

Diazepam Attenuation of Somatostatin Binding and Effect of Somatostatin on Accumulation of Inositol 1,4,5-Trisphosphate in the Rat Frontoparietal Cortex

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Numerous reports in both humans and animals have confirmed that benzodiazepines produce amnesia; however, mechanisms mediating this effect are not clear. In view of the important role of brain somatostatin (SRIF) in the cognitive function of rats, this study sought to determine if the benzodiazepine, diazepam, alters somatostatinergic system in the rat frontoparietal cortex. Intraperitoneal (IP) administration of diazepam (5 mg/kg/day) to male Wistar rats (200–250 g) for 3 or 7 days decreased the number of SRIF receptors (26 and 37%, respectively) in synaptosomes from the frontoparietal cortex, without influencing their apparent affinity. This decrease in the tracer binding was not attributable to a direct effect of diazepam on SRIF receptors, because no decrease of SRIF binding was induced by a large concentration of diazepam (10^{-4} M) when the drug was added to a preparation of synaptosomes from frontoparietal cortex of untreated rats. To determine if the effect of diazepam on SRIF binding is related to the binding of diazepam to its recognition site on the GABA_A receptor, a benzodiazepine antagonist, 2-phenylpyrazolo[3,4-c]quinolin-3(5H)-one (CGS 8216) was administered before the diazepam injection. Pretreatment with CGS 8216 (20 mg/kg/day, IP) blocked completely the diazepam-induced decrease in the number of

SRIF receptors. CGS 8216 alone had no observable effect. The decrease in the number of ^{125}I -Tyr¹¹-SRIF receptor induced by diazepam was accompanied by a decrease in the effect of SRIF, after 15 seconds of stimulation, on inositol 1,4,5-trisphosphate (IP₃) mass accumulation in the rat frontoparietal cortex at 3 (64%) or 7 days (59%) after its administration. Diazepam alone had no observable effect on mass accumulation of IP₃. After 14 days of daily diazepam injections, the levels of binding of ^{125}I -Tyr¹¹-SRIF in the frontoparietal cortex returned to control values, coinciding with the tolerance that develops to this benzodiazepine agonists when administered chronically. The decrease in IP₃ levels was still observed after 14 days (57%) diazepam administration. Diazepam and CGS 8216 did not affect SRIF-like immunoreactivity levels in the frontoparietal cortex at the three time intervals studied (3, 7 or 14 days). The alteration of frontoparietal cortex SRIF receptor-effector system after 3 or 7 days of diazepam treatment suggests that somatostatinergic neurotransmission plays a role in the mechanism of diazepam action on memory.

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Clinical and experimental studies have shown that benzodiazepines cause temporary memory impairment (Brown et al. 1994; Ghoneim and Mewaldt 1975). Benzodiazepines that are endowed with full positive allosteric modulatory (FAM) activity on GABA_A receptors cause anterograde amnesia in both, animals and humans (Haefely et al. 1985). It was demonstrated that in rats subjected to a delayed object recognition test, diazepam endowed with FAM activity, exerts an amnesic action (Ghoneim and Mewaldt 1975). Nabeshima et al. (1990) suggest that benzodiazepines induce disruptive effects on learning and memory through their recognition site on the GABA receptor and that benzodiazepine-induced learning and memory impairment of is, at least in part, the result of the dysfunction of the cholinergic neuronal system. However, because of the important role of brain tetradecapeptide somatostatin (SRIF) in the cognitive functions of rats (Cacabelos et al. 1988; Dournaud et al. 1996; Matsuoka et al. 1994; Schettini et al. 1988; Vécsei et al. 1984), and interaction between somatostatinergic and GABAergic neuronal systems (Boyano-Adánez et al. 1995; Hendry et al. 1984; Kawaguchi and Kubota 1998; Llorens-Cortes et al. 1992; Xie and Sastry 1992) it is possible to assume that the somatostatinergic system is implicated in the mechanism of action of diazepam. SRIF is widely distributed in the central nervous system (CNS) and peripheral tissues (see Reichlin 1983). SRIF induces its biological actions by interacting with membrane-bound receptors that are sensitive to guanine nucleotides (Law et al. 1991) of which at least five subtypes have been cloned (Hoyer et al. 1994). All five receptors increase phospholipase C (PLC) activity; although, the SS1/SRIF1 subclass induces greater increase of inositol-1,4,5-trisphosphate (IP₃) (Akbar et al. 1994). SRIF in brain acts as a neurotransmitter and/or neuromodulator by inhibiting neuronal firing and plays a role in the modulation of such complex behaviors as motor activity and cognition (see Epelbaum 1986). Cacabelos et al. (1988) found that SRIF is effective in improving learning in rats after intraventricular injection. In a similar paradigm, the SRIF-depleting substance cysteamine, impaired the learning performance of rats in an active avoidance behavior model (Vécsei et al. 1984). SRIF could also reverse the impairment of cognitive functions induced by cysteamine depletion of brain SRIF (Schettini et al. 1988). Dournaud et al. (1996) suggest that frontal somatostatinergic interneurons are likely to participate in learning behavior and in the regulation of spatial amnesic processes. These authors also suggest that parietal somatostatinergic interneurons are involved in spatial memory processes. In addition, it has been demonstrated that the brain somatostatinergic system is one of the

most severely affected systems in patients with Alzheimer's disease, suggesting its possible involvement in alterations of memory capacity of these patients (Davies et al. 1980).

