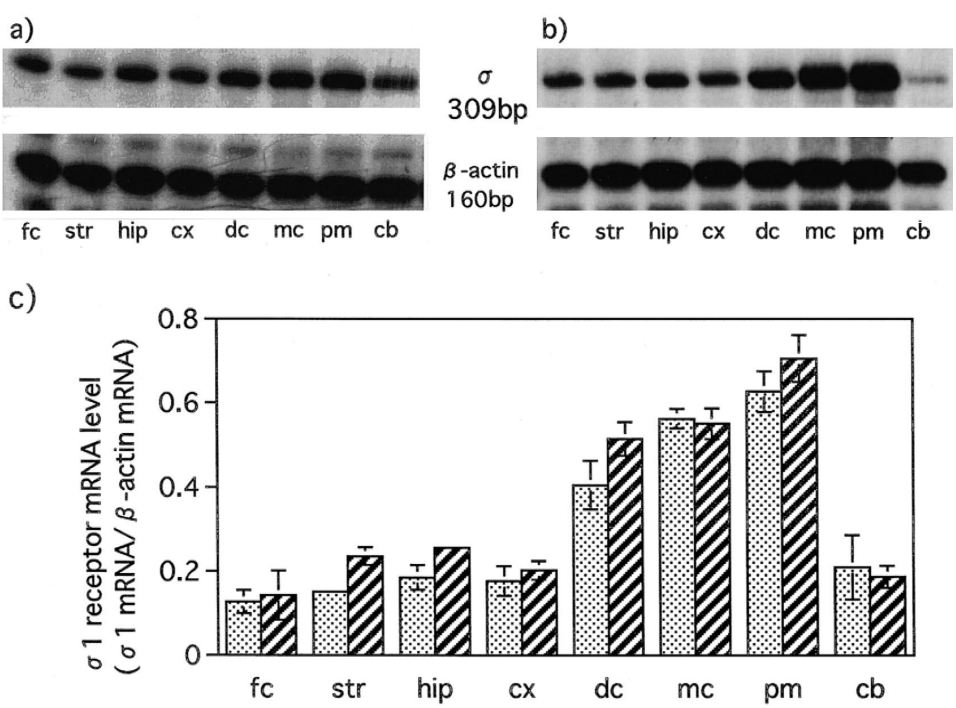

Fig. 4. Effects of chronic treatment with haloperidol on the level of σ_1 receptor mRNA in guinea pig brain regions. (a, b) Typical bands for σ_1 receptor and β-actin mRNA of guinea pigs treated with vehicle (a) and haloperidol (b) analyzed by ribonuclease protection assay. (c) Relative hybridization levels for σ receptor mRNA are expressed as the ratio of the band intensity (intensity of σ_1 receptor mRNA/intensity of β-actin mRNA). Guinea pigs were administered vehicle (square with vertically dotted lines) or haloperidol (2 mg/kg) (▨) intraperitoneally for 21 days. The guinea pigs were decapitated 24 h after the last injection. The brain was dissected into the eight regions as follows: fc, frontal cortex; str, striatum; hip, hippocampus; cx, cortex; dc, diencephalon; mc, mesencephalon; pm, pons and medulla; cb, cerebellum. Each column represents the mean ± S.E.M. of three to seven independent experiments.

