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Expression pattern of the type 1 sigma receptor in the brain and identity of critical anionic amino acid residues in the ligand-binding domain of the receptor

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Abstract

The type 1 σ receptor (σR1) has been shown to participate in a variety of functions in the central nervous system. To identify the specific regions of the brain that are involved in σR1 function, we analyzed the expression pattern of the receptor mRNA in the mouse brain by in situ hybridization. σR1 mRNA was detectable primarily in the cerebral cortex, hippocampus, and Purkinje cells of cerebellum. To identify the critical anionic amino acid residues in the ligand-binding domain of σR1, we employed two different approaches: chemical modification of anionic amino acid residues and site-directed mutagenesis. Chemical modification of anionic amino acids in σR1 with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide reduced the ligand-binding activity markedly. Since it is known that a splice variant of this receptor which lacks exon 3 does not have the ability to bind σ ligands, the ligand-binding domain with its critical anionic amino acid residues is likely to be present in or around the region coded by exon 3. Therefore, each of the anionic amino acids in this region was mutated individually and the influence of each mutation on ligand binding was assessed. These studies have identified two anionic amino acids, D126 and E172, that are obligatory for ligand binding. Even though the ligand-binding function was abolished by these two mutations, the expression of these mutants was normal at the protein level. These results show that σR1 is expressed at high levels in specific areas of the brain that are involved in memory, emotion and motor functions. The results also provide important information on the chemical nature of the ligand-binding site of σR1 that may be of use in the design of σR1-specific ligands with potential for modulation of σR1-related brain functions.

Keywords: Sigma receptor; In situ hybridization; Brain; Chemical modification; Anionic amino acid; Ligand-binding domain

1. Introduction

σ receptors, which interact with several psychoactive agents such as haloperidol, phencyclidine, neurosteroids, benzomorphans and cocaine, are defined as

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non-opiate and non-phencyclidine binding sites [1–3]. These binding sites have been identified in the brain as well as in a variety of other organs [3]. Based on the ligand-binding characteristics and biochemical features, the σ ligand binding sites fall into several subtypes [4]. Of these different subtypes, the type 1 σ receptor (σR1) represents the best characterized subtype at the functional and structural level. Autoradiographic studies with subtype-specific radiolabeled ligands have shown that σR1 is expressed at high levels in specific regions of the brain, primarily in those areas that are involved in motor, sensory, and endocrine functions, and in emotion and memory [5–8]. This subtype has been shown to play vital roles in learning and memory in animal models of amnesia [9], in cocaine-induced behavioral changes [10,11], and in opiate-induced analgesia [12,13]. In the periphery, σR1 is expressed in the placenta, liver, immune cells, gastrointestinal tract, and steroid-producing organs [1–3,14]. Progesterone is believed to be one of the endogenous ligands to σR1 [15]. Since several σR1 ligands are known to be immunosuppressive [16], it has been speculated that progesterone, by acting as a pregnancy-specific ligand to σR1, might provide at least one of the mechanisms for the maternal tolerance of the fetal/placental allograft [17]. σR1 has been shown to be involved in the modulation of several second messenger systems [2,3]. Of these, the role of the receptor in Ca^{2+} signaling has received much attention in recent years [18–22]. The interaction between σR1 and Ca^{2+} signaling is supported by the findings that the receptor is located in close proximity to the IP_3 receptor in the endoplasmic reticulum [23].

Until recently, studies involving the function and distribution of σ receptors have been carried out using ligands that are supposedly specific for different subtypes of the receptor. Since many of these ligands also interact with other receptors in addition to σ receptor subtypes, it has been difficult to obtain a direct correlation between the ligands' pharmacological actions and their binding to specific σ receptor subtypes. Consequently, despite the large volume of information available on the pharmacology of a number of σ ligands, the exact roles of different σ receptor subtypes have remained elusive. Furthermore, there was no information available until recently on the molecular identity of any of these receptor subtypes and this has also slowed the progress in defining the structural and functional features of these receptors. However, currently there is a renewed interest in σ receptors and their physiological functions and pharmacological and therapeutic potential because of the recent success in the cloning and molecular characterization of one of the σ receptor subtypes. σR1 was first cloned from the guinea pig liver [24]. Subsequently, we and others have cloned the human, rat, and mouse homologues [25–29]. Interestingly, the cloned σR1 exhibits no significant homology to any of the mammalian proteins thus far cloned. However, this receptor is significantly homologous to a sterol metabolizing enzyme in yeast [24]. This yeast protein is a C_8-C_7 isomerase involved in the ergosterol biosynthetic pathway. However, a mammalian C_8-C_7 isomerase has been recently cloned [30,31], but surprisingly this protein shows no structural similarity to either the yeast C_8-C_7 isomerase or mammalian σR1.

