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Research report

Activity of the hippocampal somatostatinergic system following daily administration of melatonin

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Abstract

If melatonin or its analogs are to be used therapeutically in humans, their chronic effects on responsiveness of melatonin target cells need to be assessed. We have previously demonstrated that acute melatonin treatment regulates the somatostatinergic system in the rat hippocampus. In the present study, we have investigated the effects of subchronic and chronic daily treatment with melatonin on the somatostatinergic system in the rat hippocampus. Male Wistar rats (200–250 g) were injected with melatonin (25 μg/kg body weight, subcutaneously) daily for 4, 7 or 14 days and sacrificed 24 h after the last injection. Melatonin administration for 4 days induced a decrease in the hippocampal somatostatin (SRIF)-like immunoreactivity content as well as a decrease in the number of SRIF receptors and an increase in their apparent affinity. The decreased number of SRIF receptors in the melatonin (4 days)-treated rats was associated with a decreased capacity of SRIF to inhibit both basal and forskolin-stimulated adenylyl cyclase activity. These melatonin-induced effects reversed to control values after 7 or 14 days of treatment. Hippocampal membranes from control and melatonin-treated rats showed similar Gi and Gs activities. Melatonin treatment altered neither the functional Gi activity nor the Giα1 or Giα2 levels at any of the time periods studied. The present results suggest that chronic exposure to melatonin results in a tolerance of the hippocampus to this hormone.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Receptor modulation, up- and down-regulation

Keywords: Somatostatin receptors; Melatonin; Adenylyl cyclase; G proteins; Hippocampus; Rat

1. Introduction

In mammals, melatonin (*N*-acetyl-5-methoxytryptamine) is mainly produced by the pineal gland [3] and, to some extent, by other extrapineal tissues such as the retina and gastrointestinal tract [34]. It is involved in regulating neuroendocrine function and acts directly on the biological clock in the suprachiasmatic nucleus to modulate the circadian rhytmicity of diverse biological functions such as sleep, hormonal and temperature cycles [42,43]. To date, only the Mel1a and Mel1b receptors have been detected in mammals [43]. Some of the responses of melatonin have been reported to be mediated through GABA-benzodiazepine receptors in the central nervous system [23]. In addition, melatonin is known to possess free radical scavenging and antioxidant properties [35]. There is increasing interest in the clinical applications of melatonin as therapeutic agents in humans [4]. If melatonin or its analogs are to be used therapeutically in humans, the effects of chronic exposure to these antagonists on the responsiveness of melatonin target cells need to be assessed. However, the functional consequence of chronic melatonin treatment has received little attention, and it is unknown whether such a treatment can subsequently alter the sensitivity of different brain areas to melatonin. Previous studies carried out by our group have demonstrated that acute melatonin treatment regulates the rat hippocampal somatostatinergic system in vivo [19]. In the present study, we have investigated the effects of subchronic and chronic daily melatonin administration on somatostatin (SRIF)-like immunoreactivity (SRIF-LI) levels and on [^{125}I]-Tyr^{11}-SRIF binding to its specific receptors in the rat hippocampus. We have also evaluated the effect of melatonin on SRIF-mediated inhibition of adenylyl cyclase

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(AC) activity in the rat hippocampus. In addition, the AC catalytic subunit was measured by stimulating the enzyme with the diterpene forskolin. The functional activity of Gi proteins was also determined.

2. Materials and methods

2.1. Chemicals and reagents

Melatonin, phenylmethylsulfonyl fluoride (PMSF), 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin (fraction V) (BSA), guanosine triphosphate (GTP), forskolin, pre-stained protein markers and other reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) were purchased from Sigma (Madrid, Spain). Specific antiserum against αi1 (MAB3075) or αi2 (MAB3077) G protein subunits was obtained from Chemicon International (California, USA). Nitrocellulose membranes as well as the chemiluminescence Western blotting detection system were purchased from Amersham (Buckinghamshire, UK). All other reagents were of the highest purity commercially available.

