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Acutely administered melatonin decreases somatostatin-binding sites and the inhibitory effect of somatostatin on adenylyl cyclase activity in the rat hippocampus

Abstract: Melatonin is known to increase neuronal activity in the hippocampus, an effect contrary to that of somatostatin (somatotropin release-inhibiting factor, SRIF). Thus, the aim of this study was to investigate whether the somatostatinergic system is implicated in the mechanism of action of melatonin in the rat hippocampus. One group of rats was injected a single dose of melatonin [25 μg/kg subcutaneously (s.c.)] or saline containing ethanol (0.5%, s.c.) and killed 5 hr later. Melatonin significantly decreased the SRIF-like immunoreactivity levels and induced a significant decrease in the density of SRIF receptors as well as in the dissociation constant (K_d). SRIF-mediated inhibition of basal and forskolin-stimulated adenylyl cyclase activity was markedly decreased in hippocampal membranes from melatonin-treated rats. The functional activity of Gi proteins was similar in hippocampal membranes from melatonin-treated and control rats. Western blot analyses revealed that melatonin administration did not alter Giα1 or Giα2 levels. To determine if the changes observed were related to melatonin-induced activation of central melatonin receptors, a melatonin receptor antagonist, luzindole, was administered prior to melatonin injection. Pretreatment with luzindole (10 mg/kg, s.c.) did not alter the melatonin-induced effects on the above-mentioned parameters and luzindole, alone, had no observable effect. The present results demonstrate that melatonin decreases the activity of the SRIF receptor–effector system in the rat hippocampus, an effect which is apparently not mediated by melatonin receptors. As SRIF exerts an opposite effect to that of melatonin on hippocampal neuronal activity, it is possible that the SRIFergic system could be implicated in the mechanism of action of melatonin in the rat.

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Introduction

Melatonin (*N*-acetyl-5-methoxytriptamine) is the principal secretory product produced by the pineal gland. It is currently thought that melatonin reaches its neural targets via the peripheral circulation [1]. In addition, cerebrospinal fluid melatonin seems to originate directly from the pineal gland. Thus, melatonin could reach its targets through the cerebroventricular system [2]. To date, only MT_1 and MT_2 receptors have been detected in mammals [3]. The distribution pattern of these melatonin receptors in the central nervous system (CNS) has been extensively studied in different species in recent years [3, 4]. Some of the responses of melatonin have been reported to be mediated through GABA-benzodiazepine receptors in the CNS [5–7]. In addition, melatonin is known to possess free radical scavenging and antioxidant properties [8, 9].

Melatonin has been shown to inhibit the excitability of the CNS [10]. However, it has been recently shown that melatonin increases neuronal activity in hippocampal neurons [11, 12]. These effects are contrary to that of the tetradecapeptide somatotropin (somatostatin release-inhibiting factor, SRIF) in this brain area [13, 14]. In the hippocampus, immunohistochemical studies have revealed many SRIF-containing interneurons and a profuse network of intrinsic and extrinsic SRIF-containing fibers that appear to project to pyramidal and granule neurons [15]. The hippocampus has been found to be highly enriched with SRIF receptors concentrated in the molecular layer of the dentate gyrus and the dendritic fields of hippocampal CA1 pyramidal cells [16] suggests that the SRIF receptors are localized on intrinsic neurons of the hippocampus. Candidates are the pyramidal and granule cells, which correlates well with the majority of the immunohistochemical studies suggesting the presence of SRIF terminals in close vicinity to these cells [17]. Five SRIF receptor subtypes, designated SSTR1–5, have been cloned and shown to belong to the seven transmembrane domain receptor family [18]. All SRIF receptor subtypes are linked to guanine nucleotide-binding proteins (G proteins) [19]

and lead to adenylyl cyclase (AC) inhibition following hormone binding [20]. The SRIF receptors also regulate other effectors via G proteins, which include inhibition of calcium channels, stimulation of potassium channels and stimulation of serine/threonine and tyrosine phosphatases [21].