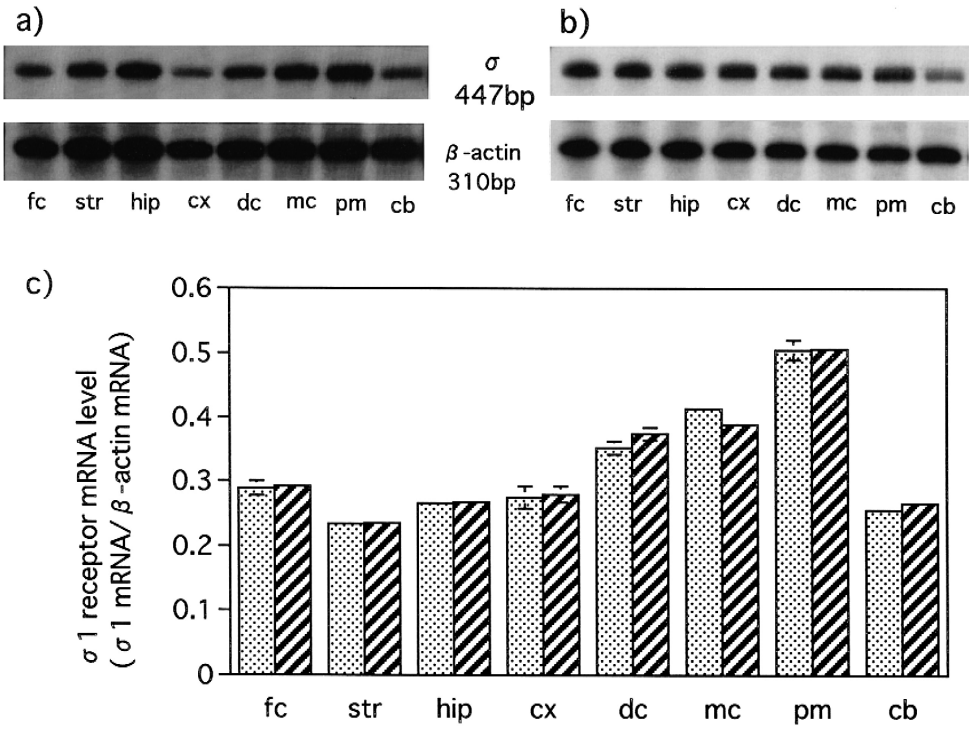

Fig. 5. Effects of chronic treatment with haloperidol on the levels of σ_1 receptor mRNA in rat brain regions. (a, b) Typical bands for σ_1 receptor and β-actin mRNA of rats treated with vehicle (a) and haloperidol (b) analyzed by ribonuclease protection assay. (c) Relative hybridization levels for σ receptor mRNA are expressed as the ratio of the band intensity (intensity of σ_1 receptor mRNA/intensity of β-actin mRNA). Rats were administered vehicle (square with vertically dotted lines) or haloperidol (2 mg/kg) (▨) intraperitoneally for 21 days. The rats were decapitated 24 h after the last injection. The brain was dissected into the eight regions as follows: fc, frontal cortex; str, striatum; hip, hippocampus; cx, cortex; dc, diencephalon; mc, mesencephalon; pm, pons and medulla; cb, cerebellum. Each column represents the mean ± S.E.M. of three to seven independent experiments.

shown in Fig. 3. As in the guinea pig, chronic treatment of rats with haloperidol had no effect on the levels of σ_1 receptor mRNA in any brain region examined.

4. Discussion

We discriminated between σ_1 and σ_2 binding sites by binding assay using [^{3}H](+)-pentazocine and [^{3}H]DTG. As reported previously (De Costa et al., 1989; DeHaven-Hudkins et al., 1992; Akunne et al., 1997), in our preliminary experiments [^{3}H](+)-pentazocine binding was completely inhibited by 100 nM (+)-pentazocine, whereas [^{3}H]DTG binding was only partially inhibited. DTG shows similar high affinities for both σ_1 and σ_2 binding sites. Thus, σ_2 binding sites were labeled with [^{3}H]DTG in the presence of 100 nM (+)-pentazocine to mask σ_1 binding sites. To obtain the values of non-specific binding, we used 10-μM haloperidol as a displacer in [^{3}H](+)-pentazocine and [^{3}H] DTG binding experiments. Haloperidol completely inhibited binding of both ligands at a concentration of 1 μM with K_i values of 28 nM for [^{3}H] (+)-pentazocine binding and 92 nM for [^{3}H] DTG binding.

