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Evidence that the σ_1 receptor is not directly coupled to G proteins ☆

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Abstract

Sigma (σ) receptors have been implicated in psychosis, cognition, neuroprotection, and locomotion in the central nervous system. The signal transduction mechanisms for σ receptors have not been fully elucidated. In this study, we examined the possible coupling between σ_1 receptors and heterotrimeric guanine nucleotide-binding proteins (G proteins) in rodent brain. In σ_1 receptor-rich cerebellar membrane preparations, the competitive binding curves of two σ_1 agonists, (+)pentazocine and 1*S*,2*R*-(−)-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)cyclohexylamine (BD737), were unaffected by the addition of 10 μM guanosine-5′-*O*-(γ-thio)-triphosphate (GTPγS). Neither (+)pentazocine (1–100 μM) nor BD737 (0.01–10 μM) stimulated GTPase activities significantly above basal levels in agonist-stimulated GTPase activity assays in cerebellar membranes. Furthermore, when using the method of agonist-stimulated [^{35}S]GTPγS binding as assessed by autoradiography, we did not observe significant stimulation of [^{35}S]GTPγS binding in rat brain sections by either (+)pentazocine or BD737. The above results demonstrate that the σ_1 receptor is not likely be directly coupled to G proteins.

Keywords: σ Receptor; (+)Pentazocine; Haloperidol; Cerebellum; GTPase; [^{35}S]GTPγS autoradiography

1. Introduction

Sigma (σ) receptors were proposed by Martin et al. (1976) to account for the psychotomimetic effects of the benzomorphan *N*-allylnormetazocine (SKF 10,047) in the chronic spinal dog. Sigma receptors are widely distributed in the central nervous system and peripheral organs such as the adrenal gland, ovary, liver and kidney (Walker et al., 1990). In the central nervous system, sigma receptors are enriched in the limbic and motor areas including the hippocampus and the cerebellum (Gundlach et al., 1986). Extensive research has indicated that σ receptors may be involved in psychosis, cognition, neuroprotection, and locomotion in the central nervous system (Walker et al., 1990). The antipsychotic haloperidol binds with high affinity to σ receptors. It is not clear whether its antipsychotic effects may be partly elicited through σ receptors.

The existence of multiple σ receptor subtypes has been proposed (Quirion et al., 1992). The subtype I σ receptor (σ_1) receptor has a nanomolar K_i value for (+)pentazocine and (+)SKF 10,047, and is stereoselective for the (+)isomer of benzomorphans in general. The signal transduction mechanisms for σ receptors, however, have not been fully elucidated. Because several early studies showed that the binding parameters of σ ligands were altered by GTP and its analogs in radioligand binding experiments (Beart et al., 1989; Itzhak, 1989; Connick et al., 1992), the σ_1 receptor was hypothesized to be coupled to G proteins. A recent paper reported the molecular cloning of a σ_1-binding protein from a guinea pig liver complementary DNA (cDNA) library (Hanner et al., 1996). The predicted structure of this 223-amino acid protein has a single transmembrane-domain, different from the common seven transmembrane-domain structure of G protein-coupled receptors. Proteins highly homologous with this σ_1-binding protein were later cloned from human, rat and mouse cDNA libraries (Jbilo et al., 1997; Pan et al., 1998; Seth et al., 1998). All these proteins are too small to form seven transmembrane-domain structures.

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In early radioligand binding studies, [^{3}H](+)-3-(3-hydroxyphenyl)-*N*-(*n*-propyl)piperidine ((+)-3-PPP) and [^{3}H]1,3-di-*o*-tolylguanidine (DTG) were used to label the σ_1 receptor. High-affinity ligands with higher selectivity for the σ_1 receptor have since been identified. In previous studies from our lab, we have confirmed the selectivity of σ_1 receptor agonists and antagonists in a neurotransmitter release assay system. The σ_1 receptor agonists (+)pentazocine and 1*S*,2*R*-(−)-*cis*-*N*-[2-(3,4-dichlorophenyl)-ethyl]-*N*-methyl-2-(1-pyrrolidinyl)cyclohexylamine (BD737) inhibited *N*-methyl-D-aspartate (NMDA)-stimulated neurotransmitter release from rodent brain slices; the σ_1 receptor antagonists 1-(cyclopropylmethyl)-4-[2′-4″-fluorophenyl)-2′-oxoethyl]piperidine HBr (Dup734), haloperidol and DTG reversed the inhibition by (+)pentazocine and BD737 (Gonzalez-Alvear and Werling, 1995a).

