

Modulation of the GABA_A Receptor by Srf in Rat Thalamus

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Received 2012-08-20, Revised 2012-09-08; Accepted 2012-09-14

ABSTRACT

Somatostatin has been reported to modulate GABA_A receptor complex in many brain structures. This study was conducted to investigate somatostatin modulation of the GABA_A receptor binding in several rat diencephalic structures, focusing primarily on the thalamus, as this structure plays important roles. Animals were assigned to control conditions. Changes in specific binding of GABA_A receptor as labelled with [³⁵S]-Butylbicyclophosphorothionate (TBPS) were assessed by in vitro quantitative autoradiography with the aid of a computer assisted image analysis system. Our results reveal the presence of higher densities in several thalamic structures located principally in the part of thalamus. We demonstrate for the first time the presence of a modulatory effect of somatostatin on the GABA_A receptor complex in this brain region in rats. Indeed, the peptide affected in a concentration-dependent manner; the binding of [³⁵S]-Tertiary Butylbicyclophosphorothionate (TBPS) to the convulsant site of the GABA_A receptor complex in thalamic structures with an affinity in the micromolar range (10^{-3} to 3.10^{-6} M). The inhibitory effect of somatostatin is observed in all thalamic structures analyzed. The absence of a specific region effect of somatostatin on the binding of [³⁵S]-TBPS, suggests the presence of a homogenous subunit GABA_A receptor composition. Furthermore, GABA and muscimol, a GABA_A receptor agonist, enhanced the affinity of somatostatin effect on [³⁵S]-TBPS. This suggests that somatostatin allosterically modifies [³⁵S]-TBPS binding through a mechanism similar to that of GABA.

Keywords: Somatostatin, In Vitro Quantitative Autoradiography, [³⁵S]-Tbps Binding, GABA_A Receptor Complex

1. INTRODUCTION

Gamma-Amino Butyric Acid (GABA) is widely accepted as an inhibitory neurotransmitter in the mature central nervous system (Purves *et al.*, 2012; Bowery and Smart, 2006). GABA-mediated inhibition passes via two major types of receptors, GABA_A and GABA_B receptors (Mehta and Ticku, 1999; Bowery, 2010). GABA_A are hetero-oligomeric ligand-gated ion channel proteins. They are distributed widely in the central nervous system of many vertebrate species (Edgar and Schwartz, 1990; Fubara *et al.*, 1996). These receptors are primarily

responsible for fast inhibitory neurotransmission in the central nervous system (Macdonald and Olsen, 1994). One of the unique features of these receptors is that, in addition to the GABA binding site, the receptor has several allosteric modulatory sites. Indeed, they are regulated by many positive and negative allosteric modulators including neurotransmitters, neuromodulators and neurohormones (Majewska, 1992; Sapp *et al.*, 1992; Macdonald and Olsen, 1994; Sieghart, 2012; Huidoboro-Toro *et al.*, 1996). Interestingly, we reported for the first time that a neuropeptide i.e., somatostatin, was able to modulate allosterically the

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GABA_A receptor complex. Indeed, the neuropeptide induced a dose-dependent inhibition of [³⁵S]-Tertiary Butylbicyclophosphorothionate (TBPS) binding in several forebrain regions in rats (Vincens *et al.*, 1998; Chigr *et al.*, 1999; 2002). However, the effects of somatostatin on GABA_A receptors in other brain regions remain to be established. One region that has not been closely studied is the thalamus. This is surprising since this structure plays important functional roles (Saalman and Kastner, 2011; Wang *et al.*, 2011). Furthermore, this brain region is enriched in somatostatin containing neuronal elements (Wang *et al.*, 2000) GABA (Geis and Borst, 2012) and GABA receptors (Edgar and Schwartz, 1990; Halonen *et al.*, 2009). Somatostatin and GABA_A receptors in this region play important roles in a variety of physiological functions (Leresche *et al.*, 2000; Wang, 2011; Wang *et al.*, 2011). Therefore, the principal goal of the present study was the assessment of somatostatin effects on the *in vitro* autoradiographic labeling of [³⁵S]-TBPS site on the benzodiazepine/GABA chloride ionophore receptor complex by receptor autoradiography in rat thalamic structures.