The aim of the present study was to investigate the effect of diazepam treatment on specific binding of ¹²⁵I-Tyr¹¹-SRIF to its receptors in synaptosomes from frontoparietal cortex, given that frontal and parietal somatostatinergic interneurons are involved in learning and memory (Dournaud et al. 1996). These interneurons are also rich in somatostatinergic neuronal elements (Johansson et al. 1984). In addition, the effect of this benzodiazepine on SRIF-stimulated accumulation of IP₃ and SRIF immunoreactivity levels in rat frontoparietal cortex was also examined with the aim of evaluating the functionality of SRIF receptors. The effect of pretreatment with the benzodiazepine antagonist, 2-phenylpyrazolo[3,4-c]quinolin-3(5H)-one (CGS 8216), was used to evaluate whether the possible effects of diazepam on the somatostatinergic system are related to GABA_A receptor activation.

MATERIALS AND METHODS

Materials

Synthetic Tyr¹¹-SRIF and SRIF tetradecapeptide were purchased from Universal Biologicals Ltd (Cambridge, UK); diazepam (Valium) from Roche (Madrid, Spain); 2-phenyl-pyrazolo [3,4-c]-quinolin-3(5H)-one (CGS 8216) was kindly donated by Ciba-Geigy (Spain); bacitracin and bovine serum albumin (fraction V) from Sigma (Madrid, Spain); dextran was from Serva, Feinbiochemica (Heidelberg, Germany), and carrier-free Na ¹²⁵I (IMS 30, 100 mCi/ml) from the Radiochemical Centre (Amersham, UK).

The rabbit antibody, used in the radioimmunoassay technique, was purchased from the Radiochemical Centre (Amersham, U.K.). This antiserum was raised in rabbits against SRIF-14, conjugated with bovine serum albumin, and it is specific for SRIF, but because SRIF-14 constitutes the C-terminal portions of both SRIF-25 and SRIF-28, the antiserum does not distinguish between these three forms.

Experimental Animals

Wistar rats (*n* = 90) of 200–220 g were used in the present investigation by feeding on a stock diet throughout the experiment. All animals received food and tap water ad libitum. Room temperature was kept at 22°C and day–night cycle of 12 h was maintained. Diazepam (Valium 5 mg/kg) or an equivalent volume of vehicle (saline) was injected intraperitoneally (IP) once daily for 3, 7, or 14 days at a dose sufficient to impair spatial memory in the absence of sensorimotor disrup-

tion (McNamara et al. 1993). The second experimental group of rats received the benzodiazepine antagonist, CGS 8216, in a suspension of Tween 80, at a dose of 10 mg/kg, IP at 9:00 and 18:00 h as previously described (Eisenberg 1987) for 3, 7, or 14 days. A third experimental group of animals was employed to test the hypothesis that the effect of diazepam on the somatostatinergic system depended upon its binding to its receptor; the antagonist CGS 8216 was administered as described above, and, in addition, diazepam (5 mg/kg, IP) was injected at 12 AM on each day. Control animals for each group were injected with saline, Tween 80 or Tween 80 plus saline as appropriate. Animals were sacrificed by decapitation 1 h after the last injection, as described previously (Popova et al. 1988), the brains were removed, and the frontoparietal cortex rapidly dissected, as described by Glowinski and Iversen (1966).

Tissue Extraction and Somatostatin Radioimmunoassay

For measurements of immunoreactivity, the frontoparietal cortex was rapidly homogenized using a Brinkman polytron (setting 5, 30 s), in 1 ml 2 M acetic acid. Extracts were boiled for 5 min in a water bath, chilled in ice, and aliquots (100 μ l), were removed for determination of protein (Lowry et al. 1951). Subsequently, homogenates were centrifuged at $15,000 \times g$ for 15 min at 4°C, and the supernatant was neutralized with 2 M NaOH. Extracts were immediately stored at -70°C until assay. The level of immunoreactivity was determined in tissue extracts by a modified radioimmunoassay method (Patel and Reichlin 1978), with a sensitivity limit of 10 pg/ml. The possibility that substances present in the tissue extracts might interfere with antibody-antigen binding and give rise to erroneous results, was checked by performing serial dilutions of selected extracts in the assays and comparing the resulting changes in hormonal immunoreactivity with those of the diluted standards. In addition, known standard amounts of the hormone were added to varying amounts of the extracts, and serial dilutions were again assayed to determine if this exogenously added hormonal immunoreactivity could be measured reliably in the presence of tissue extracts. Incubation tubes, prepared in duplicate, contained 100 μ l samples of unknown or standard solutions of 0–500 pg cyclic SRIF tetradecapeptide, diluted in phosphate buffer (0.05 M, pH 7.2 containing 0.3% bovine serum albumin, 0.01 M ethylene diamine tetra acetic acid [EDTA]), 200 μ l appropriately diluted anti-SRIF serum, 100 μ l freshly prepared 125 I-Tyr¹¹-SRIF, diluted in buffer to yield 6,000 cpm (equivalent to 5–10 pg) and enough buffer to give a final volume of 0.8 ml. All reagents, as well as the assay tubes, were kept chilled in ice before incubation at 4°C for 48 h. Separation of bound and free hormone was ac-