Although it has been reported that σ_1 receptor radioligand binding was increased in cell lines transfected with the cloned σ_1-binding protein (Hanner et al., 1996; Pan et al., 1998; Seth et al., 1998), pharmacological evidence that this protein per se can serve as a functional receptor has been lacking. The putative non-seven-transmembrane-domain structure of the cloned σ_1-binding protein does not preclude the possibility that the σ_1 receptor may be coupled to G proteins, if the σ_1-binding protein is only one component of a multi-subunit σ_1 receptor. Besides aforementioned radioligand binding experiments, several studies have shown certain cellular effects of σ_1 ligands were inhibited by pretreatment of pertussis toxin or cholera toxin, suggesting involvement of G proteins in these effects of σ_1 receptors (Monnet et al., 1994; Soriani et al., 1999). In this study, we set out to thoroughly investigate whether or not the σ_1 receptor is *directly* coupled to G proteins using three different kinds of pharmacological assays.

2. Methods

2.1. Chemicals

The following chemicals were purchased or obtained from the following sources: [^{3}H]haloperidol (15 Ci/mmol), [^{3}H](+)pentazocine (28 Ci/mmol) and [^{35}S]guanosine-5′-*O*-(γ-thio)-triphosphate ([^{35}S]GTPγS) (1228 Ci/mmol), New England Nuclear (Boston, MA, USA); haloperidol and spiroperidol hydrochloride, Research Biochemicals International (Natick, MA, USA); [γ-^{32}P]GTP(25 Ci/mmol) and GDP, ICN Biochemicals (Irvine, CA, USA); adenosine 5′-(β,γ-imino-triphosphate) (App[NH]p), Fluka (Milwaukee, WI, USA); (+)pentazocine, Research Technology Branch, National Institute on Drug Abuse (Rockville, MD, USA). BD737 was kindly provided by Dr. Wayen Bowen, National Institute of Digestive Disorders and Kidney (Bethesda, MD, USA). Endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzamidazole-1-carboxamide hydrochloride (BIMU-8) was kindly provide by Dr. Douglas W. Bonhaus, Department of Neurosciences, Syntex Discovery Research (Palo Alto, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

2.2. Radioligand binding studies

Membrane preparations were made as previously described (Gonzalez-Alvear et al., 1995). Briefly, the liver and cerebellar tissue from male Hartley guinea pigs and the cerebellar tissue from male Sprague–Dawley rats (Hilltop Lab, Scottdale, PA, USA) were homogenized in ice-cold Tris buffer (10 mM Tris–HCl, pH 7.4) with 0.32 M sucrose, and centrifuged at $1000 \times g$ for 10 min. The supernatant was collected and centrifuged at $31{,}000 \times g$ for 15 min. The pellets were resuspended in Tris buffer and centrifuged again at $31{,}000 \times g$ for 15 min, and this step was repeated once. The final pellets were resuspended in Tris buffer at a concentration of 4 mg protein/ml, and stored at −80°C until use. Protein concentrations were determined by Peterson's method (Peterson, 1977).

Radioligand competition assays were done in Tris buffer (10 mM Tris–HCl, pH 7.4). Four nanomolar [^{3}H]haloperidol or 2 nM [^{3}H](+)pentazocine were used as radioligands to label σ_1 receptor binding in membranes. When [^{3}H]haloperidol was used, 100 nM spiroperidol was included in binding reactions to block dopamine D_2 and serotonin 5-HT_2 receptor binding; 100 nM σ_2 receptor antagonist BIMU-8 was also included to block σ_2 receptor binding. Non-specific binding was measured in the presence of 1 μM haloperidol. When [^{3}H](+)pentazocine was used, 100 nM BIMU-8 was included to block σ_2 receptor binding. Non-specific binding was measured in the presence of 10 μM (+)pentazocine. The σ_1 receptor agonists (+)pentazocine and BD737 were used to compete for σ_1 receptor binding in the presence or absence of 10 μM GTPγS. After a 2 h incubation at 25°C, binding assays were terminated by addition of ice-cold Tris buffer and filtration through 0.1% polyethyleneimine-soaked Whatman GF/B filters using a BRANDEL cell harvester. Filters were then washed three times with ice-cold Tris-buffer, dried, and counted by liquid scintillation spectroscopy to measure bound radioactivity. Data were analyzed by the MacLigand 4.97 (ABC Software, Baltimore, MD, USA) and Prism 2.01 programs (GraphPad Software, San Diego, CA, USA).

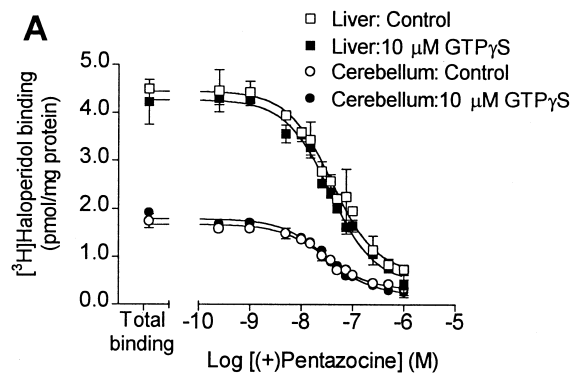
2.3. GTPase assays

GTPase assays were done using the methods of Cassel and Selinger (1976) and Koski et al. (1982). Rat cerebellar membranes were prepared by the same method as above except that the tissues were homogenized and resuspended in 10 mM Tris buffer (pH 7.5) with 0.1 mM EDTA and