The distribution patterns of σ_1 binding sites were very similar in rat and guinea pig brains; i.e. brain stem > cortex, cerebellum > striatum and hippocampus. In a previous experiment with [^{3}H]3-(3-hydroxyphenyl)-*N*-(i-propyl) piperidine [^{3}H]3-PPP), Gundlach et al. (1986) suggested that σ_1 binding sites in the brain stem were reduced by treatment with 6-hydroxydopamine. Therefore, σ_1 binding sites that were abundant in the brain stem would play an important role in dopaminergic neurotransmission.

Differences in proportion of σ_1 to σ_2 binding sites were observed across the brain regions of the rat and the guinea pig. In the guinea pig brain, numbers of σ_1 binding sites were equivalent to or higher than those of σ_2 binding sites, whereas in the rat brain, σ_2 binding sites were prevalent especially in the cortex and cerebellum. The distributions of σ_2 binding sites were similar to those of σ_1 binding sites in the guinea pig brain. These results were in agreement with those reported previously (Leitner et al., 1994) and in apparent contrast with the study of Bouchard and Quirion (1997) in some points. The latter authors showed the regional distribution of σ_1 and σ_2 binding sites in a rat brain using in vitro receptor autoradiography. Their results regarding the distribution of [^{3}H](+)-pentazocine binding sites are similar with our data, while the ratio and distribution of [^{3}H]DTG binding to σ_2 sites are different. They showed that the apparent levels of σ_2 sites are generally lower than that of σ_1 sites in almost all brain areas except a few. This discrepancy, as the authors men-

tioned there, may be due to the different experimental approaches (homogenate binding vs. autoradiography) as well as the concentration of (+)-pentazocine used to mask σ_1 sites (100 nM vs. 5 μM). Recently, Sanchez et al. (1997) reported that the selective σ_2 ligand Lu 28–179 has potent anxiolytic-like effects in rodents, and Walker et al. (1993) and Bouchard and Quirion (1997) reported that the σ_2 receptor was involved in motor function since these receptors appeared to be responsible for at least one class of motor effects produced by σ ligands. Furthermore, in the human brain, σ_2 binding sites were reported to be prevalent. In this study, we demonstrated that σ_2 binding sites were abundant in the rat cortex but not in that of the guinea pig confirming that there are species-specific differences in terms of σ_2 receptor distribution.

We have demonstrated in this study that chronic haloperidol treatment induces a marked decrease in [^{3}H](+)-pentazocine binding in all brain regions in the guinea pig and rat. The reduction of σ_1 binding activities by chronic treatment with haloperidol has been demonstrated previously using [^{3}H](+)-SKF10047 or [^{3}H](+)-3-PPP (Itzhak and Alerhand, 1989; Itzhak and Stein, 1991; Matsumoto et al., 1990; Karbon and Naper, 1990; Klein et al., 1994). In the present study, we confirmed that the effects of haloperidol are mediated only via σ_1 receptors, and compared the extent of the effect in guinea pigs with that in rats. In the rat, σ_1 binding was reduced by 20–30% similar to previous reports (Itzhak and Alerhand, 1989; Itzhak and Stein, 1991; Matsumoto et al., 1990; Karbon and Naper, 1990), and this effect was weaker than that in guinea pigs where σ_1 binding almost disappeared after chronic treatment with as little as 0.01 mg/kg of haloperidol or a single treatment at a dose of 0.1 mg/kg. This difference may be due to differences in the metabolic pathways of haloperidol in these species. Haloperidol is metabolized by both oxidative and reductive pathways to yield three metabolites. In humans, the keto-group of the hydrocarbon chain of haloperidol is reduced to a hydroxyl group to form reduced haloperidol. Reduced haloperidol is apparently only slightly less lipid-soluble than haloperidol, and it has been shown to accumulate in the brains of schizophrenia patients treated with haloperidol. In addition, reduced haloperidol exhibits higher binding affinity at the σ receptor than the dopamine D_2 receptor (Bowen et al., 1990). In the rat, haloperidol is not reduced, although reduced haloperidol is readily oxidized to haloperidol (Korpi and Wyatt, 1984). Following intraperitoneal injection into guinea pigs, haloperidol is converted to reduced haloperidol so quickly that 1 h after injection the concentration of haloperidol is only about one fifth of that of reduced haloperidol (Korpi et al., 1985). Therefore, in this study the sensitivity of haloperidol to σ_1 binding in guinea pigs may have been due to reduced haloperidol.