2. MATERIALS AND METHODS

2.1. Animals

16 Male Wistar rats (200-220 g) were housed 4-5 per cage in an animal room maintained at 22±2 and 50±10%, relative humidity and on a 12light/12 dark cycle. Food and water were available *ad libitum*. The rats were sacrificed by decapitation, their brains rapidly removed. The brains were then frozen in isopentane and dry ice and stored at -70°C until histological sectioning. 20 µm coronal sections were cut in relevant areas using a cryomicrotome (Frigocut 2800, Reichert Jung) at -20°C, thaw-mounted onto 0,5% gelatin-coated, dried overnight in a desiccator at 4°C and stored at -80°C until required.

2.2. In Vitro Quantitative Autoradiography

For autoradiography procedures, on the day of the assay, the slides were brought to room temperature and preincubated at the same temperature in 50 mM Tris-HCl (pH 7.4) for 30 min. Subsequently sections were rapidly dried in ambient air and drop incubated for 180 min at room temperature, in the same buffer supplemented with 500 mM NaCl, 10⁻⁴ M ascorbic acid and 3 nM [³⁵S]-Tertiary Butylbicyclophosphorothionate (TBPS) (specific activity 100-400 Ci/mmol, purchased from new England Nuclear, USA). The non-specific component of the total binding was determined by incubating adjacent

brain sections in the presence of 10⁻⁵ M TBPS (Sigma Chemical Co; USA). For the assessment of effects of somatostatin (Sigma Chemical Co, USA), other adjacent sections were incubated in the presence or the absence of the GABA or the neurosteroid 5 α-pregnane-3αol-one (5α3αP; Sigma Chemical Co, USA). At the end of the incubation period, slides were sequentially washed 3 times (5 min each) in fresh 50 mM Tris-HCl buffer at 4°C (pH 7.4) dipped in distilled water to remove salts and air-dried out at room temperature. Dried radiolabelled sections were placed in X-ray cassettes (Kodak), apposed against tritium-sensitive film ([³H]-Hyperfilm, Amersham) for 3-4 days. Films were then processed and developed 2 min in Kodak D 19, fixed 4 min in Kodak rapid fixer and rinsed 1 h under tap water. The selected thalamic structures in film autoradiograms were quantified by densitometry using a computerized "Biocom" (les Ulis, France) image analysis system. Data were measured in relative optical density units. These were quantified using radiolabelled standards. For each thalamic structure analyzed, [³⁵S] TBPS binding was measured in 5-6 sections of each animal and the mean of these values represent a single determination. Non specific labeling density averaged less than 5% of total [³⁵S] TBPS total binding in the present experimental conditions and was systematically subtracted from optical density readings.

3. RESULTS

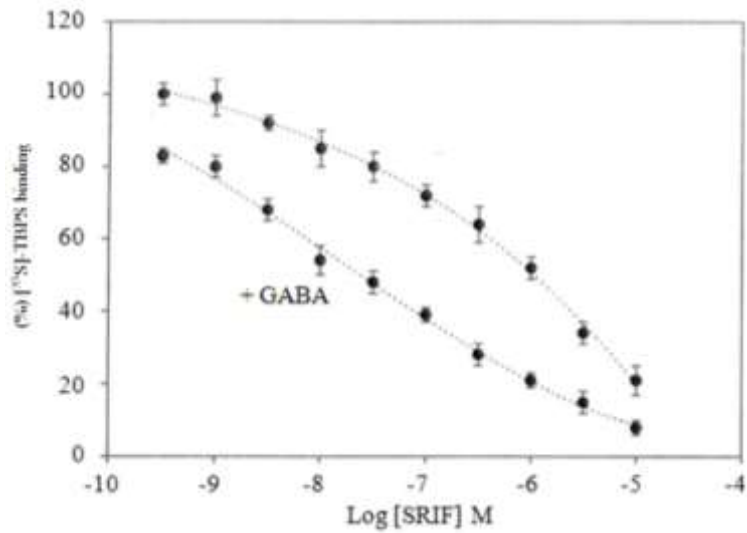
At the concentration of [³⁵S]-TBPS used in the present study, considerable amounts of the autoradiographic labeling were observed in the different thalamic structures investigated in rats. The quantitative analysis of autoradiographic images showed the presence of significantly high densities of [³⁵S]-TBPS specific binding in the anterior and in the ventral nuclei. Thus high densities were present in thalamic nuclei located in the medial part.