complished by addition of 1 ml dextran-coated charcoal (dextran: 0.2% w/v). Dilution curves for each area of brain were parallel to the standard curve. The coefficients for intra- and interassay variation were 6.5 and 8.3%, respectively.

Binding Assay

Tyr¹¹-SRIF was radioiodinated by chloramine-T iodination (Greenwood et al. 1963). Separation of iodinated SRIF from unincorporated iodine was carried out on a Sephadex G-25 (fine) column equilibrated and eluted with 0.1 M acetic acid containing bovine serum albumin (0.1% w/v). The specific activity of the radioligand was 600 Ci/mmol.

Synaptosomes from the frontoparietal cortex were prepared as previously described (Yu and Ho 1990). Experimental conditions for SRIF binding were essentially as previously described by this laboratory (Colás et al. 1990). Briefly, synaptosomes from frontoparietal cortex were separately incubated in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl₂, 30 mM NaCl, 1% bovine serum albumin, 0.1% bacitracin, and 100 pM of 125 I-Tyr¹¹-SRIF in the absence or presence of 0.01–10 nM unlabeled SRIF. After 60 min incubation at 25°C, synaptosome-bound peptide was isolated by centrifugation at $13,000 \times g$ for 1.5 min, and the radioactivity determined in a Kontron gamma counter. Nonspecific binding was obtained from the amount of radioactivity bound in the presence of 10^{-6} M SRIF and represented about 20% of the binding observed in the absence of unlabeled peptide. This nonspecific component was subtracted from the total bound radioactivity to obtain the corresponding specific binding.

Evaluation of Radiolabeled Peptide Degradation

To determine the extent of tracer degradation during incubation, we measured the ability of preincubated peptide to bind to fresh synaptosomes, as previously described (Schonbrunn et al. 1983). Briefly, 125 I-Tyr¹¹-SRIF (100 pm) was incubated with synaptosomes from rat frontoparietal cortex (1 mg protein/ml) for 60 min at 25°C. After this preincubation, aliquots of the medium were added to fresh synaptosomes and incubated for an additional 60 min at 25°C. The fraction of the added radiolabeled peptide that was specifically bound during the second incubation was measured and expressed as a percentage of the binding that had been obtained in control experiments performed in the absence of synaptosomes during the preincubation period.

Inositol 1,4,5-trisphosphate Analyses

Cross-chopped frontoparietal cortical slices (250 \times 250 μ m) were prepared from male Wistar rats (200–250 g)

and preincubated for 45 min at 37°C in Krebs bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11.7 mM glucose, 10 mM HEPES) pH 7.4 saturated with O₂/CO₂ (95/5%) with buffer replacement and gassing every 15 min (Challiss et al. 1988). Slices were allowed to sediment, and 50 µl of packed slices were added to tubes containing 200 µl of Krebs buffer and 20 µl of 1.5 mM LiCl. The samples were gassed regularly, capped, and incubated. SRIF (10⁻⁷ M) in a volume of 30 µl was subsequently added to these tubes. Incubation was terminated by addition of 500 µl of 0.5 M trichloroacetic acid (TCA). Acidified samples were left on ice for 15 min and subsequently centrifuged for 15 min at 1,800 × *g*. To remove TCA, the supernatants were washed 5 × with 1.25 ml of water-saturated diethyl ether and, finally, 200 µl of 50 mM Tris-HCl pH 8.4 were added. The protein concentration in the tissue pellets was determined, as previously described (Lowry et al. 1951).

The specific binding of [³H]IP₃ to a preparation of bovine cerebellum membranes was used as a radioreceptor assay to determine the IP₃ concentration in these slices, according to the method previously described (Bredt et al. 1989). Bovine cerebellar membranes were prepared by homogenizing bovine cerebella in a cold buffer A (50 mM Tris-HCl, 1 mM EDTA and 1 mM 2-mercaptoethanol) pH 7.7 to obtain a protein concentration of 4 mg/ml (Challiss et al. 1988). These membranes (50 µg/tube) were added to Eppendorf tubes containing 25 µl of [³H]IP₃ (5 nCi/tube) and 50 µl of unknown or standard samples containing IP₃ (0.005 µM–5 µM in buffer A, pH 8.6) or IP₆ (1% w/v in buffer A pH 8.6) to define nonspecific binding. All tubes were incubated for 10 min at 4°C. Separation of bound and free IP₃ was achieved by centrifugation at 10,000 × *g* for 5 min. After aspiration of the supernatant, 50 µl of 0.15 M NaOH were added to each tube, and the pellet was dissolved by shaking. The radioactivity was determined by liquid scintillation spectrometry. The IP₃ content was determined by interpolating the inhibition of [³H]IP₃ binding on a calibration curve with known amounts of IP₃. Nonspecific binding was 13% of the total binding.