With the current availability of molecular tools such as the cDNA probes and antibodies specific to σR1, significant developments have occurred recently in defining the structural and functional features of this receptor. With the cloned σR1, we have demonstrated unequivocally that progesterone functions as a ligand to this receptor [17]. Use of antisense approaches has demonstrated that σR1 plays a significant role in opiate-induced analgesia [28]. Studies with σR1-specific antibodies have established that σR1 is a membrane-associated protein present in intracellular organelles [32] and that the receptor is involved in protein–protein interaction with the type 3 IP_3 receptor and ankyrin B regulating Ca^{2+} signaling [33]. There is also evidence for σR1 translocation from intracellular sites to plasma membrane as a result of ligand binding [34], suggesting that there may be proteins in the plasma membrane, not yet identified, that might be targets for σR1 for protein–protein interaction.

The present study was undertaken for two purposes, first to assess the expression pattern of σR1 in the brain by in situ hybridization and second to identify the anionic amino acid residues that play an essential functional role in the ligand-binding domain of the receptor. The distribution of σR1 in the brain has been investigated so far only with ligand-binding assays. Since there is a significant degree of overlap

in ligand specificity among different subtypes of σ receptor, the assessment of the distribution of σR1 mRNA in the brain might provide a more reliable approach to determine the specific areas of the brain that express the receptor. The second aim was based on our recent findings that a splice variant of σR1 that lacks 31 amino acids coded by exon 3 has no detectable ligand-binding activity [17]. These findings suggest that the ligand-binding domain is likely to be present in or around the region coded by exon 3. Since most of the σ ligands are positively charged, we hypothesized that anionic amino acid residues located in the putative ligand-binding domain may play a role in the interaction of the receptor with the ligands.

2. Materials and methods

2.1. Materials

[^{3}H]Haloperidol and (+)-[^{3}H]pentazocine were purchased from NEN Life Science Products (Boston, MA, USA). Lipofectin and media and chemicals for cell culture were purchased from Life-Technologies (Rockville, MD, USA).

2.2. Preparation of JAR cell crude membranes

The human placental choriocarcinoma cell line JAR was used to prepare membranes for studies with native σR1 which is constitutively expressed in these cells [14]. The cells were lysed using ice-cold 5 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5, and the lysate was homogenized in an Ultra-Turrax Tissuemizer. The homogenate was centrifuged at $40\,000 \times g$ for 30 min. The final membrane pellet was suspended in 5 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5.

2.3. Heterologous expression of σR1s in MCF-7 cells

The vaccinia virus expression system was used to study the ligand-binding characteristics of the cloned σ receptors. The σR1 cDNAs were expressed heterologously in MCF-7 cells. These cells express negligible levels of σR1 constitutively [27]. Subconfluent MCF-7 cells in 24-well culture plates were first infected with a recombinant vaccinia virus (VTF_{7-3}) encoding T7 RNA polymerase and then transfected with pSPORT-cDNA construct in the presence of Lipofectin [25–27]. Twelve hours post transfection, the medium was removed by aspiration and the cells washed once with ice-cold 5 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5. 250 μl of this buffer was then added to the cells and the cells were subjected to a freeze–thaw procedure once. The lysate was centrifuged at $40\,000 \times g$ for 30 min and the membrane pellet was suspended in the binding buffer.

2.4. Treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

Crude membranes prepared from JAR cells or MCF-7 cells were treated with varying concentrations of EDC in 5 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5) for 1 h at room temperature. Following this treatment, the reaction mixture was diluted with ice-cold buffer and centrifuged at $40\,000 \times g$ for 30 min to pellet the membranes. Unreacted EDC was removed by this procedure. The membrane pellet was suspended in the same buffer and used for ligand-binding measurements.