Under the experimental conditions used in this investigation, somatostatin inhibited [³⁵S]-TBPS specific binding in a dose-dependent manner in the rat thalamic structures (**Fig. 1**). The maximum of inhibition in [³⁵S]-TBPS binding i.e., 83.5±2.37% (mean ±SEM, n = 7) was obtained at 10⁻⁵M somatostatin, the highest concentration used. Within the thalamic structures investigated, no significant interregional differences were evidenced for somatostatin inhibition of [³⁵S]-TBPS specific binding. Thus, somatostatin inhibited [³⁵S]-TBPS binding with IC₅₀ values in the micromolar range (1.3 10⁻⁶ to 2.3 10⁻⁶ M) (**Table 1**). Scatchard analysis of the displacement curves obtained with

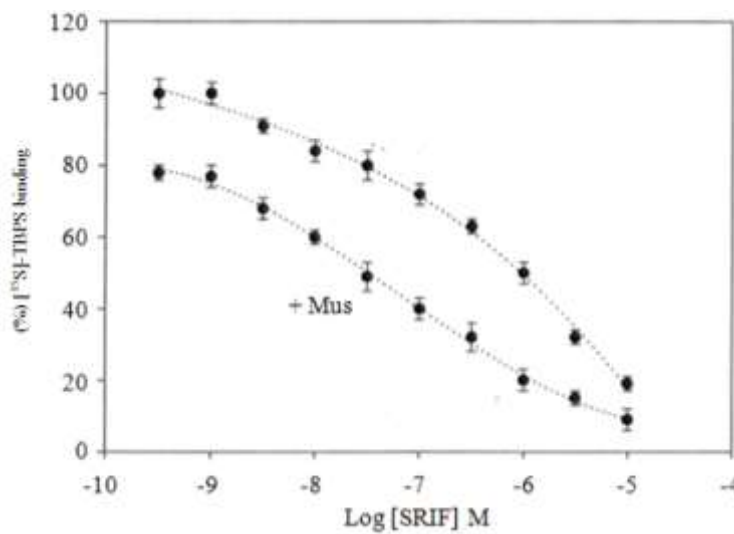
somatostatin evidenced the presence of a single high affinity site (data not shown). The apparent Dissociation constant (KD) is in the average of 29 ± 4 nM. The inhibitory effect of somatostatin on the [³⁵S]-TBPS binding was not nucleus specific in the thalamic region. The IC₅₀ values did not show significant differences between the thalamic structures analyzed (**Table 1**).

In order to determine whether the inhibitory effect of SRIF occurs by acting at the GABA_A receptor, we tested the actions of GABA and muscimol (a GABA_A receptor agonist). Furthermore, we assayed the effect of a positive modulator of GABA_A receptor complex, i.e., the

neurosteroid 5α3αP to determine any cooperativity between somatostatin and neurosteroids. Thus, in the presence of GABA (10^{-6} M) in the medium incubation, the effect of somatostatin on the binding of [³⁵S]-TBPS is more pronounced (**Fig. 1a**). Significant decrease of [³⁵S]-TBPS specific binding was observed in all the thalamic structures analyzed in rats and for all the somatostatin concentrations used (**Fig. 1a**). The IC₅₀ values were decreased tenfold ($0.12 \cdot 10^{-6}$ M– $0.23 \cdot 10^{-6}$ M) (**Table 1**). The addition of a muscimol, to the medium incubation at a concentration of 10^{-6} M, enhances also the dose-dependent inhibitory effect of somatostatin on [³⁵S]-TBPS binding.



(a)



(b)