Data Analysis

The computer program LIGAND (Munson and Rodbard 1980) was used to analyze the binding data. The use of this program enabled models of receptors that best fit a given set of binding data to be selected. The same program was also used to present data in the form of Scatchard plots (Scatchard 1949) and to compute values for receptor affinity (*K_d*) and density (*B_{max}*) that best fit the sets of binding data for each rat. Statistical comparisons of all the data were carried out by one-way

analysis of variance (ANOVA) and the Student-Newman-Keuls test. Means among groups were considered significantly different when the *p* values were < .05. Each individual experiment was performed in duplicate.

RESULTS

Diazepam administration during 3 or 7 days decreased the specific binding of SRIF to rat frontoparietal cortical synaptosomes as compared to control conditions (Table 1, Figures 1,2). After 2 weeks of daily diazepam injection, the binding of SRIF returned to control values (Table 1, Figure 3). Upon comparison of the corresponding curves of ¹²⁵I-Tyr¹¹-SRIF displacement by increasing concentrations of the unlabeled neuropeptide, the binding data were significantly lower in the diazepam-treated rats during 3 or 7 days throughout the whole range studied (Figures 1 and 2). Scatchard plots of the stoichiometric binding data were linear and essentially parallel (Figures 1,2,3; right panel). Interpretation of these data with the LIGAND computer program resulted in the best fit for a model with one type of SRIF receptor. Frontoparietal cortical synaptosomes from diazepam-treated rats exhibited a significant decrease in

Table 1. Effect of Diazepam and the Benzodiazepine Antagonist, CGS 8216, on Equilibrium Parameters of the Binding of ¹²⁵I-Tyr¹¹-Somatostatin to Frontoparietal Cortical Synaptosomes

Groups	Days of Treatment		
	3	7	14
Saline			
Kd	0.36 ± 0.02	0.54 ± 0.07	0.47 ± 0.05
Bmax	254 ± 10	268 ± 35	238 ± 33
Diazepam			
Kd	0.44 ± 0.03	0.65 ± 0.09	0.45 ± 0.05
Bmax	180 ± 11***	165 ± 22*	255 ± 35
Tween 80			
Kd	0.33 ± 0.04	0.37 ± 0.08	0.37 ± 0.03
Bmax	234 ± 35	262 ± 30	225 ± 32
CGS 8216			
Kd	0.31 ± 0.08	0.38 ± 0.08	0.37 ± 0.02
Bmax	237 ± 32	261 ± 30	227 ± 4
Tween 80 + saline			
Kd	0.34 ± 0.02	0.45 ± 0.05	0.42 ± 0.04
Bmax	242 ± 18	263 ± 20	231 ± 17
CGS 8216 + Diazepam			
Kd	0.38 ± 0.02	0.55 ± 0.08	0.47 ± 0.04
Bmax	241 ± 11	256 ± 34	239 ± 33

Binding parameters for the binding of ¹²⁵I-Tyr¹¹-somatostatin to receptors were determined by least-squares analysis of Scatchard plots of the data in Figures 1–3.

Abbreviations: Kd, dissociation constant, nM; Bmax, binding capacity from femtomoles of somatostatin bound per mg protein.

Results are expressed as means ± SEM of five rats in each group.

Statistical significance: **p* < .05; ****p* < .001.