2.5. Ligand-binding assay

The membranes were incubated with radiolabeled σ ligands (haloperidol, (+)-pentazocine) in 5 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5) [14,17]. A 3 h incubation was used for all equilibrium binding assays. Binding was terminated by the addition of ice-cold binding buffer, followed by filtration of the mixture on a Whatman GF/F glass fiber filter presoaked in 0.3% polyethylenimine. The filters were washed thrice with ice-cold binding buffer. Radioactivity associated with the filters was determined by liquid scintillation spectrometry.

2.6. Site-directed mutagenesis

The Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to introduce point mutations in the C-terminal half of the human σR1 protein sequence. The procedure was performed using *Pfu* DNA polymerase which replicates both plasmid strands with high fidelity and without displacing the mutant primers. Two syn-

thetic oligonucleotide primers (sense and antisense) specific for the human σR1 cDNA and containing the desired mutation in the middle were used. Following PCR with the wild type human σR1 cDNA as the template, the product was digested with *Dpn*I to digest the parental DNA template, leaving behind the nicked dsDNA containing the mutation introduced in the primers. The resultant product was then transformed into *Escherichia coli* for repair of the nicks and amplification. The entire coding region of the mutant cDNAs was sequenced to confirm the presence of the introduced mutations and the absence of any unwanted mutations arising from PCR.

2.7. Analysis of σR1 protein expression

The MCF-7 cells, cultured on chamber slides, were transfected with wild type or mutant σR1 cDNA using the vaccinia virus expression technique. After 12 h of transfection, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). The cells were fixed with ice-cold methanol for 5 min. The fixative was removed and the cells were permeabilized with 0.1% Triton X-100 for 5 min. The cells were washed with PBS and then treated with 4% goat serum for 30 min. The σR1-specific monoclonal antibody [32] was added to the cells and incubated for 3 h at room temperature and then overnight at 4°C. Following the incubation, the cells were washed with PBS and then incubated overnight at 4°C with FITC-coupled anti-mouse IgG antibody. After the incubation, the cells were washed, covered with Vectashield, and coverslipped. Sections were analyzed by laser-scanning confocal microscopy (Bio-Rad MRC 600 Laser Scanning Confocal Imaging System).

2.8. In situ hybridization

The brain from CBA adult mice was collected and immediately frozen in liquid nitrogen. Unfixed 12 μm serial sections were prepared on a cryostat, mounted on 2% 3-aminopropyltriethoxysilane-coated slides, air-dried for 10 min, and then stored at −70°C until required. In situ hybridization using digoxigenin UTP-labeled probes was performed on tissue sections as previously described [35,36]. For the preparation of the mouse σR1-specific riboprobe, a 0.65 kb fragment of the mouse σR1 cDNA, obtained by the digestion of pSPORT-mouse σR1 cDNA by *Sal*I-*Sma*I, was subcloned into pBluescript vector. The orientation of the cDNA insert in the pBluescript vector was determined by sequencing. Antisense and sense riboprobes were synthesized with T7 RNA polymerase or T3 RNA polymerase after linearization of the plasmid with appropriate restriction enzymes. The riboprobes were labeled using a digoxigenin-labeling kit (Boehringer-Mannheim, Indianapolis, IN, USA).

3. Results and discussion

3.1. Expression pattern of σR1 mRNA in the brain

Fig. 1 describes the in situ hybridization data for the expression of σR1 mRNA in the mouse brain. The expression of σR1 mRNA is evident throughout the brain, especially in the cerebral cortex (C), hippocampus (H), and cerebellum (CB). Within the hippocampus, the σR1 mRNA-specific hybridization signals are found in cornu ammonis pyramidal neurons (CA), the granular cells of the dentate gyrus (DG) as well as interneurons. Within the cerebellum, the single layer of Purkinje cell bodies that lie between the granular cell layer (G) and the molecular layer (ML) as well as the cell bodies in the deep cerebellar nuclei (D) are intensely positive for σR1 mRNA. The convoluted folia which are supported by the central medulla of white matter (M) does not express σR1. This expression pattern of σR1 mRNA by in situ hybridization correlates with the previous findings on the expression pattern of σR1-specific ligand binding [5–8]. McLean and Weber [5] found σR1-specific ligand-binding sites to be highly concentrated in the Purkinje cell layer of the cerebellum and the pyramidal cell layer of the hippocampus. Gundlach et al. [6] have analyzed in greater detail the expression pattern of σR1 in the hippocampus by ligand binding. Their results have shown that σR1 is present in pyramidal neurons rather than in the terminals of input neurons from the entorhinal cortex and lateral septum. The high level of expression of σR1 in the cerebellum and hippocampus suggests a role for this receptor in motor functions, emotion, and memory.