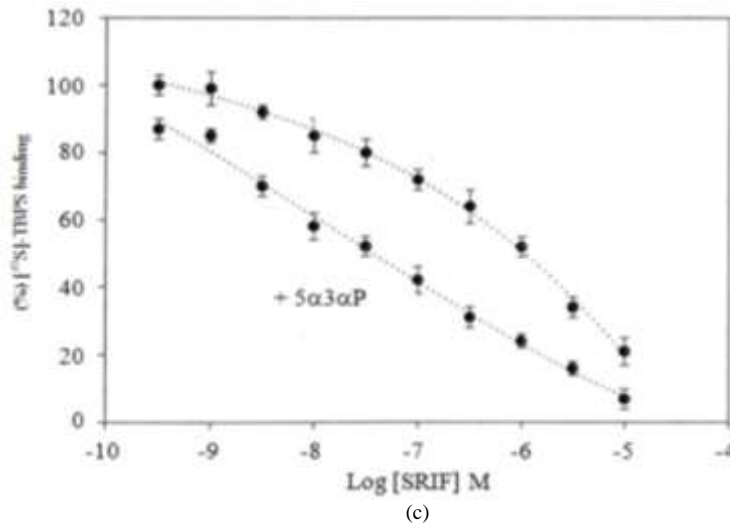


Fig. 1. Effect of somatostatin alone (upper curves in a-c) or in the presence of GABA 1µM (Panel a), Muscimol 1µM (Panel b) and the neurosteroid 5α3αP 1µM (Panel c) on the specific binding of [³⁵S]-TBPS binding in rat mediodorsal thalamic nucleus. Brain sections containing the thalamic level were incubated at room temperature for 180 min with 2nmol/L [³⁵S]TBPS and various concentrations of somatostatin (10⁻¹⁰ to 10⁻⁵ mol/L) in the absence or the presence of the modulators cited. For all experiments performed, each point corresponds to the mean ± S.E.M. of 8-10 densitometric measurements (4-6 independent experiments) and is expressed as a percentage of maximal [³⁵S] (TBPS) binding in the absence of drugs.

Table 1. Effect of GABA, muscimol and the neurosteroid 5α-pregnane-3αol-one on somatostatin modulation of [³⁵S]-tert-butylcyclophosphonothionate binding to different thalamic nuclei.

Thalamic structure	+STIF	+SRIF +GABA (10 ⁻⁶ mol/L)	IC ₅₀ (µmol/L) +muscimol (10 ⁻⁶ mol/L)	+SRIF +5α3αP (10 ⁻⁶ mol/L)
Angular thalamic nucleus	3.3±0.4	0.23±0.02*	0.15±0.01*	0.13±0.02*
Central medial thalamic nucleus	1.3±0.1	0.21±0.02*	0.25±0.03*	0.23±0.03*
Centrolateral thalamic nucleus	2.3±0.4	0.23±0.04*	0.19±0.01*	0.14±0.02*
Laterodorsal thalamic nucleus	1.8±0.3	0.26±0.02*	0.16±0.03*	0.17±0.04*
Mediodorsal thalamic nucleus	1.3±0.1	0.15±0.01*	0.19±0.01*	0.19±0.02*
Paracentral thalamic nucleus	2.1±0.1	0.26±0.04*	0.18±0.01*	0.15±0.01*
Posterior thalamic nucleus	1.8±0.3	0.19±0.01*	0.21±0.04*	0.23±0.03*
Submedial thalamic nucleus	1.4±0.2	0.21±0.02*	0.22±0.03*	0.24±0.02*
Ventrolateral thalamic nucleus	1.3±0.2	0.25±0.01*	0.13±0.03*	0.21±0.03*
Ventromedial thalamic nucleus	1.8±0.3	0.12±0.02*	0.19±0.02*	0.16±0.02*
Ventral posteriolateral nucleus	1.7±0.2	0.15±0.02*	0.17±0.03*	0.11±0.02*
Reticular thalamic nucleus	1.6±0.4	0.13±0.04*	0.18±0.03*	0.18±0.02*
Rhomboid nucleus	1.9±0.3	0.24±0.02*	0.19±0.01*	0.12±0.01*

Data are the mean ± SEM of six animals from four to six independent experiments. Each experiment was repeated three times. *P<0.01 compared with the effect of SRIF only, in post hoc analyses. Specific binding of [³⁵S]-tert-butylcyclophosphonothionate (TBPS) was measured in the presence of increasing concentrations of somatostatin (SRIF) in the absence or the presence of GABA, muscimol or 5α-pregnane-3αol-one (5α3αP) under equilibrium. In each case, the concentration required to achieve 50% of TBPS binding (IC₅₀) was determined.