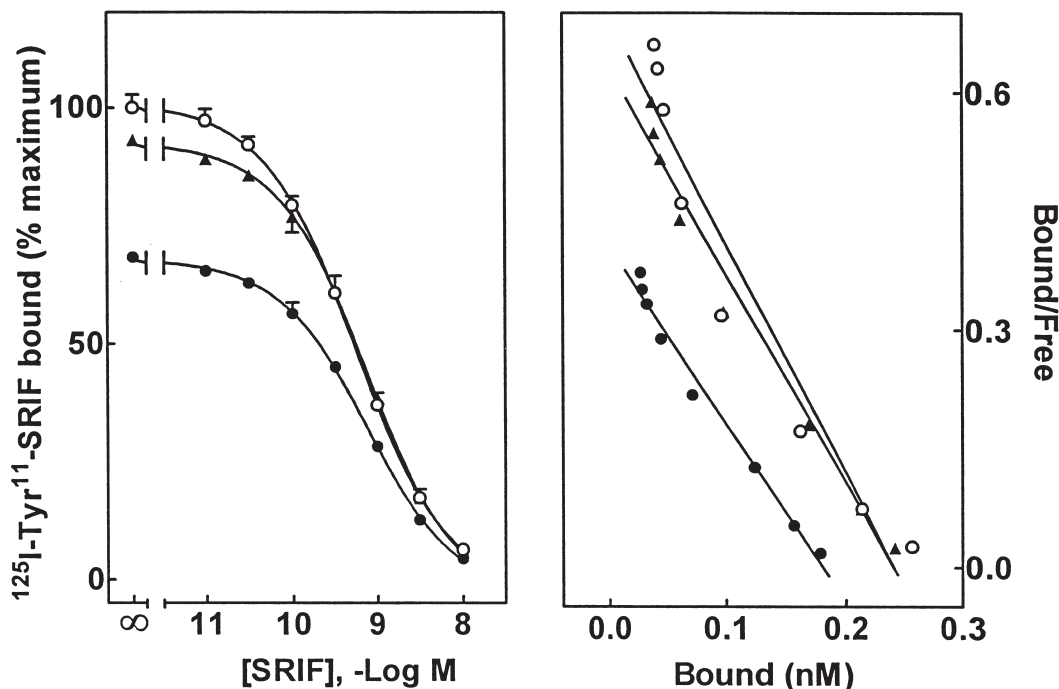


Figure 1. The effect of short-term (3 day) daily administration of diazepam and the benzodiazepine antagonist CGS 8216 on the binding of somatostatin (SRIF) to frontoparietal cortical synaptosomes. The animals were decapitated 60 min after the last injection. Left panel: synaptosomes (1 mg protein/ml) were incubated for 60 min at 25°C in the presence of 100 pM [^{125}I]Tyr 11 -SRIF and increasing concentrations of native peptide. The points correspond to values for the animals in the control group (\circ , $n = 5$), diazepam-treated group (\bullet , $n = 5$), and CGS 8216 plus diazepam-treated group (Δ , $n = 5$). The results express the value of the pooled control injection groups, because the B_{max} and the K_d values of the control groups were not affected by the saline or Tween 80 media. Right panel: Scatchard analysis of the same data.

the maximum SRIF-binding capacity at 3 and 7 days of administration of diazepam (Table 1, Figures 1,2). The corresponding K_d values, remained unchanged after diazepam administration.

To assess whether diazepam exerts a direct action on SRIF receptors, diazepam (10^{-4} M) was included in the incubation medium at the time of the binding assay, with frontoparietal cortical synaptosomes from control rats. The addition of diazepam had no effect on the SRIF receptors (data not shown).

To determinate if the above-mentioned changes are related to the binding of diazepam to their recognition site on the GABA $_A$ receptor, a benzodiazepine antagonist, CGS 8216, was administered before the diazepam injection. Pretreatment with CGS 8216 (20 mg/kg/day, IP) completely blocked the diazepam-induced changes in the number of SRIF receptors (Table 1, Figures 1, 2). The administration of CGS 8216 alone did not produce any change in the $^{125}\text{I-Tyr}^{11}\text{-SRIF}$ binding in frontoparietal cortical synaptosomes (Table 1).

Degradation of peptide was determined in all the preparations to rule out the possibility of different degrading activities of SRIF, which might have affected the interpretation of the results. The percentage of labeled SRIF degraded by synaptosomes from frontopari-

etal cortex during the binding experiments was similar in both treated and untreated animals, being 8.1, 9.4, and 10.2%, respectively, in animals treated with diazepam-, CGS 8216-, and CGS 8216 plus diazepam-treated animals.

Because SRIF receptors are coupled to PLC, we examined the effect of SRIF on accumulation of IP $_3$ in frontoparietal cortical slices of control rats and diazepam-treated rats. SRIF rapidly increased IP $_3$ accumulation in frontoparietal cortex of control rats (Figure 4). This effect was maximal at 15 seconds of incubation and decreased subsequently. The SRIF effect on IP $_3$ accumulation was significantly lower in diazepam-treated rats than in control rats at 3 (64%), 7 (59%), or 14 days (57%) after its administration. The administration of diazepam did not modify basal IP $_3$ levels in the frontoparietal cortex (Figure 4).

Levels of SRIF immunoreactivity in the frontoparietal cortex were unaffected by the administration of diazepam, either alone or after treatment with CGS 8216 (Table 2).

DISCUSSION

The present results show that administration of the benzodiazepine, diazepam, for 3 or 7 days decreased the

