Effects of subchronic and chronic melatonin treatment on somatostatin binding and its effects on adenylyl cyclase activity in the rat frontoparietal cortex

Abstract: Melatonin and somatostatin are known to exert similar effects on locomotor activity. We have previously demonstrated that acute melatonin treatment regulates somatostatin receptor function in the rat frontoparietal cortex. However, the effects of subchronic and chronic melatonin treatment on the somatostatin receptor-G protein-adenylyl cyclase system in the rat frontoparietal cortex are unknown. Melatonin was administered subcutaneously at a daily dose of 25 µg/kg for 4 days, 1 wk or 2 wk. Twenty-four hours after the last injection, the animals were sacrificed. Melatonin did not alter the somatostatin-like immunoreactivity content in the frontoparietal cortex from control and melatonin-treated rats during any of the previously indicated periods. Four days of melatonin administration induced both an increase in the number of [¹²⁵I]-Tyr¹¹-somatostatin receptors and a decrease in the affinity of somatostatin for its receptors in frontoparietal cortical membranes. The increased number of somatostatin receptors in the melatonin-treated rats was associated with an increased capacity of somatostatin to inhibit basal and forskolin-stimulated adenylyl cyclase activity. Melatonin administration for 4 days induced a higher adenylyl cyclase activity both under basal conditions and after direct stimulation of the enzyme with forskolin. No significant differences were observed in the function of Gi proteins in the 4-day melatonin-treated rats. Western blot analyses showed that the 4-day melatonin treatment reduced Gi₂ levels, without altering the amount of Gi₁. These melatonin-induced changes reverted to control values after 7 or 14 days of treatment. Altogether, the present findings suggest that subchronic melatonin treatment modulates the somatostatin receptor/effector system in the rat frontoparietal cortex.

**Rosa María Izquierdo-Claros,
María del Carmen Boyano-
Adánez and Eduardo Arilla-
Ferreiro**

Departamento de Bioquímica y Biología
Molecular, Facultad de Medicina, Universidad
de Alcalá, Madrid, Spain

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Address reprint requests to Eduardo Arilla-
Ferreiro, Departamento de Bioquímica y Bio-
logía Molecular, Facultad de Medicina, Carre-
tera Madrid-Barcelona Km. 33,600,
Universidad de Alcalá, E-28871 Alcalá de
Henares, Madrid, Spain.
E-mail: carmen.boyano@uah.es

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Introduction

Somatostatin (SRIF) is a tetradecapeptide that is widely distributed throughout the central nervous system [1, 2]. Immunohistochemical studies have demonstrated complex systems of SRIF-positive neurons in the cerebral cortex [3, 4]. More recently, SRIF mRNA was identified in the rat cerebral cortex [5]. These studies, together with others, suggest that SRIF is intrinsic to the cerebral cortex *in vivo* [6]. Endogenous neurotransmitters influence SRIF levels and SRIF release in fetal rat cerebral cortical cells in culture [7]. In the rat cortex and hippocampus, SRIF-containing interneurons, which are a subset of GABAergic neurons, may also contain NPY [8, 9] and stain positively for NADPH diaphorase [10]. In the brain, SRIF has been shown to exert both neuromodulatory and transmitter-like functions [6]. The frontoparietal cortex contains specific receptors for SRIF, termed SSTR1–SSTR5, which belong to the family of seven transmembrane domain G-protein-coupled receptors. All five receptor subtypes bind the

natural peptides, SRIF-14 and SRIF-28, with similar affinity and are coupled to the adenylyl cyclase (AC) system via a guanine nucleotide binding inhibitory protein (Gi) [11]. These SRIF receptors are widely distributed in the brain. In the rat, mRNA for SSTR1–SSTR5 has been localized in the cerebral cortex [12]. Recently, our group has shown that a single dose of the pineal indolamine melatonin increased the total number and function of SRIF receptors in rat frontoparietal cortex 5 hr after administration [13]. Therefore, the somatostatinergic system might be one of the mechanisms mediating the acute actions of melatonin in the central nervous system. In this regard, melatonin and SRIF have been shown to decrease locomotor activity after short-term administration [14–18]. On the other hand, Wolden-Hanson et al. [19] have shown that long-term melatonin administration increases locomotor activity. However, the subchronic and chronic effects of melatonin on the somatostatinergic system in the brain are presently unknown. Therefore, the aim of the present study was to determine the subchronic and chronic effects of melatonin treatment