The aim of this study was to investigate if the SRIFergic system is implicated in the mechanism of action of melatonin in the rat hippocampus. The effects of melatonin on SRIF-like immunoreactivity (SRIF-LI) content and ^{125}I-Tyr^{11}-SRIF binding to its specific receptors in the rat hippocampus were evaluated. The integrity of SRIF receptor function was determined by assaying the ability of SRIF to inhibit AC activity. In addition, we assessed the functional activity of the guanine nucleotide-binding inhibitory protein (Gi) and determined the levels of the αi1 and αi2 G protein subunits by Western blot using isoform-specific antibodies.

Materials and methods

Materials

Synthetic Tyr^{11}-SRIF and SRIF tetradecapeptide were purchased from Universal Biologicals Ltd (Cambridge, UK). Melatonin, bacitracin, bovine serum albumin (fraction V) (BSA) phenylmethylsulfonyl fluoride (PMSF), 3-isobutyl-1-methylxanthine (IBMX), guanosine triphosphate (GTP), forskolin (FK), prestained protein markers and other reagents for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from Sigma (Madrid, Spain). Luzindole was supplied by Alexis Corporation (Nottingham, UK). Dextran was obtained from Serva Feinbiochemica (Heidelberg, Germany) and carrier-free Na[^{125}I] (IMS 30, 100 mCi/mL) from the Radiochemical Centre (Amersham, Buckinghamshire, UK). Specific antiserum against αi1 (MAB3075) or αi2 (MAB3077) G protein subunits was obtained from Chemicon International (Temecula, CA, USA). Nitrocellulose membranes as well as the chemiluminescence Western blotting detection system were purchased from Amersham. The rabbit antibody used in the radioimmunoassay technique was purchased from the Radiochemical Centre. This antiserum was raised in rabbits against SRIF-14 conjugated with BSA and 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. Male Wistar rats weighing 200–250 g were used in the present study. All animals received food and tap water ad libitum. Room temperature was kept at 22°C and a 12 hr day–night cycle was maintained. Melatonin was dissolved in saline containing ethanol (0.5%) and administered subcutaneously (s.c.) at 09:00 hr in a volume of 100 μL according to the method described by Alexiuk and Vriend [22] at a dose of 10, 25, 50 or 100 μg/kg (s.c.) and the rats were killed at 14:00 hr. A second experimental group of rats was injected a single dose of melatonin (25 μg/kg, s.c.) at 09:00 hr and killed at 14:00 hr. A third experimental group of rats received the melatonin receptor antagonist luzindole, dissolved in saline, at a dose of 10 mg/kg (s.c.) [23] at 09:00 hr and killed at 14:00 hr. A fourth experimental group of rats was injected luzindole as described above, 30 min prior to melatonin (25 μg/kg, s.c.) injection and were killed at 14:30 hr. Control animals for each group were injected with saline containing ethanol. The brains were removed and the hippocampus was rapidly dissected as described by Glowinski and Iversen [24].

Binding assay

Tyr^{11}-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 × 100 cm) equilibrated with 0.1 M acetic acid containing BSA 0.1% (w/v). The specific activity of the purified labelled peptide was about 600 Ci/g.

Hippocampal membranes were prepared as previously described by Reubi et al. [26]. Protein concentration was assayed by the method of Lowry et al. [27], with BSA as a standard. Specific SRIF binding was measured according to the modified method of Czernik and Petrack [28]. Briefly, the membranes (0.15 mg protein/mL) were incubated in 250 μL of a medium containing 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.2% (w/v) BSA and 0.1 mg/mL bacitracin with 250 pM [^{125}I]-Tyr^{11}-SRIF either in the absence or presence of 0.01–10 nM unlabeled SRIF. After a 60-min incubation at 30°C, bound and free ligand were separated by centrifugation at 11,000 g for 2 min. The supernatant was discarded and the pellet was washed with Tris (50 mM)–sucrose (0.9%) buffer (pH 7.4). The radioactivity in the pellet was measured in a Kontron gamma counter. Non-specific 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 unlabeled peptide. This non-specific component was subtracted from the total bound radioactivity in order to obtain the corresponding specific binding.