2.2. Experimental animals

All procedures regarding handling and treatment of laboratory animals were carried out in accordance to the guidelines established by our Animal Care and Use Committee and were approved by the mentioned Committee before implementation. Efforts were made to minimize animal's suffering and to use only the exact number of animals necessary to secure reliable scientific data. In the present study, male Wistar rats weighing 200–250 g were used. All animals received food and tap water ad libitum. Room temperature was kept constant at 22 °C and 12-h day–night cycles were maintained. Melatonin was dissolved in saline containing ethanol (0.5%) and administered subcutaneously (s.c.) at 0900 h in a volume of 100 μl according to the method described by Alexiuk and Vriend [2] at a dose of 25 μg/kg [1] daily for 4, 7 or 14 days. Control animals selected for each experimental group were injected with saline containing ethanol. All animals were sacrificed by decapitation 24 h after the last injection. The brains were removed and the hippocampus was immediately dissected as described by Glowinski and Iversen [14].

2.3. Binding assay

Tyr^{11}-SRIF was radioiodinated by chloramine-T iodination according to the method of Greenwood et al. [15]. 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. [36]. Protein concentration was assayed by the method of Lowry et al. [25], with BSA as a standard. Specific SRIF binding was measured according to the modified method of Czernik and Petrack [7]. 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 unlabelled 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)–sacarose (0.9%) buffer (pH 7.4). The radioactivity in the pellet was measured in a Kontron gamma counter. 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.

2.4. 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.

2.5. 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 [25]. Extracts were immediately stored at − 80 °C until assay. The immunoreactivity content was determined in tissue extracts by a modified radioimmunoassay method [31], 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-LI 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 h. 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.

2.6. Adenylyl cyclase assay

Hippocampal membranes were prepared as previously described by Reubi et al. [36]. Protein concentration was assayed by the method of Lowry et al. [25] with BSA as a standard. AC activity was measured as previously described [18], 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 forskolin) 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) was added and the suspension was centrifuged. The supernatant was then removed for assay of cyclic AMP (cAMP) by the method described by Gilman [12]. The SRIF concentration used was that deemed necessary to achieve inhibition of rat [39] AC activity. Similarly, forskolin was used at a concentration that could effectively stimulate the catalytic subunit of rat AC [39].

2.7. 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 [21]. 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 during 1.5 h 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 h at 4 °C. After washing, the bound immunoreactive proteins were detected by a chemiluminescent (ECL) Western blotting detection system.

2.8. Data analysis

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 experiment was performed in duplicate.

3. Results

Preliminary experiments confirmed that the specific binding of [^{125}I]-Tyr^{11}-SRIF to hippocampal membranes changed linearly with protein concentration and was time-dependent in all experimental groups. An apparent equilibrium was observed between 50 and 180 min at 30 °C (data not shown). All subsequent binding experiments were therefore conducted at 30 °C for 60 min. Our group [20] has studied the specific binding of [^{125}I]-Tyr^{11}-SRIF to hippocampal membranes 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 to the control group, with no changes being observed at the higher doses. Since the maximal effect of melatonin on SRIF receptors was detected at the dose of 25 μg/kg, subsequent studies were carried out at this dose. Stoichiometric experiments were performed on rat hippocampal membranes using

Table 1
Effect of melatonin at a daily dose of 25 μg/kg s.c. during 4, 7 or 14 days on the equilibrium parameters for [^{125}I]-Tyr^{11}-SRIF binding to rat hippocampal membranes

	SRIF receptors	
	B_{max}	K_d
Control	543 ± 12	0.51 ± 0.04
Melatonin 4 days	399 ± 23***	0.35 ± 0.04*
Control	478 ± 9	0.48 ± 0.02
Melatonin 7 days	456 ± 12	0.52 ± 0.03
Control	488 ± 10	0.40 ± 0.02
Melatonin 14 days	476 ± 30	0.39 ± 0.01

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 ± S.E.M. of five separate experiments, each performed in duplicate. Statistical comparison versus control: *$p < 0.05$; ***$p < 0.001$.