on SRIF receptors, the AC system and SRIF-like immunoreactive levels in the rat frontoparietal cortex. Low concentrations of the stable GTP analogue 5'-guanylimidodiphosphate [Gpp(NH)p] were employed to detect functional Gi activity by inhibiting basal AC activity that had been amplified by forskolin (FK). The levels of the α_1 and α_2 G protein subunits were determined by Western blot using isoform-specific antibodies. As it has been proposed that one mechanism of melatonin action may be through melatonin antagonism to calmodulin [20], the role of calmidazolium (a calmodulin antagonist) on SRIF-receptors was also evaluated in order to investigate the possible mechanism underlying the regulation of SRIF receptors by melatonin.

Materials and methods

Materials

Synthetic Tyr¹¹-SRIF and SRIF tetradecapeptide were purchased from Universal Biologicals Ltd. (Cambridge, UK). Melatonin, bacitracin, phenylmethylsulfonyl fluoride (PMSF), 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin (fraction V) (BSA), guanosine triphosphate (GTP), Gpp(NH)p and FK were purchased from Sigma (Madrid, Spain). Specific antisera against α_1 (MAB3075) or α_2 (MAB3077) G protein subunits were obtained from Chemicon International (California, USA). Nitrocellulose membranes as well as the chemiluminescence Western blotting detection system were purchased from Amersham (Buckinghamshire, UK). Dextran was obtained from Serva Feinbiochemica (Heidelberg, Germany) and carrier-free Na¹²⁵I (IMS 30, 100 mCi/mL) from the Radiochemical Centre (Amersham, Buckinghamshire, UK). Autoradiography was performed with intensifying screens (X-OMAT, UV Film Kodak).

The tracer-specific radioactivity was about 350 Ci/g. The rabbit antibody used in the radioimmunoassay technique was purchased from the Radiochemical Centre (Amersham). This antiserum was raised in rabbits against SRIF-14 conjugated with BSA and it is specific for SRIF but as 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

All procedures conform to the guidelines set by our Animal Care and Use Committee and were approved by the Committee before implementation. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Wistar rats of 200–250 g were used in this specific study. All animals received food and tap water *ad libitum*. Room temperature was kept at 22°C and a 12 hr-day and 12 hr-night cycle was maintained. Melatonin was dissolved in saline containing 0.5% of ethanol and administered subcutaneously (s.c.) in a volume of 100 μ L according to the method described by Alexiuk and Vriend [21] at a dose of 25 μ g/kg for 4, 7 or 14 days. Control animals for each

group were injected with saline containing ethanol. Animals were sacrificed by decapitation 24 hr after the final injection. The brains were removed and the frontoparietal cortex was rapidly dissected as described by Glowinski and Iversen [22].

Tissue extraction and somatostatin radioimmunoassay

For measurements of SRIF-like immunoreactivity content, the frontoparietal cortex was rapidly homogenized in 1-mL 2 M acetic acid using a Brinkman polytron (setting #5 for 30 s). Extracts were boiled for 5 min in a water bath and chilled in ice. Subsequently, homogenates were centrifuged at 15,000 g for 15 min at 4°C. The pellet was discarded and 25 μ L of the supernatant was taken for protein analysis [23]. Extracts were immediately stored at –80°C until the time of assay. The level of immunoreactivity was determined in tissue extracts by a modified radioimmunoassay method [24]. Incubation tubes contained 100- μ L samples of unknown or standard solutions of 0–500 pg cyclic SRIF-14, diluted in phosphate buffer (0.1 M, pH 7.2 containing 0.2% BSA, 0.1% sodium azide), 200 μ L of appropriately diluted anti-SRIF serum, 100 μ L of freshly prepared ¹²⁵I-Tyr¹¹-SRIF, diluted in buffer to yield 6000–10,000 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 at 4°C for 24 hr before incubation. The separation of bound and free hormones was accomplished by addition of 1 mL of dextran-coated charcoal (dextran: 0.2% w/v). The coefficients for intra- and inter-assay variation were 6.5 and 8.3%, respectively.