Evaluation of radiolabelled peptide degradation

The inactivation of [^{125}I]-Tyr^{11}-SRIF in the incubation medium after exposure to membranes was studied by measuring the ability of preincubated peptide to rebind to fresh membranes. Briefly, [^{125}I]-Tyr^{11}-SRIF (250 pM) was incubated with membranes from rat hippocampus (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 radiolabelled 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.

Tissue extraction and somatostatin radioimmunoassay

For measurements of SRIF-LI content, the hippocampus was rapidly homogenized in 1 mL of 2 M acetic acid using a Brinkman polytron (setting 5, 30 s). Extracts were boiled for 5 min in a water bath and chilled on ice. Subsequently, homogenates were centrifuged at 15,000 g for 15 min at 4°C. The pellet was discarded and 25 μL of the supernatant were taken for protein analysis [27]. Extracts were immediately stored at −80°C until assay. The immunoreactivity content was determined in tissue extracts by a modified radioimmunoassay method [29], 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 SRIF 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, in order 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 μL samples of unknown or standard solutions of 0–500 pg cyclic SRIF tetradecapeptide, diluted in phosphate buffer (0.1 M, pH 7.2 containing 0.2% BSA, 0.1% sodium azide), 200 μL of appropriately diluted anti-SRIF serum, 100 μL of freshly prepared [^{125}I]-Tyr^{11}-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 before incubation at 4°C for 24 hr. Separation of bound and free hormone was accomplished by addition of 1 mL dextran-coated charcoal (dextran: 0.2% w/v). Dilution curves for each brain area were parallel to the standard curve. The coefficients for intra- and inter-assay variation were 6.5 and 8.3%, respectively.

AC assay

Hippocampal membranes were prepared as previously described by Reubi et al. [26]. Protein concentration was assayed by the method of Lowry et al. [27] with BSA as a standard. AC activity was measured as previously described [30], with some minor modifications. Briefly, hippocampal membranes (0.06 mg/mL) were incubated with 1.5 mM ATP, 5 mM $MgSO_4$, 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 a 15-min incubation at 30°C, the reaction was stopped by heating the mixture at 100°C for 3 min. Once cooled, 0.2 mL of an aluminum slurry (0.75 g/mL in triethanolamine/HCl buffer, pH 7.4) were added and the suspension was centrifuged. The supernatant was then removed for assay of cyclic AMP (cAMP) by the method described by Gilman [31]. The SRIF concentration used was that deemed necessary to achieve inhibition of rat AC activity [32]. Similarly, FK was used at a concentration that could effectively stimulate the catalytic subunit of rat AC [32].

Immunodetection of G protein αi subunits

Membranes (100 μg) were solubilized in SDS sample buffer and the resulting proteins were then run on a 12% SDS–polyacrylamide gel as described by Laemmli [33]. 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 Tris-buffered saline with Tween 20 [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20) containing 5% (w/v) (TTBS)] non-fat dry milk during 1.5 hr at 4°C. Nitrocellulose membranes were subsequently immunoblotted with anti-Giα1 or anti-Giα2 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 an enhanced chemiluminescent detection system ECL™ kit.

Data analysis

The computer program LIGAND [34] 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 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's Newman–Keuls test. Mean among groups were considered significantly different when the P values were less than 0.05. Each individual experiment was performed in duplicate.

Results

Melatonin administration at a single dose decreased SRIF-LI content in the rat hippocampus as compared with the control group (Fig. 1). The specific binding of [^{125}I]-Tyr^{11}-SRIF to hippocampal membranes was studied in rats treated with 10, 25, 50 or 100 μg/kg (s.c.) of melatonin. The doses of 10 and 25 μg/kg significantly decreased the specific binding of [^{125}I]-Tyr^{11}-SRIF to rat hippocampal membranes as compared with the control group (Table 1), with no changes being observed at the higher doses. As the maximal effect of melatonin on SRIF receptors was detected at the dose of 25 μg/kg (Table 1), subsequent studies were therefore carried out at this dose. Stoichiometric experiments were performed on rat hippocampal membranes using a fixed concentration of [^{125}I]-Tyr^{11}-SRIF and increasing doses of unlabeled SRIF at 30°C for 60 min. A single dose of melatonin decreased the number of SRIF receptors and increased their apparent affinity in the hippocampal membranes at 5 hr of its administration.