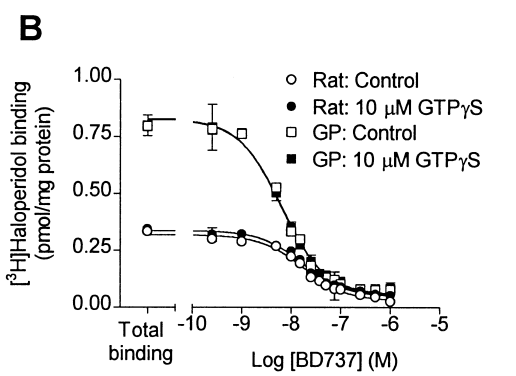
2.5 mM dithiothreitol. Membrane protein (2 μg) was added to each aliquot (100 μl) of reaction mixture containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 1 mM ATP, 200 μM App[NH]p, 1 mM ouabain, 10 mM creatine phosphate, 25 units/ml creatine phosphokinase, 500 nM GTP and ~15 nM [γ-^{32}P] GTP. The reaction was initiated by transferring the tubes to a 37°C water bath. After a 10-min incubation, tubes were returned to ice, and 0.9 ml of 20 mM phosphoric acid (pH 2.3, containing 5% activated charcoal) were then added to each tube. After centrifugation at 12,000 × *g* for 10 min, 200-μl aliquots of the supernatant were taken to measure radioactivity. GTPase activities were measured as the release of ^{32}Pi from [γ-^{32}P] GTP. Low-affinity GTPase activity was measured in the presence of 50 μM unlabeled GTP. High-affinity GTPase was calculated by subtracting the low affinity GTPase from the total GTPase activity.

2.4. [^{35}S]GTPγS autoradiography

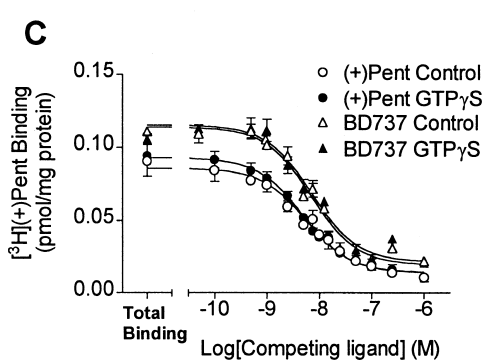
This was done following the method by Sim et al. (1995) and Selley et al. (1997). Male Sprague–Dawley rats were sacrificed by decapitation. The brains were immediately embedded into Tissue-Tek OCT compound on dry ice. Horizontal, sagittal or coronal sections (20 μm) were



	(+)Pentazocine K_i (nM)		
	Control	GTPγS	
GP liver	16 ± 3.9	12 ± 1.4	n=4
GP cerebellum	9.5 ± 1.0	14 ± 1.7	n=3



	BD737 K_i (nM)		
	Control	GTPγS	
GP cerebellum	1.9 ± 0.2	1.8 ± 0.2	n=2
Rat cerebellum	4.7 ± 0.7	4.1 ± 0.5	n=2



Competing ligand	K_i (nM)		
	Control	GTPγS	
(+)Pentazocine	6.3 ± 0.9	5.6 ± 0.8	n=3
BD737	4.2 ± 1.7	4.1 ± 1.2	n=2

Fig. 1. Effects of GTPγS on σ_1 receptor binding in rodent liver and cerebellar membrane preparations. (A) The competition binding curves of (+)pentazocine against [^{3}H]haloperidol in guinea pig (GP) liver and cerebellar membranes are not sensitive to GTPγS. (B) The competition binding curves of BD737 against [^{3}H]haloperidol in rat and guinea pig cerebellar membranes are not sensitive to GTPγS. To selectively label σ_1 receptors, 4 nM [^{3}H]haloperidol was used in the presence of 100 nM spiroperidol to block dopamine D_2 and serotonin 5-HT_2 receptor binding, and a 100 nM concentration of the σ_2 receptor antagonist BIMU-8 to block σ_2 receptor binding. (C) The competition curves of (+)pentazocine ((+)pent) and BD737 against [^{3}H](+)pentazocine in rat cerebellar membranes are not sensitive to GTPγS. Shown are representative competition binding curves from a single experiment with triplicates (mean ± S.D.) analyzed with the GraphPad Prism 2.01 program. Some filled symbols are obscured by open symbols due to very similar values. Where error bars can not be discerned, it is because the size of the error bar is smaller than the symbol. K_i values from pooled experiments of the indicated numbers (*n*) were determined with the MacLigand 4.97 program.