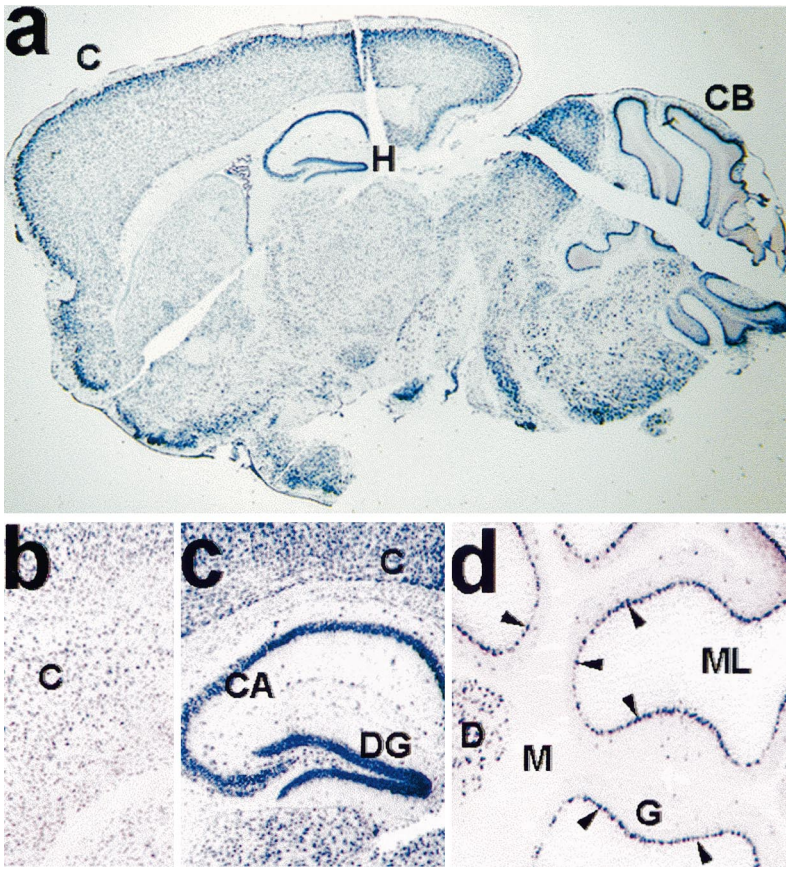

Fig. 1. In situ hybridization for analysis of regional distribution pattern of σR1 mRNA in mouse brain. (a) Sagittal section of an adult mouse brain hybridized with an antisense σR1 probe (C, cortex; H, hippocampus; CB, cerebellum). (b) Higher power image of cerebral cortex. (c) Higher power image of hippocampus (C, cerebral cortex; CA, cornu ammonis neurons; DG, dentate gyrus). (d) Higher power image of cerebellum (G, granular layer; ML, molecular layer; M, medulla of white matter; D, deep cerebellar nuclei; arrows, Purkinje cell bodies). Use of sense σR1 probe did not yield detectable hybridization signals under identical conditions in any of the brain regions (data not shown).