The IC₅₀ values are in the range of 0.15 10⁻⁶ M to 0.22 10⁻⁶ M (Fig. 1b, Table 1). Equivalent pattern concerning the allosteric modulation of the tetradecapeptide was obtained when the neurosteroid 5α3αP (10⁻⁶ M) was added to incubation medium (Fig. 1c). Similarly, the IC₅₀

values were decreased tenfold (0.11 10⁻⁶ M–0.24 10⁻⁶ M) (Table 1). Taken together, these data show that in all experimental situations, the dose-dependent inhibition effect of somatostatin on [³⁵S]-TBPS binding is enhanced tenfold and the average of is around of 10⁻⁷ M.

4. DISCUSSION

The present study was designed to investigate the regional distribution of [³⁵S] TBPS binding sites and to assess the effect of the tetradecapeptide somatostatin on the GABA_A receptor complex in the rat brain diencephalon and particularly in the thalamus. [³⁵S]-TBPS was chosen as the ligand in this study for the following reasons. First, [³⁵S]-TBPS is an ideal ligand for autoradiographic studies, because of its high specific activity, low non-specific binding, high resolution in autoradiographic analysis (Giorgi *et al.*, 1994; Atack *et al.*, 2007) and convenient quantification using commercially available standards. Second, modulation of [³⁵S]-TBPS binding by many positive-GABA agonists such as neurosteroids has been used as of a highly of allosteric interactions of neurosteroids with the GABA_A receptor (Vincens *et al.*, 1992; 1993; Atack *et al.*, 2007; Halonen *et al.*, 2009).

We have found that [³⁵S]-TBPS bound in slide-mounted rat diencephalon to a single class of receptors with an equilibrium-dissociation KD value of 29±4 nM. The kinetic parameters and pharmacological specificity measured in mounted-tissue sections obtained from rat thalamic level are quite similar to those found with slide mounted cerebral cortex in rat (Edgar and Schwartz 1990; Chigr *et al.*, 2002). Furthermore, these values are consistent with data obtained with cerebral homogenate membrane preparations (Atack *et al.*, 2007).

The present study examined the effects of the tetradecapeptide somatostatin on the in vitro autoradiography [³⁵S]-TBPS binding at or near the chloride channel. This investigation represents the first detailed investigation of somatostatin modulation of [³⁵S]-TBPS binding to GABA_A receptors in the rat thalamus. Indeed, a gradually and significant decrease in the density of [³⁵S]-TBPS binding sites, in the rat thalamic structures, following the addition of increasing concentrations of somatostatin was observed (**Fig 1a**). The potentiating effect of exogenous GABA or the GABA_A receptor agonist, muscimol, required to produce this effect could suggest that somatostatin exerts its effects with more potent facilitatory effects which were GABA sensitive. These finding add experimental support to the multiplicity of allosteric modulators of the different components of the GABA_A receptor complex.

The inhibition of [³⁵S]-TBPS binding observed at higher concentrations of somatostatin, may represent a direct activation of the GABA-dependent Cl⁻ channels, as suggested for some neuromodulators acting at the GABA_A receptor complex (Sieghart, 2012; Samochocki

and Stroszndjer, 1993; Concas *et al.*, 1994; Quinn and Harris, 1995; Martin *et al.*, 1996; Sanna *et al.*, 1996). This suggestion is consistent with the evidence that somatostatin-induced inhibition of [³⁵S]-TBPS binding, is blocked by bicuculline (Concas *et al.*, 1994). Furthermore, the fact that in the presence of exogenous GABA, low concentrations of somatostatin were able to decrease [³⁵S]-TBPS binding suggests that in the absence of GABA, these low concentrations do not directly open the GABA_A receptor-operated Cl-channel as is the case for other neuromodulators (Concas *et al.*, 1994). It appears from our experiments that the tetradecapeptide, affects the [³⁵S]-TBPS binding in all thalamic structures analyzed. Furthermore, the peptide inhibition of the [³⁵S]-TBPS binding is not region specific in thalamus. These data are in good accord with previous findings describing neurosteroids in forebrain regions in different mammalian species (Vincens *et al.*, 1993). Such homogeneity suggests that the peptide binding site and the barbiturate binding site belong to the same functional entity and presumably are not depending on the demonstrated molecular heterogeneity of brain GABA_A receptors (Levitan *et al.*, 1988; Simon *et al.*, 2004). This absence of brain regional differences may favor the efficacy of allosteric coupling between GABA and channel sites, which could indicate the absence of differences in the agonist efficacy. This could be due to gamma 2 subunit, responsible for the coupling between GABA and ion channel. Indeed, a deficit in this subunit implies a deficient coupling (Sinkkonen *et al.*, 2004). Furthermore, it well known that the gamma subunit is highly sensitive to GABA and neurosteroids (Brown *et al.*, 2002; Smith *et al.*, 2007). These two compounds inhibit the [³⁵S]-TBPS binding in similar manner to somatostatin (Chigr *et al.*, 2002). These findings are different from previous data showing that a proportion of GABA_A receptors in the thalamus displaying atypical allosteric coupling between the agonist and channel sites: the so-called GABA-insensitive [³⁵S]-TBPS binding (Sinkkonen *et al.*, 2001a; 2001b; 2004; Halonen *et al.*, 2009).