cut on a cryostat at −20°C and thaw-mounted onto gelatin-coated slides. Slides were then dried and stored at −80°C until use. Slides were first incubated in assay buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.1 mg/ml bovine serum albumin, pH 7.4) at 25°C for 10 min, and then in assay buffer containing 2 mM GDP for 15 min. Slides were then transferred to assay buffer containing 2 mM GDP and 80 pM [^{35}S]GTPγS. Adjacent sections were incubated with various drugs. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. After a 2-h incubation at 25°C, the slides were rinsed twice for 2 min each in ice-cold Tris buffer (50 mM Tris–HCl, pH 7.4), briefly dipped into deionized water, and dried by gentle blowing and suction. Slides were then dried overnight in a vacuum-sealed desiccator, and exposed to Amersham Hyperfilm-βMax films (Amersham Life Science, Arlington Heights, IL, USA) for 36–48 h. Films were digitized with a MTI camera (Amersham Research Analysis System) and analyzed with the Inquiry program (Loats Associates, Westminster, MD, USA), using Amersham ^{14}C standards for densitometric quantitation. All experiments were carried out in accordance with the guidelines and the approval of the George Washington University Institutional Animal Use and Care Committee.

3. Results

3.1. Radioligand binding studies

We studied the effects of GTPγS, the non-hydrolyzable analog of GTP, on the binding parameters of σ_1 receptor ligands in rodent liver and cerebellar membrane preparations. The σ_1 receptor is enriched in both preparations. To selectively label σ_1 receptors, we used 4 nM [^{3}H]haloperidol in the presence of 100 nM spiroperidol to block binding to dopamine D_2 and serotonin 5-HT_2 receptors, and 100 nM BIMU-8 to block σ_2 receptors. Non-specific binding measured in the presence of 1 μM haloperidol was usually 5–15% of total binding. (+)Pentazocine and BD737, two σ_1 receptor agonists, competed for [^{3}H]haloperidol binding in both liver and cerebellar membranes in a concentration-related manner, with 90% of competition occurring over 2 to 2.5 order of magnitude. Their K_i values, however, were not significantly changed in the presence of 10 μM GTPγS (Fig. 1A and B).

We also tested the effects of GTPγS on σ_1 receptor binding in rat cerebellar membranes using the radiolabeled agonist [^{3}H](+)pentazocine. Similar as the results above, K_i values of competing ligands (+)pentazocine and BD737 were not significantly changed in the presence of 10 μM GTPγS (Fig. 1C). Whether measuring against labeled antagonist or labeled agonist, the K_i values for (+)pentazocine and BD737 were consistent when compared within the same species. Interestingly, the total binding values of BD737 for [^{3}H]haloperidol binding (average 0.34 pmol/mg protein) were higher than those for [^{3}H](+)pentazocine binding (average 0.11 pmol/mg protein).

3.2. GTPase assays

We tested whether σ_1 receptor ligands could activate G protein-coupled receptors using an assay measuring the high-affinity GTPase activity (Cassel and Selinger, 1976; Koski et al., 1982) in rat cerebellar membrane preparations. The high-affinity GTPase activity was expressed as the ratio of drug-stimulated GTPase activity (in pmol/

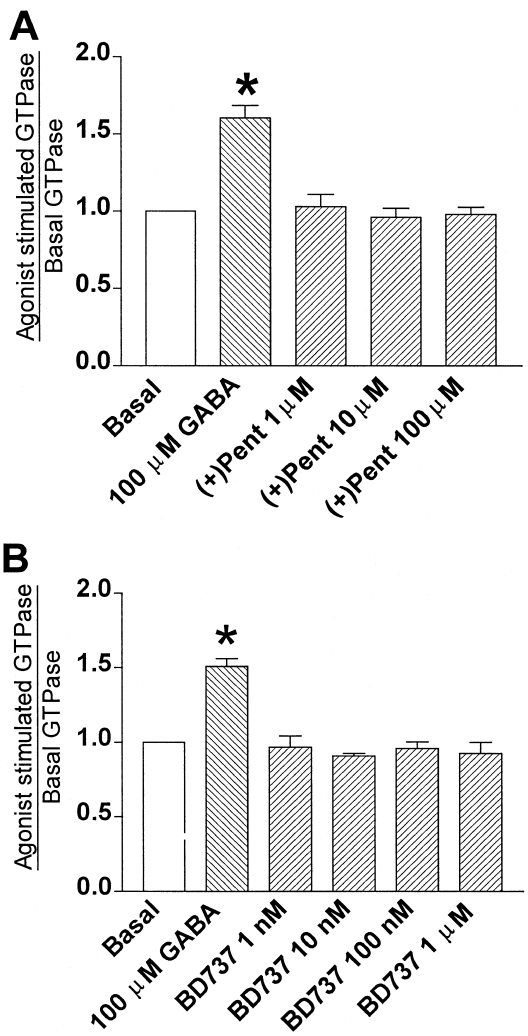


Fig. 2. Effects of σ_1 receptor agonists on the high-affinity GTPase activity in rat cerebellar membrane preparations. (A) Effects of increasing concentrations of (+)pentazocine ((+)pent). (B) Effects of increasing concentrations of BD737. The high-affinity GTPase activity was expressed as the ratio of drug-stimulated GTPase activity (in pmol/min/mg protein) over the basal (no drug added) GTPase activity. Data were mean ± S.E.M. from indicated number of experiments, with triplicates in each experiment ($n = 5$ except $n = 4$ for 1 nM BD737; $^*P < 0.01$ significantly higher than basal by one-way analysis of variance and post-hoc Dunnett's test).