Haloperidol is both a σ ligand and dopamine D_2 receptor antagonist. To determine the mechanism of reduction of σ_1 binding activity produced by haloperidol treatment, we examined the effects of other σ ligands ((+)-SKF10047, DTG and rimcazole) and dopamine D_2 receptor antagonists (chlorpromazine, sulpiride and spiperone) in the guinea pig. As σ ligands, (+)-SKF10047 (40 mg/kg) has been reported to decrease proenkephalin mRNA through σ_1 sites (Angulo et al., 1991), and DTG (5 mg/kg) and rimcazole (11 mg/kg) have been reported to increase the maximal [^{3}H](+)-3-PPP binding (B_{max}) in rats (Breat et al., 1989). Repeated methamphetamine treatment produced an increase in [^{3}H] (+)-pentazocine binding (Itzhak, 1993), and repeated haloperidol treatment up-regulated D_2 binding and down-regulated σ_1 binding. The finding that haloperidol and methamphetamine, which have opposite effects on dopamine neurotransmission, regulated the σ_1 receptor in opposite directions supported the involvement of dopaminergic transmission in σ_1 receptor function. With the exception of haloperidol, the σ and dopamine receptor ligands used in this study had no effect on σ_1 binding, suggesting that haloperidol may not exert its effects as a σ and/or dopaminergic agent in vivo.

Recently, Hanner et al. (1996) cloned a σ_1 receptor from the guinea pig liver, a tissue that expresses this receptor at high levels. Subsequently, human (Kekuda et al., 1996), mouse (Seth et al., 1997; Pan et al., 1998) and rat (Seth et al., 1998) σ_1 receptor cDNAs were cloned. Although the promoter regions of the human (Presad et al., 1998) and mouse homologues were determined (Seth et al., 1997), the regulation of σ_1 receptor expression is still unknown. We analyzed σ_1 receptor mRNA level by ribonuclease protection assay to examine whether the decrease of σ_1 binding sites produced by haloperidol treatment followed a decrease in σ_1 receptor mRNA level. The regional distribution of σ_1 receptor mRNA was similar to that of binding sites in both the guinea pig and rat brain. However, in contrast to the results of the binding experiments, haloperidol treatment did not induce the reduction of σ_1 receptor mRNA level. These results indicated that the decrease in σ_1 binding sites produced by haloperidol treatment was not the result of a decrease in σ_1 receptor gene transcription. The number of σ_1 binding sites is regulated not only by mRNA transcription but also by processes such as translation, transport, insertion, internalization and degradation. Haloperidol might induce internalization of the ligand-receptor complex without affecting σ_1 receptor transcriptional activity. Receptor internalization is generally initiated by the agonist binding. Although the definition of an agonist–antagonist relationship at σ receptor is not clear so far, the reaction of haloperidol on σ_1 binding sites in guinea pigs does not seem to be a classic agonist-induced down-regulation, because other ligands that were predicted to behave in the same manner did not induce receptor down-regulation even in chronic treatment. In this study, we could not examine the effects of reduced haloperidol in vivo. However, formation of a stable complex between reduced haloperidol and σ_1 receptor protein in vivo, as suggested by Klein et al. (1994), could explain

the differences in responsiveness to haloperidol between rats and guinea pigs, and the lack of influence of haloperidol treatment on σ_1 receptor mRNA level.

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