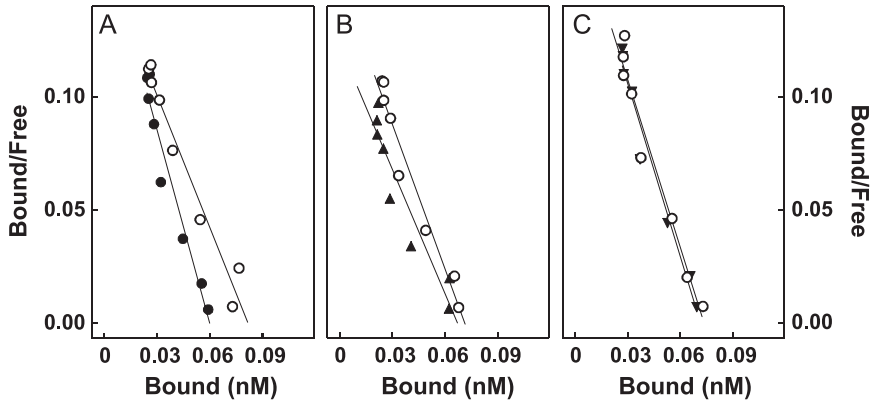

Fig. 1. Scatchard plots of the specific binding of [^{125}I]-Tyr^{11}-SRIF to rat hippocampal membranes. Membranes (0.15 mg of protein/ml) were incubated for 60 min at 30 °C in the presence of 250 pM [^{125}I]-Tyr^{11}-SRIF and increasing concentrations of SRIF. Points correspond to control rats (○), and rats treated with melatonin for 4 days (●) (A), 7 days (▲) (B) and 14 days (▼) (C). The animals were sacrificed 24 h after the last injection.

a fixed concentration of [^{125}I]-Tyr^{11}-SRIF and increasing doses of unlabelled SRIF at 30 °C for 60 min. Melatonin administration during 4 days produced a significant decrease in [^{125}I]-Tyr^{11}-SRIF binding to rat hippocampal membranes as compared to controls. This decrease was due to a decrease in the maximal number of SRIF receptors as revealed by Scatchard plots of the binding data (Table 1; Fig. 1). In addition, a significant increase in the affinity of these receptors was observed (Table 1; Fig. 1). No changes in the SRIF receptor number or affinity were observed, however, at 7 or 14 days of melatonin administration (Table 1).

Daily melatonin administration for 4 days decreased SRIF-LI content in the rat hippocampus as compared with the control group (Fig. 2). No changes were observed after 7 or 14 days of melatonin administration (Fig. 2).

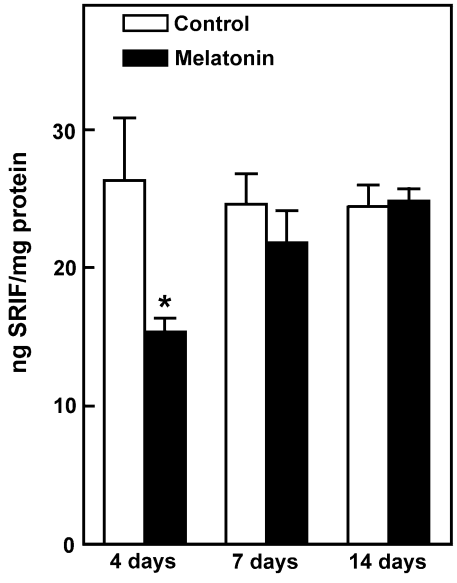

Fig. 2. Effect of melatonin (25 μg/kg) treatment for 4, 7 or 14 days on somatostatin (SRIF)-like immunoreactivity in the rat hippocampus. Values are expressed as the mean ± S.E.M. of five experiments, each performed in duplicate. Statistical comparison versus control: *$p < 0.05$.

One of the signalling pathways coupled to SRIF receptors is the inhibition of AC. Therefore, in order to investigate whether the functional effect of SRIF 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-treated groups, the degree of SRIF inhibition of both basal and forskolin-stimulated AC activity was significantly lower than in the control group after 4 days of its daily administration (Table 2). After 7 or 14 days of melatonin treatment, no significant differences were observed in this parameter (data not shown).

To test if the observed changes were related to modifications in the expression of AC, the response of the enzyme to the diterpene forskolin (10^{-5} M), which is assumed to act directly upon the catalytic subunit, was measured. No significant differences were detected in the fold forskolin stimulation over basal AC activity between the control group and melatonin-treated rats (Table 2).