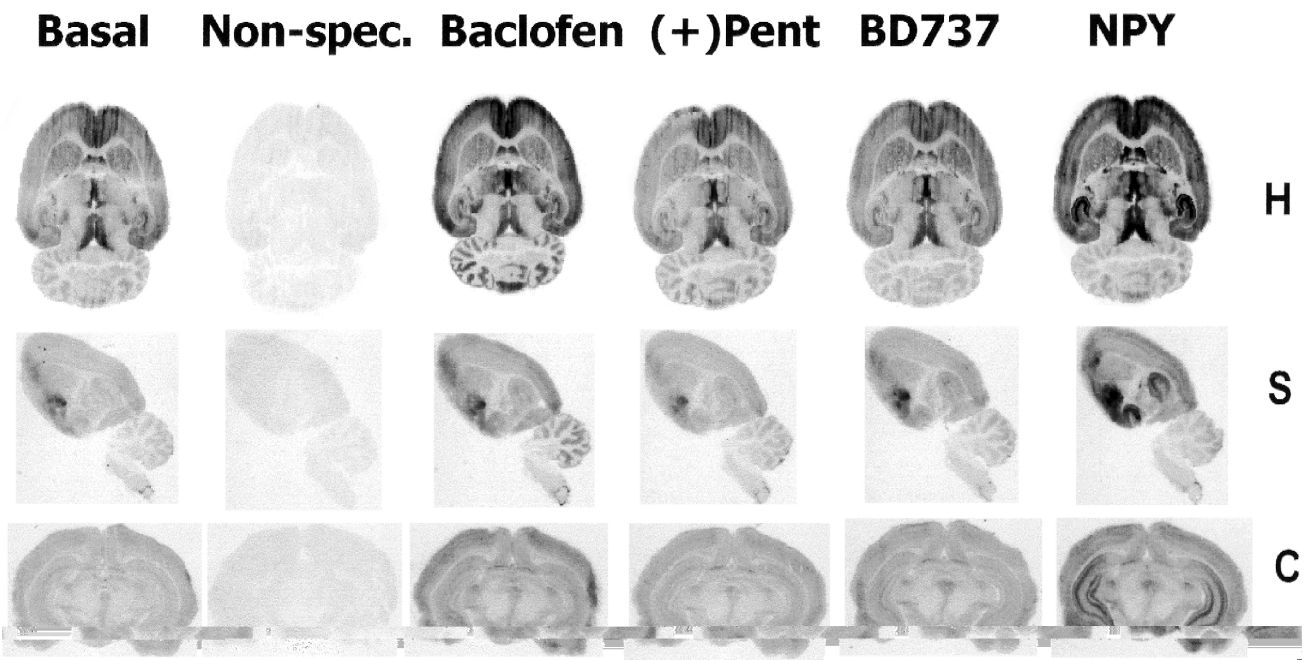


Fig. 3. Effects of (+)pentazocine, BD737, neuropeptide Y and baclofen on [^{35}S]GTPγS binding in rat brain sections. Adjacent sections were treated with different drugs. Concentrations of drugs used were: basal, no drug added; baclofen, 300 μM in the horizontal (H) section, 100 μM in the sagittal (S) and coronal (C) sections; (+)pentazocine ((+)Pent), 100 μM; BD737, 10 μM; NPY (neuropeptide Y), 500 nM in H section, 200 nM in S and C sections. Non-specific binding (non-spec.) was measured in the presence of 10 μM unlabeled GTPγS. Representative autoradiograms from five rat brains with triplicate sections in each. The light gray rectangle areas are due to film background.

min/mg protein) over basal (no drug added) GTPase activity. Gamma-aminobutyric acid (GABA) was used as a control stimulator since it activates the $GABA_B$ receptor, which is a known G protein-coupled receptor. At concentrations saturating the σ_1 receptor, (+)pentazocine up to 100 μM and BD737 up to 1 μM did not stimulate the high-affinity GTPase activity in rat cerebellar membrane preparations (Fig. 2A and B).