3.2. *Inhibition of ligand-binding function of human σR1 by carboxy group-specific chemical modification*

To determine whether or not the ligand-binding site of the human σR1 possesses essential anionic amino acid residues, the JAR cell membranes which contain the constitutively expressed σR1 and the MCF-7 cell membranes which contain the heterologously expressed human σR1 were subjected to chemical modification with EDC. This reagent is specific for the carboxy groups and causes covalent modification of the carboxy groups of anionic amino acid residues (aspartate and glutamate) in proteins. Following treatment with this reagent, the membranes were used for binding of σ ligands (haloperidol and (+)-pentazocine). Initial experiments to optimize the effect of this reagent employed increasing concentrations of the reagent. These studies showed that 1 mM EDC produced the maximal effect (data not shown). Figs. 2 and 3 describe the inhibition of σ ligand binding to native (JAR cell membranes) and cloned σR1 (MCF-7 cell membranes) by EDC treatment. Treatment of the JAR cell membranes with EDC (1 mM) caused 65–70% inhibition of the binding of (+)-pentazocine and haloperidol (Fig. 2). Similarly, MCF-7 cell membranes containing the heterologously expressed human σR1 showed 60–65% inhibition of the binding of the σ ligands as a result of EDC treatment (Fig. 3). Membranes from MCF-7 cells transfected with the pSPORT vector alone showed negligible σ ligand binding and this binding was not affected by EDC treatment. This is in agreement with our earlier findings that MCF-7 cells ex-

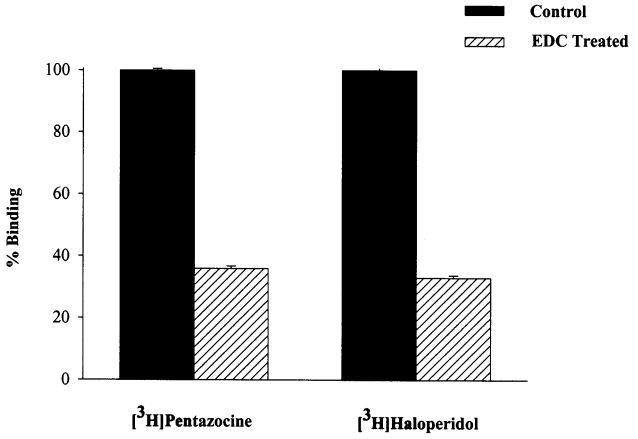

Fig. 2. Effect of EDC treatment on σ ligand binding in JAR cell membranes. Membranes were treated with or without (control) 1 mM EDC for 1 h. Control and treated membranes were used for equilibrium binding of the σ ligands (+)-pentazocine (2 nM) and haloperidol (1 nM). Results are given as percent of binding of respective ligands in control membranes. The control value was 0.30 ± 0.01 pmol/mg of protein for (+)-pentazocine and 2.10 ± 0.09 pmol/mg of protein for haloperidol.

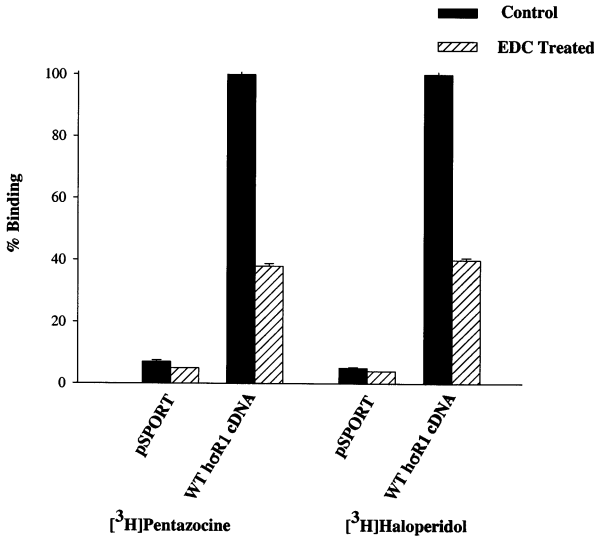

Fig. 3. Effect of EDC treatment on ligand binding to the cloned σR1. The human σR1 cDNA was expressed heterologously in σR1-negative MCF-7 cells. Cells transfected with pSPORT vector alone were used as negative control. Membranes were prepared from cells transfected with either empty vector or σR1 cDNA and then treated in the presence or absence of 1 mM EDC for 1 h. Equilibrium binding of (+)-pentazocine (2 nM) and haloperidol (1 nM) was measured in these membranes. Results are given as percent of binding of respective ligands in membranes treated in the absence of EDC. This value was 0.31 ± 0.01 pmol/10^6 cells for (+)-pentazocine and 2.06 ± 0.51 pmol/10^6 cells for haloperidol.

press negligible levels of σR1 [27]. These results demonstrate that the ligand-binding site of human σR1 contains anionic amino acid residues that are essential for the ligand-binding function.