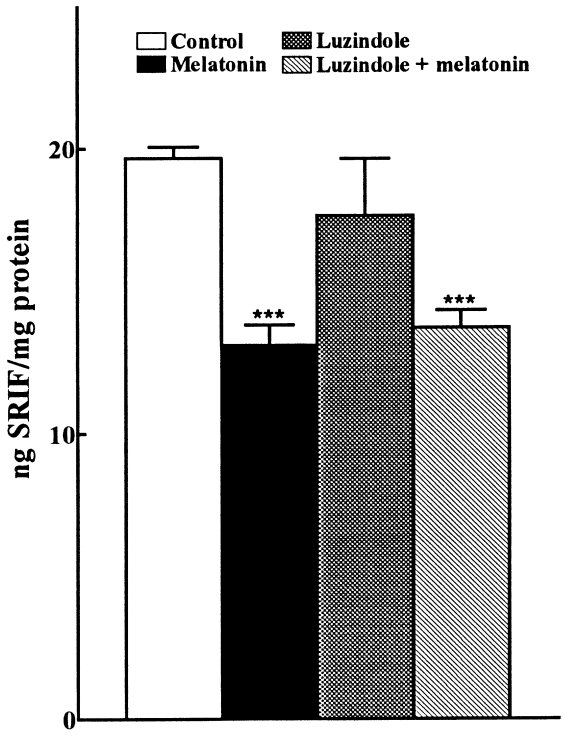

Fig. 1. Effect of melatonin (25 μg/kg) treatment for 5 hr on somatostatin (SRIF)-like immunoreactivity in the rat hippocampus. Values are expressed as the mean ± S.E.M. of five experiments, each performed in duplicate. Statistical comparison vs. control: ***$P < 0.001$.

Table 1. Effect of increasing melatonin concentrations (10, 25, 50 or 100 μg/kg, s.c.) on equilibrium parameters for [^{125}I]-Tyr^{11}-SRIF binding to hippocampal membranes

	SRIF receptors	
Groups	B_{max}	K_d
Control	486 ± 42	0.46 ± 0.03
Melatonin		
10 μg/kg	346 ± 13**	0.34 ± 0.02*
25 μg/kg	322 ± 17**	0.32 ± 0.02**
50 μg/kg	467 ± 29	0.36 ± 0.06
100 μg/kg	486 ± 14	0.41 ± 0.03

Binding parameters were calculated from Scatchard plots by linear regression.
K_d and B_{max} are expressed as nanomoles and femtomoles, respectively, of SRIF bound per milligram of protein.
The results are represented as the mean ± S.E.M. of five separate experiments performed by duplicate in individual rats.
Statistical comparison vs. control: *$P < 0.05$; **$P < 0.01$.

Hippocampal membranes from melatonin-treated (5 hr) rats exhibited a significant decrease in the maximum SRIF-binding capacity, with changes in the corresponding K_d values (Fig. 2, right panel; Table 2) as compared with the control group. The in vitro addition of increasing concentrations of melatonin (10^{-11}–10^{-5} M) to the incubation medium at the time of the binding assay had no effect on hippocampal SRIF receptors (data not shown). Pretreatment with the melatonin receptor antagonist luzindole (10 mg/kg, s.c.) did not alter the melatonin-induced effect on SRIF-LI content and SRIF binding (Fig. 2, right panel; Table 2). Administration of luzindole alone had no observable effect on these parameters (Table 2).

In order to investigate whether SRIF function was affected by melatonin treatment, hippocampal membranes from control and melatonin-treated rats were assayed for SRIF-induced inhibition of AC activity. In the melatonin- or luzindole plus melatonin-treated groups, the degree of SRIF inhibition of both basal and FK-stimulated activity was significantly lower than in the control group after 5 hr of a single dose of melatonin (Table 3).