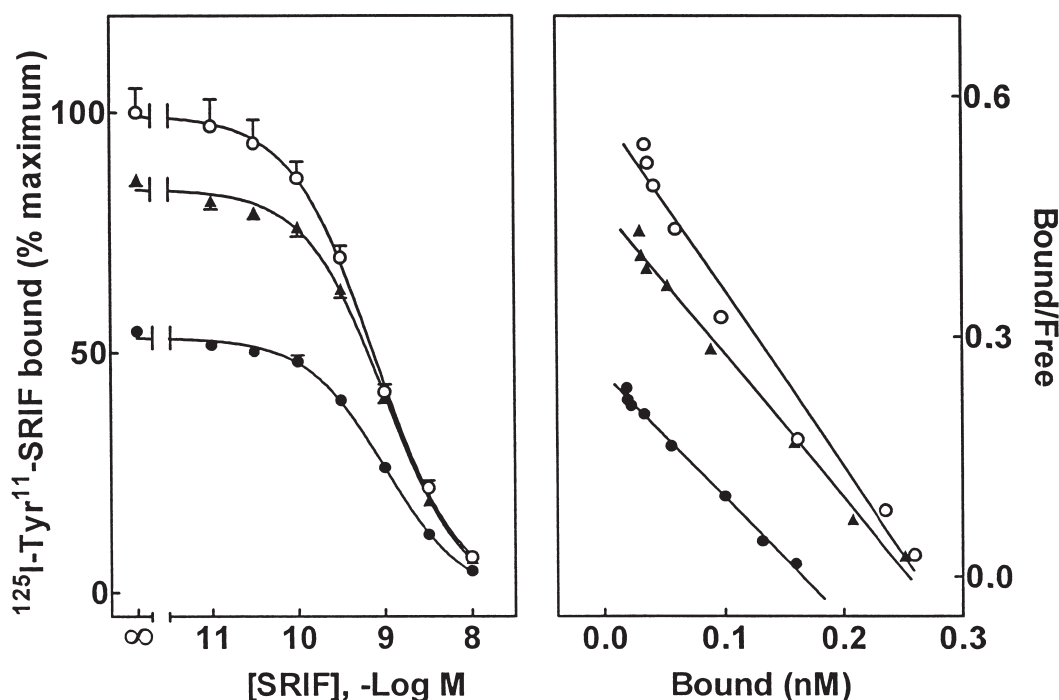


Figure 2. The effect of moderate duration (7 day) daily administration of diazepam and the benzodiazepine antagonist CGS 8216 on the binding of somatostatin (SRIF) to frontoparietal cortical synaptosomes. The animals were decapitated 60 min after the last injection. Left panel: synaptosomes (1 mg protein / ml) were incubated for 60 min at 25°C in the presence of 100 pM [^{125}I]Tyr¹¹-SRIF and increasing concentrations of native peptide. The points correspond to the values for the animals in the control group (\circ , $n = 5$), diazepam-treated group (\bullet , $n = 5$), and CGS 8216 plus diazepam-treated group (Δ , $n = 5$). The results express the value of the pooled control injection groups, because the B_{max} and K_d values of the control groups were not affected by the saline or Tween 80 media. Right panel: Scatchard analysis of the same data.

number of specific SRIF receptors in the rat frontoparietal cortex; whereas, the affinity of the receptors was unaltered. In addition, diazepam decreased SRIF-mediated accumulation of IP_3 and had no effect on SRIF immunoreactive content in frontoparietal cortex.

In the control rats, the SRIF immunoreactive content was similar to that previously reported by other authors (Pitkanen et al. 1986). Diazepam administration did not produce changes in SRIF immunoreactive content in frontoparietal cortex. To our knowledge, there are no data available concerning the effect of diazepam alone on SRIF immunoreactivity. The only study on the effect of GABA_A receptor stimulation on somatostatinergic system was carried out with diazepam plus muscimol, a GABA_A agonist in mouse brain (Hendry et al. 1984; Llorens-Cortes et al. 1992). These drugs were injected concomitantly, because previous data demonstrated that muscimol or diazepam was poorly effective when given alone; whereas diazepam markedly potentiated muscimol effect (Bourgoin et al. 1982; Duka et al. 1980). This 7-day treatment induced a 20% decrease in cortical SRIF content.

The equilibrium parameters of the SRIF receptors in the frontoparietal cortex of control rats were similar to those previously reported by other authors (Srikant and

Patel 1981; Epelbaum et al. 1982). Although Scatchard plots of the stoichiometric data seem to be linear, it cannot be taken as a proof of receptor homogeneity. Recently, it has been demonstrated that there are five cloned SRIF receptors that seem to be expressed in the brain (Hoyer et al. 1994), and that all have similar high affinity for Tyr¹¹-SRIF (Bell and Reisine 1993). Thus, a linear Scatchard plot only indicates that the labeled sites have similar affinity for the radioligand used.

So far, the molecular mechanism that underlies the decrease in SRIF receptors in frontoparietal cortical synaptosomes of diazepam-treated rats is unknown. The decrease in the number of ^{125}I -Tyr¹¹-SRIF receptors was not attributable to a direct effect of diazepam on SRIF receptors, because no change was detected in tracer binding, following the incubation of fresh frontoparietal cortical synaptosomes with 10^{-4} M diazepam. It is possible that GABA_A receptors could mediate the action of diazepam on SRIF binding, because pretreatment with the benzodiazepine antagonist CGS 8216 prevented the decreases in the number of SRIF receptors that are induced by administration of diazepam. The benzodiazepine antagonist alone had no demonstrable effect on these parameters. A previous study conducted in our laboratory (Boyano-Adánez et al. 1995) support