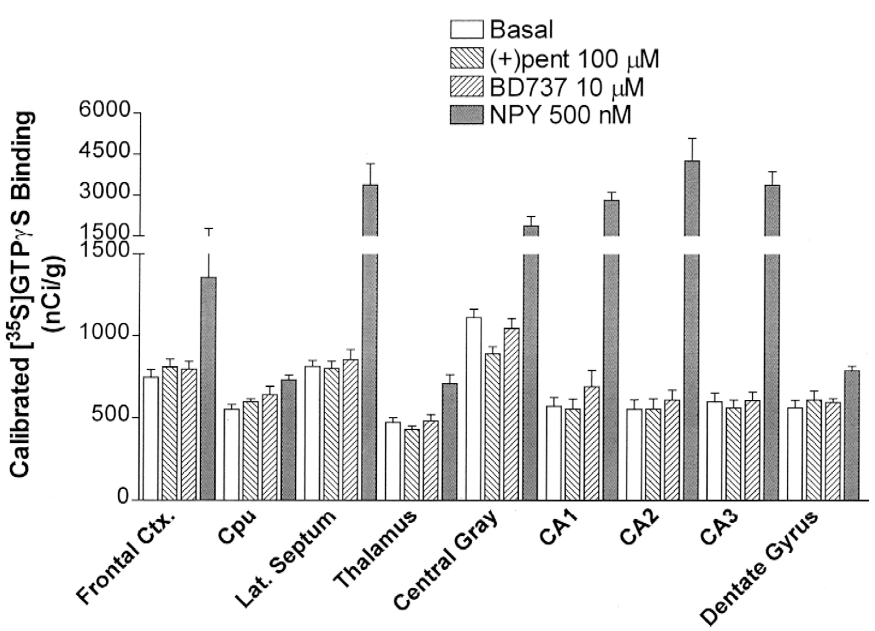


Fig. 4. Summarized quantitative results from the horizontal sections in Fig. 3. The digitization and quantitation of autoradiograms were done as described in Methods. The optic density of non-specific sections was subtracted from those of basal and drug-treated sections. Data are expressed as nanocurie/gram of tissue. Shown are mean values ± S.E.M. from triplicate sections from two rat brains. Frontal Ctx.: frontal cortex; Cpu: caudate putamen; Lat. Septum: lateral septum; CA1–3: hippocampal CA1, CA2 and CA3 regions.

3.3. [^{35}S]GTPγS autoradiography

The effects of σ_1 receptor agonists on G protein activation were again tested using [^{35}S]GTPγS binding as measured by autoradiography. Compared with the basal level, concentrations up to 100 μM (+)pentazocine or 10 μM BD737 did not significantly stimulate [^{35}S]GTPγS binding in any of the brain regions (two-way analysis of variance with Bonferroni post test performed using GraphPad Prism 3.00). Neuropeptide Y and baclofen were tested as drugs for positive control. Acting on G protein-coupled neuropeptide Y receptors, 200 nM neuropeptide Y substantially stimulated [^{35}S]GTPγS binding in areas including the CA1, CA2 and CA3 regions of the hippocampus, the superficial layers of cerebral cortex, the lateral septum and pontine central gray. Baclofen, a $GABA_B$ receptor agonist, stimulated [^{35}S]GTPγS binding in the superficial layers of cortex and molecular layer of the cerebellum (Figs. 3 and 4)

4. Discussion

We have previously shown that σ_1 receptor ligands regulate the release of NMDA-stimulated [^{3}H]-labeled dopamine and norepinephrine from rat striatal and hippocampal slices (Gonzalez-Alvear and Werling, 1995a,b). We have also shown σ_1 receptor ligands modulate [^{3}H]-labeled arachidonic acid release from rat cerebellar granule cells (Starr and Werling, 1994). In our system, (+)pentazocine and BD737 were identified as σ_1 receptor agonists, whereas haloperidol, Dup734 and DTG were identified as σ_1 receptor antagonists. It was reported that σ_1 receptor ligands such as (+)pentazocine and haloperidol modulated NMDA-induced neuronal electrical activities in dorsal hippocampal pyramidal cells (Monnet et al., 1990). The signaling pathways through which σ_1 receptor ligands elicited these effects have not yet been thoroughly investigated.

In an early radioligand binding study, competition curves of (+)SKF 10,047 and (+)-3-PPP against [^{3}H](+)-3-PPP in rat brain membranes suggested high and low affinity binding sites. The high affinity site was abolished by 0.1 mM guanosine 5′-(β,γ-imino-triphosphate) (Gpp[NH]p). Pertussis toxin appeared to abolish the effect of Gpp[NH]p on σ receptor ligand binding (Itzhak, 1989). Thus, the σ_1 receptor was hypothesized to be coupled to G proteins. However, potential problems associated with σ receptor ligands available at that time include their actions at other non-σ receptor sites, as well as their lack of selectivity on subtypes of σ receptors.