To test if the observed changes were related to modifications in the expression of AC, the response of the enzyme to the diterpene FK (10^{-5} M), which is assumed to act directly upon the catalytic subunit, was measured. No significant differences were detected in the fold FK stimulation over basal AC activity between the control group and rats treated with melatonin (Table 3). Pretreatment with the melatonin receptor antagonist luzindole (10 mg/kg, s.c.) did not alter the melatonin-induced effect on SRIF-mediated inhibition of both basal and FK-stimulated AC activity.

Further experiments were carried out in order to explore the effect of melatonin on the functionality of Gi or Gs proteins in rat hippocampal membranes by determining the ability of low and high Gpp(NH)p concentrations to inhibit FK (3×10^{-6} M)-stimulated AC activity. A characteristic biphasic response curve was obtained in all experimental groups. Gpp(NH)p concentrations ranging from 10^{-11} to 10^{-7} M decreased AC activity due to Gi activation, whereas higher nucleotide concentrations (10^{-6}–10^{-4} M) resulted in stimulation of both AC and Gs activities. Hippocampal membranes from control and melatonin-treated rats showed similar functionality of Gi and Gs (Fig. 3).

In order to investigate whether Giα1 and Giα2 levels were affected by melatonin, Western blot analyses were performed. Melatonin administration did not significantly alter the amount of the Giα1 or Giα2 subunits in hippocampal membranes after 5 hr of a single dose (data not shown).

Discussion

The present study demonstrates that a single dose of melatonin decreases the activity of the rat hippocampal somatostatinergic system. The doses of melatonin tested herein were in the range of those used by other authors for similar studies of melatonin actions in the CNS [35]. The SRIF-LI levels in the control animals, as determined by radioimmunoassay, were similar to those previously reported by our group and other authors [29, 36]. The SRIF-LI content was decreased in the hippocampus 5 hr after administration of 25 μg/kg of melatonin.

The equilibrium parameters of the SRIF receptors in the hippocampus of control rats were similar to those previously reported by our group and others [36, 37]. Melatonin produced a significant decrease in the number of ^{125}I-Tyr^{11}-SRIF receptors in the hippocampus. Pretreatment with luzindole, a competitive antagonist of the melatonin receptors, did not alter the melatonin-induced effect on SRIF-LI content and SRIF binding. In addition, administration of luzindole alone had no observable effect on these two parameters. These results suggest that the effects of