Further experiments were carried out to explore the effect of melatonin on the functionality of Gi or Gs proteins in

Table 2
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 and melatonin (25 μg/kg, s.c.)-treated rats for 4 days

	Control	Melatonin
Basal activity	234.8 ± 4.2	263.0 ± 5.9
+ 10^{-4} M SRIF	191.5 ± 4.3	236.6 ± 6.2
+ 10^{-5} M FK	638.6 ± 4.2	634.7 ± 10.9
+ 10^{-5} M FK + 10^{-4} M SRIF	515.0 ± 17.1	574.4 ± 12.2
Fold FK stimulation over basal	2.7 ± 0.12	2.4 ± 0.08
%SRIF inhibition of basal activity	18.4 ± 1.7	10.0 ± 1.8**
%SRIF inhibition of FK stimulation	18.8 ± 3.7	9.5 ± 0.6*

Values represent the mean ± S.E.M. of five separate experiments, each performed in duplicate. Statistical comparison versus control: *$p < 0.05$; **$p < 0.01$.

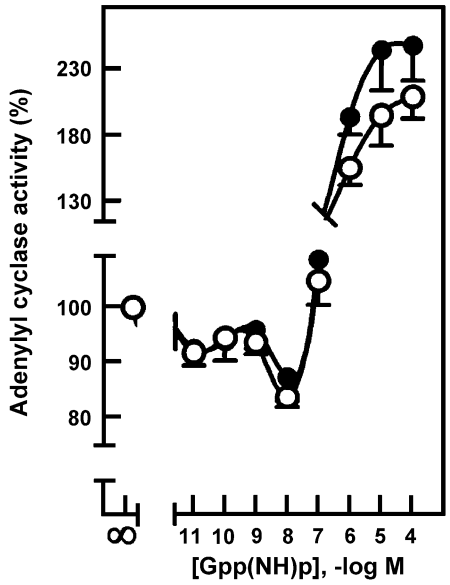

Fig. 3. Dose–effect curves for 5-guanylylimidodiphosphate [Gpp(NH)p]-mediated inhibition of adenylyl cyclase activity in rat hippocampal membranes from control (○, $n=5$) and melatonin (25 μg/kg)-treated (●, $n=5$) rats. The effect of Gpp(NH)p on adenylyl cyclase activity was studied in the presence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

rat hippocampal membranes by determining the ability of low and high Gpp(NH)p concentrations to inhibit forskolin (3×10^{-6} M)-stimulated AC activity. Characteristic biphasic response curves were obtained in all the 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 functional activity Gi and Gs (Fig. 3).

In order to investigate whether the levels of the inhibitory G protein (Giα1 and Giα2) were affected by melatonin, Western blot analyses were performed. Melatonin administration for 4, 7 or 14 days did not significantly alter the amount of the Giα1 or Giα2 subunits in hippocampal membranes (data not shown).

4. Discussion

The present study has examined the effects of short- and long-term melatonin (25 μg/kg, s.c.) administration on the rat hippocampal somatostatinergic system. Previous studies have shown that subcutaneous melatonin injection results in a rapid increase in the serum levels of this indol 30 min after its administration [26] and a gradual reduction in these levels 90 or 180 min later. Circulating melatonin passes the blood barrier and binds to its receptors, as demonstrated by systemic injection of radioactive melatonin in rodents [13,44]. A minimal dose of melatonin such as 25 μg/kg body weight during 4 days was enough to decrease both the SRIF-LI content in the rat hippocampus and the specific binding of [^{125}I]-Tyr^{11}-SRIF to rat hippocampal membranes as compared to the control group. The SRIF-LI content and equilibrium parameters of the SRIF receptors in the hippocampus of control rats were similar to those previously reported by others [32,41].