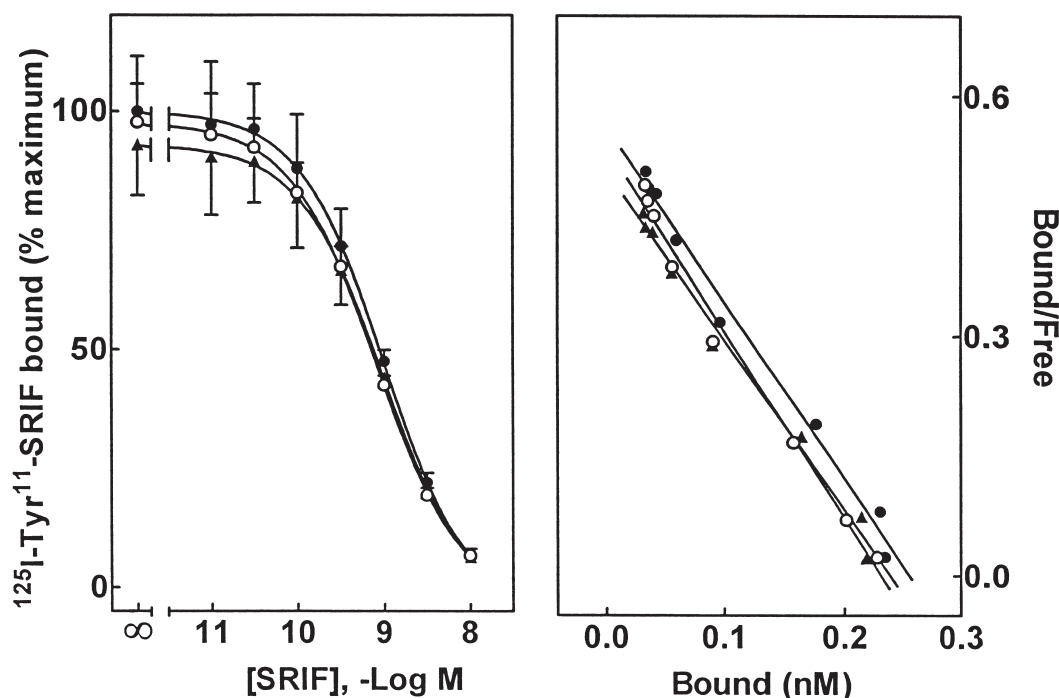


Figure 3. The effect of long-term (14 day) daily administration of diazepam and the benzodiazepine antagonist CGS 8216 on the binding of somatostatin (SRIF) to frontoparietal cortical synaptosomes. The animals were decapitated 60 min after the last injection. Left panel: synaptosomes (1 mg protein/ml) were incubated for 60 min at 25°C in the presence of 100 pm $^{125}\text{I-Tyr}^{11}\text{-SRIF}$ and increasing concentrations of native peptide. The points correspond to the values for the animals in the control group (\circ , $n = 15$), diazepam-treated group (\bullet , $n = 5$) and CGS 8216 plus diazepam-treated group (Δ , $n = 5$). The results express the value of the pooled control injection groups, because the B_{max} and the K_d values of the control groups were not affected by the saline or Tween 80 media. Right panel: Scatchard analysis of the same data.

the hypothesis that benzodiazepines recognition site on the GABA_A receptor is implicated in the decrease of the number of SRIF receptors caused by ammonium acetate administration, because the specific benzodiazepine receptor antagonist, CGS 8216, is capable of reversing this decrease of SRIF receptor back to control values in the frontoparietal cortex and hippocampus of rats treated with ammonium acetate.

Diazepam interacts with a specific site on the GABA /benzodiazepine/barbiturate receptor complex (Haefely et al. 1985). As a result of this interaction, an allosteric modulation takes place in the complex. This phenomenon permits a greater influence of GABA on the specific site of interaction by increasing the gating probability of the chloride channel in response to GABA . It is possible that hyperpolarization, mediated by chloride is an important factor in the action of diazepam on the SRIF receptor/effector system. At present, there is evidence supporting the hypothesis that changes in membrane polarization may induce modifications in the number of receptors present in membrane (Liles and Nathanson 1987). Alternatively, the regulation of the somatostatinergic system by diazepam may be indirect. In this regard, it is shown that diazepam decreases acetylcholine release from the cerebral cortex

(Petkov et al. 1983) and studies undertaken in our laboratory had shown that the reduction of the activity of cholinergic system produced a significant decrease in the $^{125}\text{I-Tyr}^{11}\text{-SRIF}$ binding in membranes from the frontoparietal cortex (Barrios et al. 1990).

The finding that the number of SRIF receptors returned to control values following long-term administration of diazepam agrees with the fact that chronic administration of diazepam induces tolerance to its clinical, behavioral, and electrophysiological actions (Gallager et al. 1985; Pesold et al. 1997).