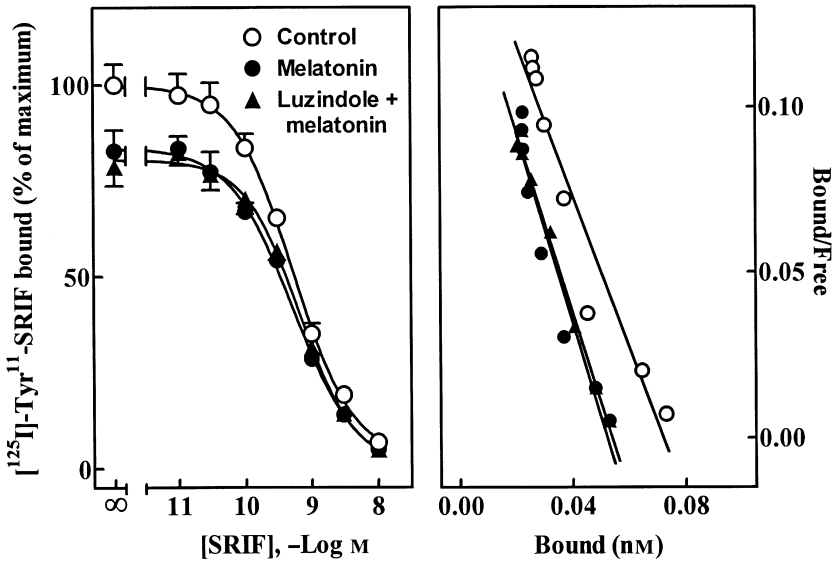

Fig. 2. Binding of [^{125}I]-Tyr^{11}-SRIF to rat hippocampal membranes. Left panel: Displacement of [^{125}I]-Tyr^{11}-SRIF binding by increasing concentrations of unlabelled SRIF. Specific binding was calculated as described in Materials and methods. Membranes (0.15 mg of protein/mL) were incubated for 60 min at 30°C in the presence of 250 pM [^{125}I]-Tyr^{11}-SRIF and increasing concentrations of SRIF. Points correspond to values for the animals in the control group (○, n = 5), the melatonin (25 μg/kg, s.c.)-treated group (●, n = 5) and the luzindole (10 mg/kg, s.c.) plus melatonin (25 μg/kg, s.c.)-treated group (▲, n = 5). The animals were killed 5 hr after the last injection. The control group corresponds to a pool of the control rats, as the B_{max} and K_d values of the different controls were not affected by the vehicle. Each point is the mean of five separate experiments, each performed in duplicate. Right panel: Scatchard analysis of the binding data.

Table 2. Effect of a single dose of melatonin (25 μg/kg), luzindole (10 mg/kg) or luzindole (10 mg/kg) plus melatonin (25 μg/kg) on the equilibrium parameters for [^{125}I]-Tyr^{11}-SRIF binding to rat hippocampal membranes

	SRIF receptors	
Groups	B_{max}	K_d
Control	518 ± 20	0.46 ± 0.03
Melatonin	349 ± 24***	0.35 ± 0.03*
Luzindole	480 ± 57	0.50 ± 0.04
Luzindole + melatonin	360 ± 14***	0.36 ± 0.02*

Binding parameters were calculated from Scatchard plots by linear regression.
K_d and B_{max} are expressed as nanomoles and femtomoles, respectively, of SRIF bound per milligram of protein.
The results are represented as the mean ± S.E.M. of five experiments performed in duplicate.
Statistical comparison vs. control: *$P < 0.05$; ***$P < 0.001$.

melatonin on the SRIFergic system are not mediated by melatonin receptors. Li et al. [6] showed that the effects of melatonin on isolated carp retinal neurons were not blocked by luzindole. Wan et al. [12] showed that melatonin inhibits GABA-induced Cl^- currents in the hippocampus, unlike other brain areas where melatonin potentiates GABA-induced Cl^- currents. Several studies indicate that there is a binding site for melatonin on the $GABA_A$ receptor [38]. This binding site may not distinguish subtle difference between melatonin and its competitive antagonist luzindole. Luzindole modulates the GABA-induced currents in a way similar to melatonin [6]. Several experiments indicate that there is a binding site of melatonin on the $GABA_A$ receptor [38]. Therefore, it is possible that the modulatory effect of melatonin on the $GABA_A$ receptors may be due to an allosteric action caused by melatonin binding to a modulatory site on these receptors. This may explain why the melatonin-induced effect on SRIF binding was not blocked by luzindole, a competitive antagonist of the melatonin receptor, and why luzindole modulates the GABA-induced currents in a way similar to that of melatonin [6]. Previous studies of our group suggest that stimulation of GABAergic neurotransmission decreases the number of SRIF receptors in the rat frontoparietal cortex [39]. Immunochemical studies have revealed GABAergic innervation of SRIF-containing neurons in the hippocampus [40], as well as the presence of GABA receptors on SRIF neurons in this brain area [41]. In addition, colocalization of GABA and SRIF and reciprocal modulation of SRIF and GABA release have been described [42, 43]. GABA tonically inhibits SRIF release [44] and synthesis of SRIF [45]. Therefore, if melatonin inhibits GABA receptor function in the rat hippocampus [41] this may lead to an increase in SRIF release and therefore a decrease in SRIF content in the hippocampus. Increased SRIF release might lead to down-regulation of SRIF receptors from adjacent SRIF-containing neurons. Receptor density has been previously shown to be modulated by changes in tissue SRIF concentration [46–48].