Binding assay

Tyr¹¹-SRIF was radioiodinated by Chloramine-T iodination according to the method of Greenwood *et al.* [25]. The tracer was purified in a Sephadex G-25 fine column (1 $\times$ 100 cm) equilibrated with 0.1 M acetic acid containing BSA 0.1% (w/v). The specific activity of the purified labeled peptide was about 600 Ci/mmol. Frontoparietal cortical membranes were prepared as previously described by Reubi *et al.* [26]. Protein concentration was assayed by the method of Lowry *et al.* [23], with BSA as a standard. Specific SRIF binding was measured according to the modified method of Czernik and Petrack [27]. Briefly, the membranes (0.15 mg protein/mL) were incubated in 250 μ L of a medium containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM MgCl₂, 0.2% (w/v) BSA and 0.1 mg/mL bacitracin with 250 pM ¹²⁵I-Tyr¹¹-SRIF either in the absence or presence of 0.01–10 nM unlabelled SRIF. After 60 min incubation at 30°C, bound and free ligands were separated by centrifugation at 11,000 g for 2 min and the radioactivity in the resultant pellet was measured. Nonspecific binding was obtained from the amount of radioactivity bound in the presence of 10^{–7} M SRIF and represented about 20% of the binding observed in the absence of unlabelled peptide. This nonspecific component was subtracted from the total bound radioactivity in order to obtain the corresponding specific binding.

Evaluation of radiolabeled peptide degradation

The inactivation of ^{125}I -Tyr¹¹-SRIF in the incubation medium after exposure to membranes was studied by measuring the ability of the preincubated peptide to rebinding to fresh membranes [28]. Briefly, ^{125}I -Tyr¹¹-SRIF (250 pM) was incubated with membranes from the rat frontoparietal cortex (0.15 mg protein/mL) for 60 min at 30°C. After this preincubation, aliquots of the medium were added to fresh membranes and incubated for an additional 60 min at 30°C. The fraction of the added radiolabeled peptide, which 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 membranes during the preincubation period.

Slice preparation

Cross-chopped frontoparietal cortical slices (250 × 250 µm) 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 gassed every 15 min [29].

Adenylyl cyclase assay

AC activity was measured as previously reported [30], with minor modifications. Briefly, frontoparietal cortical membranes (0.06 mg/mL) were incubated with 1.5 mM ATP, 5 mM MgSO₄, 10 µM GTP, an ATP-regenerating system (7.5 mg/mL creatine phosphate and 1 mg/mL creatine kinase), 1 mM IBMX, 0.1 mM PMSF, 1 mg/mL bacitracin, 1 mM EDTA, and test substances (10⁻⁴ M SRIF or 10⁻⁵ M FK) in 0.1 mL of 0.025 M triethanolamine/HCl buffer (pH 7.4). After 15 min of incubation at 30°C, the reaction was stopped by heating the mixture for 3 min. After cooling, 0.2 mL of an alumina slurry (0.75 g/mL in triethanolamine/HCl buffer, pH 7.4) was added and the suspension was centrifuged. The supernatant was taken for assay of cyclic AMP (cAMP) by the Gilman method [31].