3.3. Conserved anionic amino acid residues in the C-terminal half of the σ receptor

Human σR1 is a small protein consisting of 223 amino acids. Anionic amino acids constitute approx. 10% of the total amino acids in the protein (15 glutamate residues and six aspartate residues) and each one of these 21 anionic amino acid residues is conserved in σR1s derived from different animal species (Fig. 4). σR1 is an integral membrane protein and contains a single putative transmembrane domain that is located in the region comprising the amino acid residues 92–113. We have previously identified an alternative splice variant of σR1 that lacks exon 3 and has no ability to bind σ ligands [17]. The region deleted in the splice variant consists of the amino acid residues 119–149. Thus, the putative membrane-spanning domain lies upstream from the region coded by exon 3. Since the splice variant lacks the ligand-binding function, we speculated that the ligand-binding site is present in the C-terminal half of the protein downstream from the membrane-spanning domain in the wild type σR1. This region contains 12 anionic amino acid residues and one or more of these residues is/are likely to be involved in ligand binding. The location of the putative transmembrane

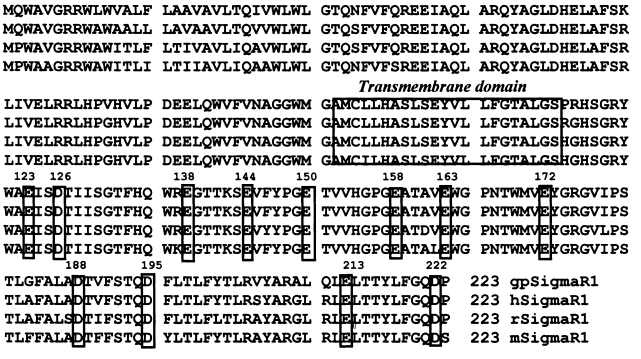

Fig. 4. Location of the putative transmembrane domain and the anionic amino acid residues in the C-terminal half of the σR1 conserved across the species. gpSigmaR1, guinea pig σR1; hSigmaR1, human σR1; rSigmaR1, rat σR1; mSigmaR1, mouse σR1.

domain and the conserved anionic amino acid residues in the C-terminal half of the σR1 protein are shown in Fig. 4.

3.4. Ligand-binding function and protein expression of the mutants

To identify the anionic amino acid residues essential for the ligand-binding function, we mutated each of the 12 anionic amino acid residues in the C-terminal half of the σR1 and assessed the influence of the mutations on the ligand-binding function. In each case, the anionic amino acid residue was converted to the neutral amino acid glycine. Table 1 describes the ligand-binding function of the mutants and the wild type σR1. The binding activity of most of these mutants varied in the range of 75–120% compared to the wild type σR1. However, two of the mutants (D126G and E172G) showed very little ligand binding ($<10\%$ of the wild type σR1 activity). These data indicated that D126 and E172 are involved in the binding of the σ ligands to σR1, but an alternative explanation may be that the mutation of these two amino acids decreases the stability of the protein and that the observed loss of ligand-binding activity is due to enhanced degradation of the protein. Therefore, we investigated the protein expression of the two mutants using a σR1-specific monoclonal antibody (Fig. 5). We expressed the wild type and the two mutant σR1s in σR1-negative MCF-7 cells heterologously. Cells transfected with vector alone served as the negative control. As expected, there was no immunodetectable protein signal in MCF-7 cells transfected with vector alone. However, an immunopositive signal was detectable in the nuclear envelope in cells expressing the wild type σR1. More importantly, the subcellular localization as well as the intensity of the immunopositive signal were comparable in cells expressing the wild type σR1 and in cells expressing either of the two mutant σR1s. These results show that the expression of σR1 is not altered as a consequence of the mutations D126G and E172G. The nuclear envelope localization of the heterologously expressed human σR1 in MCF-7 cells agrees with the data on the localization of constitutively expressed σR1 in human immune cells [32]. Taken collectively, these data demonstrate the obligatory nature of D126 and E172 for the ligand-binding function of σR1.