At present, there is evidence supporting the hypothesis that changes in membrane polarization may induce modifications in the number of receptors present in the membrane of neuronal cells [49]. As melatonin inhibits GABA-induced Cl^- currents in the hippocampus, inhibition of GABA-induced neuronal hyperpolarization might be implicated in the action of melatonin on the SRIFergic system. In this regard, our group has shown that glycine, which causes neuronal hyperpolarization upon binding to

Table 3. Effect of somatostatin (SRIF) (10^{-4} M) and forskolin (FK) (10^{-5} M) on brain adenylyl cyclase activity (pmol cAMP/min/mg protein) in hippocampal membranes from control- (n = 15), melatonin- (n = 5), luzindole- (n = 5) and luzindole plus melatonin-treated (n = 5) rats

	Control	Melatonin	Luzindole	Luzindole + melatonin
Basal activity	287.6 ± 10.3	299.3 ± 11.7	253.9 ± 29.8	329.0 ± 36.7
+ 10^{-4} M SRIF	211.5 ± 8.3	260.3 ± 24.0	181.6 ± 23.0	295.0 ± 38.9
+ 10^{-5} M FK	740.7 ± 32.3	792.2 ± 108.4	692.7 ± 74.4	815.1 ± 87.0
10^{-5} M FK + 10^{-4} M SRIF	553.6 ± 13.3	693.3 ± 90.3	528.0 ± 84.7	704.9 ± 44.5
Fold FK stimulation over basal	2.58 ± 0.11	2.65 ± 0.26	2.73 ± 0.3	2.48 ± 0.06
% SRIF inhibition of basal activity	26.5 ± 2.4	13.04 ± 4.61[a]	28.0 ± 0.5	10.32 ± 2.07[c]
% SRIF inhibition of FK stimulation	25.3 ± 3.6	12.48 ± 0.59[b]	24.6 ± 2.8	13.53 ± 3.27[d]

Experiments were performed as described in Materials and methods.
Values represent the mean ± S.E.M. of five experiments, each performed in duplicate.
Statistical analysis was performed by ANOVA.
[a]$P < 0.05$ and [c]$P < 0.001$: comparison of the % SRIF inhibition of basal activity between control and melatonin-treated rats and between control and luzindole + melatonin-treated rats, respectively.
[b]$P < 0.01$ and [d]$P < 0.05$: comparison of the % SRIF inhibition of FK stimulation between control and melatonin-treated rats and between control and luzindole + melatonin-treated rats, respectively.

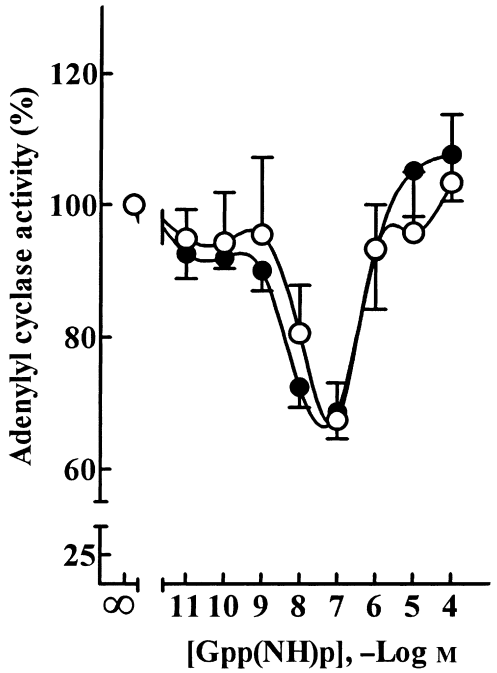

Fig. 3. Dose–effect curves for 5-guanylylimidodiphosphate [Gpp(NH)p]-mediated inhibition of adenylyl cyclase activity in rat hippocampal membranes from control (○, n = 5) and melatonin (25 μg/kg)-treated (●, n = 5) rats. The effect of Gpp(NH)p on adenylyl cyclase activity was studied in the presence of 3×10^{-6} M FK 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 ± S.E.M. of five separate determinations, each performed in duplicate.

its receptor, a chloride channel, increases the number of SRIF receptors and SRIF-mediated inhibition of AC system in the rat hippocampus [50].