Immunodetection of α i subunits of G proteins

Membranes (100 µg) were solubilized in SDS-sample buffer. The proteins were then run on a 12% SDS-polyacrylamide gel as described by Laemmli [32]. After separation, the proteins were transferred onto nitrocellulose membranes in a buffer consisting of 25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20% methanol and 0.05% SDS. The transferred nitrocellulose membranes were blocked with TTBS (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 5% (w/v) non-fat dry milk for 1.5 hr at 4°C. Nitrocellulose membranes were subsequently immunoblotted with anti-Gi2 or anti-Gi2 monoclonal antibodies (1 : 1000 dilution) in TTBS, and incubated overnight at 4°C. After incubation, three 5-min washes in TTBS containing 5% (w/v) non-fat dry milk were carried out. A mouse IgG-peroxidase conjugate (1 : 2000 dilution) in TTBS was then added to the membranes and incubated

for 1 hr at 4°C. After washing, the bound immunoreactive proteins were detected by a chemiluminescent (ECL) Western blotting detection system.

Data analysis

The computer program LIGAND [33] was used to analyze the binding data. The use of this program enabled models of receptors, which 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 [34] 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 less than 0.05. Each individual experiment was performed in duplicate.

Results

The administration of melatonin (25 µg/kg, s.c.) during 4, 7 or 14 days did not affect SRIF-like immunoreactive content in the rat frontoparietal cortex when compared with the control group (Table 1). Scatchard plots of the binding of ^{125}I -Tyr¹¹-SRIF to membranes indicated that 4-day melatonin treatment increased the number of SRIF receptors (Fig. 1; Table 1). In addition, a significant decrease in the affinity of SRIF for its binding sites was observed (Fig. 1; Table 1). However, no changes were observed at either 7 or 14 days of melatonin treatment (Fig. 1; Table 1).

It is known that SRIF receptors are coupled to the inhibition of AC activity [35]. Therefore, we studied SRIF-modulated AC activity by incubating frontoparietal cortical membranes with SRIF (10⁻⁴ M) either in the presence or absence of FK (10⁻⁵ M), which acts directly upon the catalytic AC subunit. As shown in Table 2, after treating animals with melatonin for 4 days, the basal AC activity increased when compared with the controls (Fig. 2). In addition, AC activity was markedly enhanced after FK

Table 1. Effect of melatonin treatment during 4, 7 or 14 days on somatostatin (SRIF) receptors and SRIF-like immunoreactivity content

	SRIF receptors		SRIF-like immunoreactive
	$B_{\max}$	K_d	
Control	444 ± 20	0.34 ± 0.01	20.16 ± 1.63
Melatonin, 4 days	655 ± 15***	0.61 ± 0.04**	18.72 ± 1.67
Control	410 ± 36	0.48 ± 0.05	17.57 ± 1.35
Melatonin, 7 days	460 ± 18	0.43 ± 0.03	15.14 ± 0.72
Control	489 ± 60	0.35 ± 0.06	18.53 ± 0.66
Melatonin, 14 days	505 ± 21	0.48 ± 0.04	17.71 ± 0.46

Binding parameters were calculated from Scatchard plots by linear regression. Units for K_d are nM, units for $B_{\max}$ are fmol of SRIF bound per mg of protein and units for SRIF-like immunoreactive are ng SRIF per mg protein. The results are represented as the mean ± S.E.M. of five separate experiments each performed in duplicate. Statistical comparison versus control: ***P* < 0.01; ****P* < 0.001.

Fig. 1. Scatchard plots of the specific binding of ^{125}I -Tyr 11 -SRIF to frontoparietal cortical membranes. Membranes (0.15 mg protein/mL) were incubated for 60 min at 30°C in the presence of 250 pM ^{125}I -Tyr 11 -SRIF and increasing concentrations of native peptide. Values are expressed as the mean $\pm$ S.E.M. of five separate experiments ($n = 5$) performed in duplicate. The corresponding equilibrium parameters are included in Table 1.