In summary, the present studies provide important

Table 1
Ligand-binding activity of the wild type and mutant σR1s

Wild type/mutant σR1	[^{3}H]Haloperidol binding	
	pmol/10^6 cells	%
Wild type	4.24 ± 0.47	100
E123G	4.55 ± 0.58	107
D126G	0.37 ± 0.02	9
E138G	3.25 ± 0.46	77
E144G	3.15 ± 0.34	74
E150G	3.32 ± 0.20	78
E158G	3.28 ± 0.45	77
E163G	4.31 ± 0.14	102
E172G	0.14 ± 0.04	3
D188G	5.13 ± 0.56	121
D195G	4.05 ± 0.40	96
E213G	4.62 ± 0.22	109
E222G	3.24 ± 0.29	76

The wild type and mutant σR1s were expressed heterologously in MCF-7 cells using the vaccinia virus expression technique. Crude membranes from these cells were used for equilibrium binding of [^{3}H]haloperidol. Concentration of the ligand during the binding assay was 3 nM.

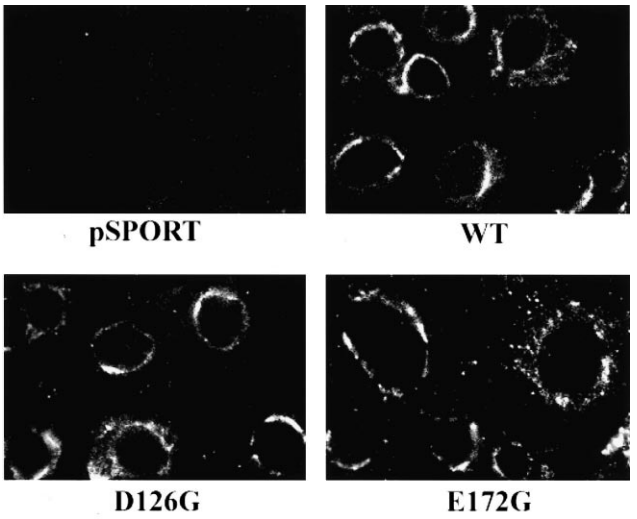

Fig. 5. Immunodetection of the expression of wild type and mutant σR1s. σR1-negative MCF-7 cells were transfected with vector alone (pSPORT), wild type σR1 cDNA (WT), D126G mutant σR1 cDNA (D126G) or E172G mutant σR1 cDNA (E172G). Twelve hours after transfection, the expressed σR1 proteins were detected with a σR1-specific monoclonal antibody and a FITC-coupled secondary antibody.

information in two areas of σR1 biology, namely the expression pattern and the structure–function relationship. These studies identify for the first time the specific brain areas that express σR1 mRNA. The present data on the expression pattern of σR1 mRNA by in situ hybridization correlate very well with the previous findings on the distribution of σR1-specific ligand-binding sites in the brain [5–8]. The receptor is expressed abundantly in the hippocampus and in the Purkinje cells of the cerebellum. Since these regions are known to be involved in important brain functions relating to memory, emotion, and motor activity, we speculate that σR1 may have a physiological role in the modulation of these critical brain functions. A number of studies have shown that σR1-specific ligands are effective as neuroprotective and anti-amnesic agents [37–42], and the widespread expression of the receptor in the brain, particularly in the hippocampus and cerebellum, may have relevance to the observed therapeutic effects of σR1-specific ligands.

σR1 is expressed not only in the brain but also in several other tissues. In addition to the previously stated therapeutic role in neuroprotection and amelioration of memory defects, σR1-specific ligands have also been shown to be effective as anti-inflammatory agents [16,32,43,44] and in the treatment of ocular hypertension associated with glaucoma [45]. These findings suggest that σR1 is a potential therapeutic target in the treatment of a multitude of clinical conditions. The design of highly selective σR1 ligands with no or little affinity towards other receptors is necessary to ensure that σR1 is the specific target for these potential therapeutic agents. The design of such selective ligands will require detailed information on the chemical nature of the ligand-binding domain of the receptor. The present studies show for the first time that anionic amino acid residues in the receptor are critical for ligand binding. In addition, these studies have identified two specific anionic amino acid residues that play an essential role in the ligand binding, suggesting that these residues are located most likely in the ligand-binding domain. This information on the molecular nature of the ligand-binding region of the receptor is potentially useful in the future design of σR1-selective therapeutic agents.

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