It is well known that the GABA_A receptors can either be synaptic or extrasynaptic (Farrant and Nusser, 2005). The extrasynaptic receptors consist of specific subunit combinations including gamma subunit and are preferentially located in several brain regions including thalamus (Cope *et al.*, 2005; Jia *et al.*, 2005). This argues strongly that somatostatin modulates principally extrasynaptic GABA_A receptors at the thalamic level. Of interest, these receptors are responsible of tonic inhibition of GABA (Farrant and

Nusser, 2005). Based on these results and the results we reported previously (Chigr *et al.*, 1999; 2002), we postulate that somatostatin modulates both synaptic and extrasynaptic GABA_A receptors in the central nervous system, with the same efficacy.

Inhibition of [³⁵S] TBPS binding by various concentrations of somatostatin and GABA yielded dose-response curves that were similarly shaped suggesting that the neuropeptide allosterically affects [³⁵S]-TBPS binding through a mechanism similar to that of GABA (Chigr *et al.*, 2002). However, other interpretations concerning the mechanism of somatostatin modulation of [³⁵S]-TBPS binding could be also exist with regard to the specific peptide receptors. Indeed, the pharmacological action of somatostatin on GABA_A receptor presents several similarities with other several neurotransmitters which influence the heterooligomeric complex. These neurotransmitters modulate the activity of GABA_A receptor complex via the different signal transduction pathways (Huidoboro-Toro *et al.*, 1996) generated by the activation of their specific receptors. Finally it is interesting to note that somatostatin effects could be a result of receptor-receptor interactions as shown for other neuromodulators (Ghosh *et al.*, 1997; Agnati *et al.*, 2003). Indeed, the action of the peptide passes via one of the five somatostatin receptor subtypes (Hoyer *et al.*, 1994) which implicate a release of endogenous GABA or GABA agonists affecting by consequent the binding of [³⁵S]-TBPS binding.

The presence of somatostatin modulation of GABA_A receptor complex as shown in the present study is of interest in regard to the functional relevance of such interactions. It is suggested that the naturally occurring peptide could play an important role in modulating GABA-mediated synaptic inhibition in certain physiological states (Green and Mason, 1996; Leresche *et al.*, 2000; Wang *et al.*, 2000). Thus, in rat thalamus, the neuropeptide abolished spontaneous bursts of GABA(A) inhibitory postsynaptic potentials in 85% and decreased (40%) the amplitude of single spontaneous GABA(A) inhibitory postsynaptic potentials in 87% of thalamic neurons respectively (Leresche *et al.*, 2000). Furthermore, the present pharmacological data are supported by previously reported anatomical findings. Immunohistochemical studies suggest the presence of dense somatostatinergic networks in GABAergic neuronal structures (Wang *et al.*, 2000). Consequently, the tetradecapeptide could modulate directly the release of GABA and thereby cause an inhibitory effect in the central nervous system.

In summary, the findings of the present data indicate that somatostatin has a modulatory effect on the central GABA_A receptor complex, which is GABA sensitive. Such findings will help to strengthen the relationship between GABAergic and somatostatinergic effects, which may be important in certain physiological conditions (Rage *et al.*, 1993; Luddens *et al.*, 1995). These findings illustrate also the importance of such interactions in somatostatin-mediated GABAergic transmission in the rat diencephalon.

5. CONCLUSION

The present results demonstrate the presence of a modulatory effect of somatostatin on the GABA_A receptor complex in rat thalamus. Furthermore, the data suggest that somatostatin allosterically modifies [³⁵S]-TBPS binding through a mechanism similar to that of GABA.

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