stimulation of membranes from rats previously treated with melatonin as compared with that from untreated controls (Fig. 2). The capacity of SRIF to inhibit basal and FK-stimulated AC activity was markedly increased in frontoparietal cortical membranes from melatonin-treated rats after 4 days of treatment when compared with control animals (Table 2). To test if the observed changes were related to modifications in the expression of the AC, we have measured the response of AC to FK. No significant differences were detected in the fold FK stimulation over basal AC activity between the control and melatonin-treated rats (Table 2). SRIF has been shown to inhibit AC activity through interaction with its receptors coupled to the enzyme via a Gi protein [35]. Therefore, functional Gi activity was examined in the frontoparietal cortical membranes of control and 4-day melatonin-treated rats in order to find out whether the increase in SRIF receptors and/or SRIF-modulated AC activity observed at 4 days of treat-

ment could be because of the alteration at the Gi protein level. In the presence of FK (3×10^{-6} M), which amplifies basal AC activity, increasing concentrations of the stable GTP analog, 5'-guanylimidodiphosphate (Gpp(NH)p), produce an inhibitory effect that was used as a measure of Gi function. Using this assay, membranes derived from control and melatonin-treated rats yield a characteristic biphasic response curve (Fig. 3). Gpp(NH)p concentrations between 10^{-11} and 10^{-8} M decreased AC activity because of Gi activation whereas higher nucleotide concentrations (10^{-7} to 10^{-4} M) resulted in stimulation of both, the AC and Gs activities. No significant differences in Gpp(NH)p-mediated inhibition of FK-stimulated AC activity were observed between the control and 4-day melatonin treated animals (Fig. 3).

Previous studies have also demonstrated that SRIF receptors are coupled to the inhibition of AC activity through Giz_1 and Giz_2 proteins, among others [35].

Table 2. Effect of somatostatin (SRIF) and forskolin (FK) on brain adenylyl cyclase activity (pmol cAMP/min/mg protein) in frontoparietal cortical membranes from control and melatonin (25 $\mu\text{g/kg}$)-treated rats for 4, 7 or 14 days

	4 days		7 days		14 days	
	Control	Melatonin	Control	Melatonin	Control	Melatonin
Basal activity	212.8 $\pm$ 14.8	297.2 $\pm$ 29.5*	238.1 $\pm$ 9.2	212.5 $\pm$ 2.5	208.0 $\pm$ 1.9	195.9 $\pm$ 8.0
+ 10^{-4} M SRIF	163.5 $\pm$ 11.9	183.9 $\pm$ 22.1	139.9 $\pm$ 2.0	133.1 $\pm$ 7.7	157.6 $\pm$ 7.0	145.2 $\pm$ 3.5
+ 10^{-5} M FK	597.8 $\pm$ 12.1	989.3 $\pm$ 59.0***	703.5 $\pm$ 64.5	664.7 $\pm$ 41.2	813.8 $\pm$ 15.1	811.6 $\pm$ 40
+ 10^{-5} M FK + 10^{-4} M SRIF	484.5 $\pm$ 25.9	659.4 $\pm$ 50.5	502.1 $\pm$ 9.9	488.5 $\pm$ 38.9	620.7 $\pm$ 29.2	648.9 $\pm$ 30.3
Fold FK stimulation over basal	2.9 $\pm$ 0.2	3.41 $\pm$ 0.34	3.0 $\pm$ 0.16	3.1 $\pm$ 0.44	3.9 $\pm$ 0.9	4.1 $\pm$ 0.13
Percentage SRIF inhibition of basal activity	23.0 $\pm$ 3.1	37.9 $\pm$ 4.7*	41.2 $\pm$ 6.9	37.4 $\pm$ 9.2	24.22 $\pm$ 2.7	25.9 $\pm$ 2.6
Percentage SRIF inhibition of FK stimulation	19.1 $\pm$ 3.1	33.49 $\pm$ 1.8**	28.6 $\pm$ 5.6	26.5 $\pm$ 1.5	23.73 $\pm$ 2.5	20.0 $\pm$ 2.9

Values represent the mean $\pm$ S.E.M. of five separate experiments each performed in duplicate. Statistical comparison versus control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. Basal and forskolin (FK)-stimulated cAMP levels in control and melatonin-treated rats after 4 days of melatonin treatment. Membrane preparations were incubated in absence (basal) or presence of 10^{-5} M FK. Data are expressed as the mean $\pm$ S.E.M. of five separate experiments ($n = 5$), each performed in duplicate. Statistical comparison versus control; * $P < 0.5$; *** $P < 0.001$.