Recently, a σ_1-binding protein was cloned from a guinea pig liver cDNA library (Hanner et al., 1996). The σ_1-binding protein, expressed in both central nervous system and peripheral organs, has a very similar binding profile with the σ_1 receptor. However, this σ_1-binding protein has a putative single transmembrane-domain structure, different from the seven transmembrane-domain structure shared by G protein-coupled receptors. This is apparently inconsistent with the conclusions from early radioligand binding studies. Although the σ_1-binding protein has not been proven to be a pharmacologically functional receptor, its binding profile strongly suggests that it is a ligand-binding component of the σ_1 receptor that may comprise other auxiliary subunits. If the σ_1 receptor is coupled to G proteins, as hypothesized by early radioligand binding studies, it has a novel signaling mechanism since examples of direct coupling between a multi-subunit receptor and G proteins have not been reported to our knowledge. We thought that it was necessary to examine the coupling between the σ_1 receptor and G proteins in light of recent advances in σ_1 receptor research. This has been facilitated by the development of selective σ_1 receptor ligands that we have used in the current study.

We first investigated the coupling between the σ_1 receptor and G proteins by examining whether σ_1 receptor binding was sensitive to GTPγS. As shown previously in work from our lab, σ_1 receptor binding was enriched in membrane preparations of rat cerebella. Two σ_1 receptor-selective agonists, (+)pentazocine and BD737 competed for [^{3}H]haloperidol binding with inhibition constants of 10 and 5 nM, respectively (Gonzalez-Alvear et al., 1995). To detect possible different characteristics of σ_1 receptor binding in the central nervous system and peripheral organs, we used both rat cerebellar membrane and guinea pig liver membrane preparations. To selectively label the σ_1 receptor with [^{3}H]haloperidol, we used 100 nM spiroperidol to block dopamine D_2 and serotonin 5-HT_2 receptors, and 100 nM BIMU-8, a σ_2 receptor antagonist with a K_i of 20 nM (Bonhaus et al., 1993), to block σ_2 receptors. To selectively label σ_1 receptor binding with [^{3}H](+)pentazocine, 100 nM BIMU-8 was also included to block σ_2 receptors.

We found that the binding parameters of two σ_1 receptor agonists, (+)pentazocine and BD737 were not altered by the addition of GTPγS in rodent cerebellar membrane preparations using either the antagonist [^{3}H]haloperidol or the selective σ_1 receptor agonist [^{3}H](+)pentazocine as a radioligand. This was different from the results of earlier radioligand binding studies in rodent brain membrane preparations, in which (+)-3-PPP and (+)-SKF 10,047 were used to compete for [^{3}H](+)-3-PPP labeled σ receptor binding. Whereas (+)-3-PPP and (+)-SKF 10,047 might not differentiate subtypes of σ receptors, we used (+)pentazocine and BD737 which have higher affinities at σ_1 than σ_2 sites. These two drugs have also been identified as σ_1 receptor agonists in neurotransmitter release assays used extensively in our lab. Consistent with our results, another study done in guinea pig brain membrane preparations showed that the binding of [^{3}H](+)pentazocine was not affected by the addition of guanine nucleotides (DeHaven-Hudkins et al., 1992). Although the binding of [^{3}H]haloperidol and [^{3}H](+)pentazocine was

done under conditions which should have limited their access to only σ_1 receptors, the total binding values for [^{3}H]haloperidol was higher than those of [^{3}H](+)pentazocine (compare Fig. 1B and C). This could indicate that haloperidol labeled more than one population of receptors defined as σ_1, and that (+)pentazocine had access to only about one-third of these receptors. In guinea pig liver membranes, we also did not observe significant changes of binding parameters of (+)pentazocine and BD737 in the presence of GTPγS. It appeared that there was a higher density of σ_1 receptors in guinea pig liver membranes than in guinea pig cerebellar and rat cerebellar membranes.

Because GTPγS did not alter the binding properties of σ_1 receptor ligands in membrane preparations, direct coupling between the σ_1 receptor and G proteins was not demonstrated. A second approach measuring agonist-stimulated GTPase activities in membrane preparations was used to confirm our results. (+)Pentazocine and BD737 at saturating concentrations did not significantly stimulate GTPase activities above basal levels in rat cerebellar membranes, suggesting that no significant activation of G proteins or increase of GTP turnover rates were induced by these two σ_1 receptor agonists. In contrast, 100 μM GABA, acting through the G protein-coupled $GABA_B$ receptor, significantly stimulated GTPase activities in the same membrane preparations. These results again suggested that the σ_1 receptor might not be directly coupled to G proteins.

Since radioligand binding and GTPase assays were done in homogenized membrane preparations suspended in artificial buffers, the integrity of membrane structures would be inevitably compromised. This could make it difficult to detect a GTP effect in membrane binding and an increase of GTPase by agonists. We then used the method of [^{35}S]GTPγS autoradiography. In this method, the coupling of receptor and G proteins is tested in situ, keeping membrane structures more intact. Distinct patterns of stimulation of [^{35}S]GTPγS binding caused by G protein activation were observed in rat brain slices when baclofen and neuropeptide Y were applied to activate G protein coupled $GABA_B$ and neuropeptide Y receptors. The distribution of activated G proteins correlated well with those of $GABA_B$ receptors and neuropeptide Y receptors. Saturating concentrations of (+)pentazocine and BD737, however, did not produce substantial stimulation of [^{35}S]GTPγS binding above the basal level in any of the brain regions. Therefore, consistent results from three different assays showed that a direct coupling between σ_1 receptors and G proteins could not be demonstrated.