Some of the responses induced by melatonin have been reported to be mediated via GABA-benzodiazepine receptors in the CNS [24,29,38,45]. Previous results from our group [19] suggest that melatonin via interaction with GABA receptors could modulate the rat hippocampal SRIFergic system. Thus, since melatonin inhibits GABA receptor function in the rat hippocampus [10] and GABA antagonists have been found to stimulate SRIF release [37], melatonin may lead to an increase in SRIF release and, therefore, to a decrease in SRIF content in the hippocampus as seen at 4 days of daily melatonin administration. An increased SRIF release might lead to a decrease in the density of the SRIF receptors in adjacent SRIF-containing neurons. Thus, regulation of SRIF receptors by SRIF appears to be similar to that of other hormone–receptor systems [8,22].

Basal and forskolin-stimulated AC activity was inhibited by SRIF in all the experimental groups studied, which is in agreement with the literature [27]. A high concentration of SRIF (10^{-4} M) was required to produce this inhibition although the same concentration was used by other researchers in their studies on SRIF-mediated inhibition of AC activity in rat brain [11,28]. In a previous study from our group, the inhibitory effect of increasing SRIF concentrations on basal and forskolin (10^{-5} M)-stimulated AC activity in control rats was analyzed [33]. 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 on AC activity. It should also be noted that abundant studies on the SRIF effect on AC activity were performed in cell lines expressing higher levels of SRIF receptors than in animal tissues. This may be due to the cell heterogeneity of the present preparations. Thus, SRIF inhibited basal AC activity in neuronal but not glial cells [5,6]. On the other hand, the synaptic concentration of SRIF is, to date, unknown. However, it is tempting to speculate that because the hippocampus is very rich in SRIF-containing neurons, the amount of SRIF released may be sufficiently great as to justify the high SRIF concentration used to inhibit AC activity. Therefore, it is possible that this concentration of SRIF is necessary to inhibit AC activity in our experimental conditions.

Melatonin administration led to a decrease in SRIF-mediated inhibition of basal and forskolin-stimulated AC activity after a short-term 4-day daily administration. However, no changes were detected in either the basal or forskolin-stimulated AC activity, suggesting that the de-

creased sensitivity of AC to SRIF inhibition was not due to an alteration of the AC catalytic subunit. Gi protein functionality, as measured by the capacity of Gpp(NH)p to inhibit forskolin-stimulated AC activity, was similar in both the melatonin-treated and control rats. On the other hand, Western blots of the Giα1 or Giα2 proteins indicate that neither protein was modified by melatonin. Taken together, the present results suggest that the decrease in SRIF-mediated inhibition of AC activity is most probably due to the decrease in the number of SRIF receptors.

The melatonin-induced changes in the hippocampal SRIFergic system reversed to control values after 7 or 14 days of daily administration. This finding is in agreement with studies from Hadley [16], who demonstrated that continuous exposure to melatonin renders the target tissue unresponsive to the hormone. It has previously been reported that the total number of melatonin receptors in the hippocampal midbrain [30] and pars tuberalis of the anterior pituitary [17] may be reduced following prolonged exposure to melatonin.

Recently, Li et al. [24] have described that melatonin induces desensitization of the $GABA_A$ receptor. Several experiments indicate that there is a binding site for melatonin on the $GABA_A$ receptor [29,38]. Allosteric modulation of GABA receptors by melatonin was demonstrated in the rat brain [29]. This allosteric modulation provides a possible mechanism for regulating GABAergic transmission leading to desensitization of GABA receptors. Therefore, it is possible that a continuous exposure to melatonin via $GABA_A$ receptor desensitization causes the SRIF system to return to control values. In this regard, it should be noted that there are $GABA_A$ receptors located in hippocampal SRIF-containing neurons [9,10] so that desensitization of $GABA_A$ receptors could blunt the release of SRIF which could explain the return of SRIF levels and SRIF receptors to control values.

The significance of the changes in the SRIF receptor–effector system induced by melatonin remains to be established. However, the fact that acute melatonin administration leads to a memory deficit is in agreement with the decrease of the SRIFergic system, which increases memory processes. In addition, the fact that memory deficits induced by melatonin revert to control values after chronic treatment with melatonin during 7 days [40] correlates with the return of activity to control values, as shown in this study. Therefore, the present results suggest that chronic exposure to melatonin results in tolerance of the rat hippocampus to this hormone.

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