Western blot analyses were carried out in order to determine whether the increase observed in the capacity of SRIF to inhibit basal and FK-stimulated AC activity in membranes from 4-day melatonin-treated rats, as compared with control rats, could be because of the alterations of G_{i1} and G_{i2} protein levels. Melatonin administration for 4 days did not change the amount of the G_{i1} subunit but reduces the amount of the G_{i2} subunit in frontoparietal cortical membranes (Fig. 4). After 7 or 14 days of melatonin treatment, there were no changes in either the inhibitory effect of SRIF on basal and FK-stimulated AC activity (Table 2) or the amount of the G_{i1} and G_{i2} subunits (Fig. 4).

In recent years, it has been proposed that one melatonin mechanism of action may be through the modulation of Ca^{2+} -activated calmodulin as melatonin is considered to be a selective calmodulin antagonist [20]. Thus, melatonin antagonism to calmodulin might be involved in the melatonin-induced effect on the rat frontoparietal cortical somatostatinergic system. To examine this hypothesis, the effect of calmidazolium, a calmodulin antagonist, was tested. The *in vitro* addition of melatonin (10^{-6} M) induced an increase in the number of SRIF receptors in membranes from frontoparietal cortical slices (Table 3) whereas calmidazolium led to a decrease in the number of these receptors (Table 3).

Discussion

There is evidence that melatonin has anti-convulsive properties and decreases locomotor activity [36]. These

Fig. 3. Dose-effect curves for 5'-guanylylimidodiphosphate [Gpp(NH)p]-mediated inhibition of adenylyl cyclase activity in rat frontoparietal cortical membranes from control ($n = 5$) and melatonin ($25 \mu\text{g/kg}$)-treated ($n = 5$) rats for 4 days. The effect of Gpp(NH)p on adenylyl cyclase activity was studied in the presence of 3×10^{-6} M forskolin and the indicated concentrations of Gpp(NH)p. Data are expressed as a percentage of forskolin-stimulated adenylyl cyclase activity in the absence of Gpp(NH)p (100%). The results are given as the mean $\pm$ S.E.M. of five separate determinations ($n = 5$), each performed in duplicate. No statistically significant differences were obtained between the control and melatonin-treated rats for 4 days.

effects are reminiscent of those produced by SRIF [17, 37, 38]. It has also been demonstrated that melatonin influences the electrical activity of the rat cerebral cortex, a brain area rich in SRIF content and receptors [39] as well as in melatonin receptors [40]. On the other hand, acute melatonin treatment increases SRIF binding in frontoparietal cortical membranes. So, it is reasonable to hypothesize that the effect of this agent could involve its action on SRIF binding sites. In the present study, a similar enhancement in the number of ^{125}I -Tyr¹¹-SRIF binding sites as that demonstrated after acute melatonin treatment [13] was observed, which supports the hypothesis that melatonin's actions involve, at least in part, a modulation of somatostatinergic activity in the rat frontoparietal cortex. Melatonin not only increases the number of SRIF receptors but also decreases their affinity. The fact that both the number and the affinity of SRIF receptors are altered suggests that the interaction of ligand-receptor-G protein is modified. In fact, melatonin treatment induced an alteration in the functionality of G_i and G_s proteins. The mechanism by which melatonin increases the number of SRIF receptors is still unknown and remains obscure and subject to speculation. So far, five different SRIF receptor subtypes have been cloned [35] and the tissue distribution of the mRNA

Fig. 4. Western blot analysis of $Gi\alpha_1$ and $Gi\alpha_2$ subunits in frontoparietal cortical membranes. Frontoparietal cortical membranes from control and melatonin (25 $\mu\text{g/kg}$)-treated rats were solubilized in SDS-sample buffer. Proteins (100 μg) were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using the specific mouse anti-Gi monoclonal antibodies, $Gi\alpha_1$ (upper panel) and $Gi\alpha_2$ (lower panel). This experiment is representative of five others. C: control rats; M: melatonin-treated rats. The results are given as the mean $\pm$ S.E.M. of five separate determinations, each performed in duplicate. Statistical comparison versus control: *** $p < 0.001$. ■ melatonin, □ control.