Our research group has previously studied the effect of different SRIF concentrations (10^{-6} – 10^{-8} M) on IP_3 accumulation in the rat brain (Muñoz-Acedo et al. 1995). In agreement with other studies (Lachowicz et al. 1994), the stimulatory effect of SRIF on IP_3 was maximum at 10^{-7} M in all rat brain areas studied (Muñoz-Acedo et al. 1995). Therefore, subsequent studies were carried out at 10^{-7} M SRIF. Diazepam had no effect on IP_3 accumulation in the frontoparietal cortex, coinciding with other studies (Raskovsky and Medina 1992). SRIF increases the IP_3 accumulation in the control rats in agreement with reports by other authors (Lachowicz et al. 1994) and our own (Muñoz-Acedo et al. 1995). Recent studies suggest that all five human SRIF receptors ex-

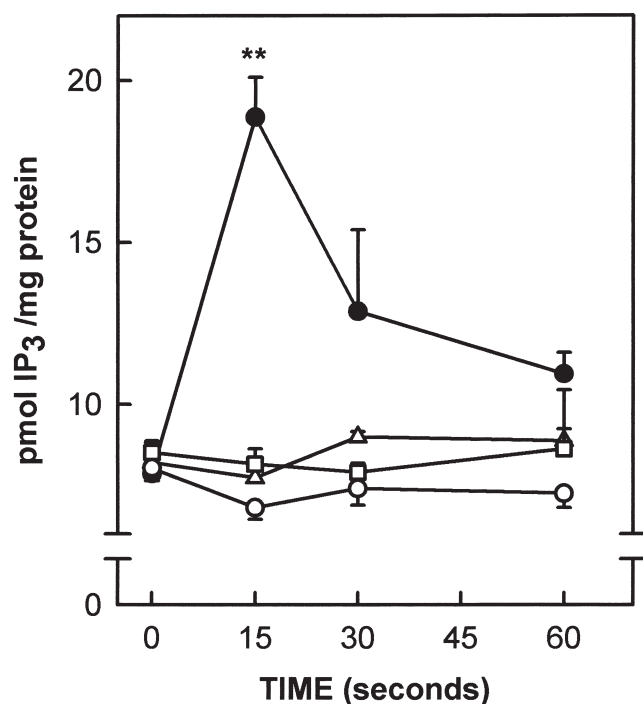


Figure 4. Time-course effect of somatostatin (10^{-7} M)-induced inositol 1,4,5-triphosphate (IP_3) accumulation in frontoparietal cortical slices from control (●) and diazepam-treated (○, 3 day; △, 7 day; □, 14 day) rats. Values are expressed as the mean \pm SEM of five separate experiments. Statistical comparison versus control: $**p < .01$.

pressed in COS-7 cells are coupled to activation of phosphoinositide (PI)-specific PLC- β (Akbar et al. 1994). A few studies have also reported that SRIF activates PLC followed by Ca^{2+} mobilization in native SRIF-receptor expressing cells, such as striatal astrocytes (Marin et al. 1991) and NG 108-15 cells (Okajima and Kondo 1992). Therefore, the decreased effect of SRIF on accumulation of IP_3 observed after diazepam administration at 3 or 7 days might be a consequence of the decrease in the number of ^{125}I -Tyr 11 -SRIF receptors observed after this treatment.

Although the number of SRIF receptors returned to control values after 14 days of treatment, the effect of SRIF on IP_3 accumulation still remains decreased. An inconsistency between changes in receptors and signal transduction is not unprecedented. For example, chronic exposure of primary cultures of mouse cortical neurons to the muscarinic antagonist atropine has been shown to increase the density of muscarinic receptors while decreasing carbachol-stimulated phosphoinositide hydrolysis (Smith et al. 1989).

In the cortex, the pyramidal cells possess SRIF and GABA $_A$ receptors (Xiang et al. 1998; Pérez et al. 1994); therefore, it is possible that binding of diazepam to its recognition site on the GABA $_A$ receptor decreases the number and functionality of SRIF receptors in the fron-

Table 2. Effect of Diazepam and the Benzodiazepine Antagonist, CGS 8216, on Somatostatin-like Immunoreactivity Concentration in Rat Frontoparietal cortex

Groups	Days of Treatment		
	3	7	14
Saline	10.49 \pm 0.76	12.10 \pm 1.37	9.98 \pm 1.06
Diazepam	12.72 \pm 0.95	12.09 \pm 2.19	8.89 \pm 3.20
Tween 80	12.09 \pm 2.20	11.41 \pm 1.02	11.35 \pm 4.70
CGS 8216	12.05 \pm 0.67	14.54 \pm 1.80	9.52 \pm 0.15
Tween 80+saline	11.29 \pm 1.48	11.75 \pm 1.58	9.75 \pm 0.60
CGS 8216+Diazepam	12.40 \pm 0.78	14.22 \pm 1.34	14.01 \pm 2.12

Determinations were made in duplicate for each experiment.

Results are expressed as ng somatostatin/mg protein and as means \pm SEM for five rats in each group.

No statistically significant differences were obtained when compared with control animal.

toparietal cortex. The fact that diazepam causes temporary memory impairment and that the frontal and parietal somatostatergic interneurons have an important role in learning behavior and regulation of spatial memory (Dournaud et al. 1996) suggests that diazepam-induced impairment of learning and memory is, at least in part, the result of the dysfunction of the somatostatergic neuronal system.

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