Studies on signaling pathways of the σ_1 receptor have produced mixed results. Different from our GTPase assay results, a small increase of GTPase activity and stimulation of [^{35}S]GTPγS binding by (+)pentazocine in membrane preparations of mouse brain prefrontal cortices were previously reported (Tokuyama et al., 1997, 1999). The stimulation of [^{35}S]GTPγS binding by (+)pentazocine was shown abolished after pertussis toxin treatment, and recovered after reconstitution with G_{i1} protein. However, (+)pentazocine as high as 10 μM produced only 10% stimulation of GTPase activities above basal levels. In an electrophysiological study testing the modulation of NMDA response in dorsal hippocampal pyramidal neurons, effects of (+)pentazocine was shown to be insensitive to pertussis toxin pretreatment (Monnet et al., 1994). In another electrophysiological study done in frog pituitary melanotrope cells, a negative modulation of potassium A-current by (+)pentazocine was shown to be sensitive to cholera toxin treatment (Soriani et al., 1999). It appeared that cholera toxin-sensitive G_s subtype G proteins were involved in the effect of (+)pentazocine in this case. However, it was reported that (+)pentazocine as high as 10 μM had no effect on either basal or forskolin-stimulated cAMP levels in human neuroblastoma BE(2)-C cells (Ryan-Moro et al., 1996). In DMS-114 tumor cells, σ receptor ligands SKF 10,047 and DTG were reported to inhibit voltage-activated potassium currents in patch clamp experiments. This inhibition was not affected by perfusion of GTP (Wilke et al., 1999), suggesting G proteins were not involved.

In our study, we did not find evidence that the σ_1 receptor was directly coupled to G proteins. However, the possibility of involvement of G proteins in σ_1 receptor signaling could not be ruled out. An early study reported that σ_1 ligands (+)pentazocine and haloperidol inhibited carbachol-stimulated phosphoinositide turnover in rat brain membrane preparations (Bowen et al., 1988). (+)Pentazocine, DTG and haloperidol were shown to attenuate NMDA-induced calcium increase in primary cultures of rat cortical neurons (Hayashi et al., 1995; Klette et al., 1997). In cultured rat cardiac myocytes, nanomolar concentrations of (+)pentazocine and BD737 were shown to affect the amplitude of cell contraction and calcium transients. BD737 caused a rapid increase of inositol 1,4,5-triphosphate production in these cells (Ela et al., 1994; Novakova et al., 1998). An recent report showed that activation of σ_1 receptors by (+)pentazocine decreased phospholipase C-mediated hypoglossal activity and induced cellular translocation of protein kinase C isoforms in isolated guinea pig brain stem preparations (Morin-Surun et al., 1999). All these studies suggested that certain cellular signal transduction components and possibly G proteins were involved upon σ_1 receptor activation.

The cloned σ_1-binding protein is a small 25-kDa polypeptide with no homologous regions with known phosphorylation or protein interaction domains. Although it shares 40% homology with the fungal sterol isomerase, it exhibits no isomerase activity (Hanner et al., 1996). Its putative single transmembrane-domain structure is different from the characteristic seven transmembrane-domain structure shared by G protein-coupled receptors. Considering its small size and distinct structure, it is tempting to speculate that the cloned σ_1-binding protein may be a part of a multi-subunit complex that is functional as a whole.

Two early studies in guinea pig and rat brain membranes using [^{3}H]azido-DTG as a photo-affinity ligand specifically labeled a 29-kDa polypeptide under denaturing conditions and a larger complex (150 kDa or more) under non-denaturing conditions (Kavanaugh et al., 1988; Schuster et al., 1995). The 29-kDa polypeptide was suggested to be a subunit of the large complex. It has yet to be determined whether this 29-kDa protein is the same as the σ_1-binding protein identified using [^{3}H](+)pentazocine as a photo-affinity ligand in guinea pig. The real σ_1 receptor is very likely a multi-subunit receptor including the σ_1-binding protein serving as the ligand-binding subunit, and other subunits involved in signal transduction. It is worth noting that the σ_1-binding protein has been found located in endoplasmic reticular membranes and nuclear membranes (Dussossoy et al., 1999), as well as plasma membranes. If the σ_1-binding protein is associated with different proteins in these membrane structures, different functions could be served. Translocation of the σ_1-binding protein itself would possibly serve as a signal for cellular processes.

In summary, we have shown that the σ_1 receptor is not directly coupled to G proteins in this study. To exert its physiological functions, the σ_1-receptor may employ a unique signal transduction pathway, which awaits further elucidation.

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