As previously shown, the basal and FK-stimulated AC activity was inhibited by SRIF in all the experimental groups [21]. A high concentration of SRIF (10^{-4} M) was required to produce this inhibition, although the same concentration was used by other investigators in their studies on SRIF-mediated inhibition of AC activity in rat brain [51, 52]. In the present study, the SRIF-mediated inhibition of AC activity was only significant at the maximal concentration tested (10^{-4} M). Thus, this concentration was chosen for subsequent studies involving AC activity. It should also be noted that abundant studies on SRIF-mediated inhibition of AC activity were performed in cell lines expressing higher levels of SRIF receptors than in animal tissues and therefore, the inhibitory effect of SRIF is obtained at lower peptide concentrations. Another possible explanation may lie in the observation that G proteins can modulate the affinity of SRIF receptors and the coupling to the effector system (AC among others). In this respect, Enjalbert et al. [53] have demonstrated that the mobilization of the G protein by GTP reduces the SRIF receptor affinity for the neuropeptide in cerebral cortical cells. Indeed, in the presence of GTP necessary to couple the SRIF receptor to the AC catalytic subunit, the SRIF receptor may shift from an apparent high-affinity state (observed in binding studies) to an apparent low-affinity state (observed in AC studies). However, the synaptic concentration of SRIF is, to date, unknown. However, it is tempting to speculate that as the hippocampus is very rich in SRIF-containing neurons, the amount of SRIF released may be sufficiently high as to justify the increased SRIF concentration that was used to inhibit AC activity. Therefore, it is possible to assume that the high concentration of SRIF was necessary to inhibit AC activity under the present experimental conditions.

Melatonin administration led to a decrease in SRIF-mediated inhibition of basal and FK-stimulated AC activity after 5 hr of a single melatonin dose. The capacity of SRIF to inhibit basal and FK-stimulated AC activity was significantly lower in the melatonin-treated rats when compared with controls. This decreased sensitivity of AC to SRIF induced by melatonin administration might be a consequence of SRIF receptor downregulation. We can exclude the possibility that it was due to a decrease in the functionality of the AC catalytic subunit as FK-stimulated AC activity was unaffected by melatonin administration.

To test the functionality of Gi proteins, the response of AC to the GTP analog Gpp(NH)p was examined. As the inhibitory effect of Gpp(NH)p on FK-stimulated AC activity

was not altered by melatonin treatment, the possibility that the decreased SRIF-mediated inhibition of basal and FK-stimulated AC activity was due to a decrease in the functional activity of the Gi proteins can also be ruled out. Finally, Western blot analyses of $Gi\alpha_1$ and $Gi\alpha_2$ proteins were performed. The results obtained indicate that melatonin treatment did not alter the amount of the $Gi\alpha_1$ or $Gi\alpha_2$ levels.

As melatonin decreases the activity of the SRIFergic system in the hippocampus and increases it in the frontoparietal cortex [54, 55], effects that were not blocked by luzindole, a competitive antagonist [56, 57], it is unlikely that the modulatory effects of melatonin on the SRIF receptors are mediated by melatonin receptors, although they are present in both brain areas [3]. Melatonin enhances GABAergic function in the cerebral cortex [5] whereas it inhibits GABA-induced Cl^- currents in the rat hippocampus. GABA tonically inhibits the release [44] and synthesis of SRIF [45]. In the frontoparietal cortex from rats treated with melatonin we previously observed a significant increase in SRIF receptors, despite the fact that no changes in SRIF content were found [55]. Although the overall content of SRIF in the frontoparietal cortex did not decrease, the rate of SRIF synthesis and release may have changed. If this were the case, decreased SRIF release or turnover might lead to upregulation of SRIF receptors in the frontoparietal cortex, although we could not detect any increase in SRIF content by radioimmunoassay. In the hippocampus, however, melatonin inhibits GABA receptor function, which affects the GABA-SRIF neurons of the hippocampus. This could lead to SRIF release, resulting in significant short-term internalization of the SRIF receptors, as has been previously demonstrated both in brain and pituitary [58, 59].

The functional significance of the melatonin-induced decrease of SRIFergic activity in the rat hippocampus remains to be established. In rats, melatonin increases neuronal activity in hippocampal neurons [11, 12]. As SRIF exerts an opposite effect to that of melatonin on hippocampal neuronal activity [13, 14], it is possible that the SRIFergic system could be implicated in the mechanism of action of melatonin in the rat. However, the precise mode of action of acute melatonin administration on SRIFergic neurotransmission warrants further investigation.

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