Table 3. Effect of melatonin (10^{-6} M) or calmidazolium (50 μM) on SRIF receptors

	Control	Melatonin	Calmidazolium
K_d	0.5 ± 0.06	$0.69 \pm 0.03^*$	$0.26 \pm 0.05^*$
B_{max}	473 ± 23	$732 \pm 21^{***}$	$316 \pm 20^{***}$

Frontoparietal cortical slices were incubated at 37°C for 2.5 min with melatonin (10^{-6} M) or calmidazolium (50 μM) for 15 min. Binding parameters were calculated from Scatchard plots by linear regression. Units for K_d are nM, units for B_{max} are fmol of SRIF bound per mg of protein. The results are represented as the mean $\pm$ S.E.M. of five separate experiments each performed in duplicate. Statistical comparison versus control: * $p < 0.05$; *** $p < 0.001$.

for each subtype has been studied in the rat [12]. The five SRIF receptor subtypes are expressed in the rat brain and have a similar affinity for SRIF [35]. Thus, a linear Scatchard plot only reveals that all SRIF receptor subtypes have similar affinity for the radioligand used in this study. The increase in $^{125}\text{I-Tyr}^{11}$ -SRIF binding was not because of a direct effect of melatonin on SRIF receptors, as no change in tracer binding was detected following incubation of fresh frontoparietal cortical membranes with melatonin. Additional studies are required to elucidate the mechanisms implicated in the observed increase of the SRIF receptors after melatonin treatment.

Melatonin enhances SRIF receptor-effector system after 4 days of treatment and reverses after 7 and 14 days to control values. This finding is in agreement with studies

from Hadley [41] who demonstrated that continuous exposure to melatonin renders the target tissue unresponsive to the hormone. Previously, it had been reported that the total number of melatonin receptors in the hippocampus midbrain [42], and pars tuberalis of the anterior pituitary [43] may be reduced following prolonged exposure to melatonin.

The present study shows that subchronic (4 days) melatonin treatment sensitizes the cAMP transduction system in the rat frontoparietal cortex, as revealed by subsequent increases in basal and FK-stimulated AC activities. These results are in agreement with the studies *in vitro* in which prolonged exposure to melatonin leads to tissue-dependent sensitization of AC in pars tuberalis cells from ovine pituitary [43]. Sensitization of AC after prolonged exposure to inhibitory agonists is a well-established phenomenon [44, 45]. The cAMP-mediated signal transduction system includes several mechanisms through which sensitization can be achieved. To determine whether the sensitization observed in the present study involved changes in the activity of G-proteins, the functional Gi protein activity was measured by studying the effects of increasing concentrations of the stable GTP analog Gpp(NH)p on FK-stimulated AC activity. In both, the control and melatonin-treated rats, Gpp(NH)p elicited a characteristic biphasic effect on FK-stimulated AC activity, with low concentrations inhibiting FK-stimulated AC activity and higher concentrations stimulating it. This biphasic effect is because of the activation of Gi and Gs proteins, respectively, owing to the higher affinity of Gi proteins for Gpp(NH)p [46]. The inhibitory effect of low Gpp(NH)p

concentrations on FK-stimulated AC activity was similar in the control and 4-day melatonin treated rats, indicating no change in functionality at the Gi protein level. Another plausible hypothesis is that the expression of G-proteins, which exert a tonic inhibitory effect upon the activation of AC, is reduced by prolonged receptor stimulation. Such a mechanism has precedent in studies of prolonged exposure to hormones in several other receptor systems [47, 48]. To test this possibility, we performed Western blot analyses of G_{i1} and G_{i2} proteins. These studies revealed that melatonin treatment induces a significant decrease in G_{i2} levels without affecting G_{i1} levels. Therefore, it is possible to speculate that sensitization of AC observed in frontoparietal cortex after subchronic exposure to melatonin may be related to the decrease in G_{i2} protein levels. Moreover, Brydon et al. [49] have demonstrated that G_{i2} and G_{i3} are coupled to melatonin receptors while G_{i1} did not coprecipitate with these receptors.

Basal and FK-stimulated AC activity was inhibited by SRIF in all the experimental groups studied, which is in agreement with the literature [35]. A relatively high concentration of SRIF was required to produce AC inhibition. The same concentration was required by other authors in their studies on SRIF-mediated inhibition of brain AC [50, 51]. A possible explanation for this SRIF concentration may lie in the cell heterogeneity of the present preparations. Separation of brain culture into specific glial and neuronal preparations has made it possible to identify and determine the cell localization of SRIF-inhibited basal AC [52, 53]. Thus, SRIF inhibited basal AC activity in neuronal but not glial cells. Therefore, owing to the different responses of neuronal and glial cells to AC inhibition by SRIF, it is possible that the cell heterogeneity of the present preparations may be the cause of the high SRIF concentration necessary to obtain inhibition of the enzyme. In addition, the synaptic concentration of SRIF is, to date, unknown. The capacity of SRIF to inhibit basal and FK-stimulated AC activity was significantly higher in the melatonin-treated rats than in the control group, probably because of the SRIF receptor up-regulation. On the other hand, and as previously mentioned, melatonin administration for 4 days sensitizes AC. Therefore, the increased sensitivity of the enzyme could explain the fact that an inhibitory peptide like SRIF has a greater inhibitory effect on AC activity. The increase in the capacity of SRIF to inhibit AC activity was not because of the increase in the activity of Gi proteins as no significant changes were observed in the functionality of these proteins. SRIF receptors mediate their inhibitory effect on AC activity by coupling to G_{i1} , G_{i2} and G_{i3} proteins [35]. Despite their functional similarity, these Gi proteins exhibit differential preference for coupling to receptors. For example, the SRIF receptor subtype 3 selectively couples to G_{i1} , but not to G_{i2} or G_{i3} [54]. As G_{i2} levels were lower after subchronic melatonin treatment, it is possible that the increase in the capacity of SRIF to inhibit AC activity was mediated by G_{i1} or G_{i3} but not by G_{i2} . After 7 or 14 days of melatonin administration, there were no significant changes in the AC activity, this observation is consistent with the possibility that the frontoparietal cortex is involved in

the mediation of melatonin-dependent phenomena in rats during subchronic periods.

Several studies have suggested that calmodulin may be involved in the mechanism of action of melatonin and suggest a role of melatonin as a selective calmodulin antagonist [20, 55]. In order to test the possibility that melatonin increases the number of SRIF receptors via antagonism of calmodulin, the calmodulin antagonist calmidazolium was used. The *in vitro* addition of calmidazolium decreased the number of SRIF receptors in frontoparietal cortical slices whereas melatonin leads to an increase in these receptors. This finding suggest that calmodulin is not implicated in the up-regulation of SRIF receptors.

Acute [13] and subchronic (4 days) melatonin administration increases the activity of the somatostatinergic system in the frontoparietal cortex. This suggests that SRIF could be implicated in the mechanism of action of melatonin. As melatonin and SRIF both decrease locomotor activity [14–16], it is tempting to speculate that SRIF could be implicated in the effect of melatonin on locomotor activity. However, the fact that chronic melatonin administration increases the spontaneous physical activity [19] but does not modify the somatostatinergic system suggests that SRIF is not implicated in this effect of melatonin on locomotor activity.

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