Melatonin is a potent modulator of dopamine release in the retina

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Melatonin, a hormone originally discovered in the pineal gland[1], has also been found in the retina of several vertebrate species[2–5]. The enzyme system for melatonin synthesis also exists in the retina[2,3,6–8], where the activity of one such enzyme, (serotonin *N*-acetyltransferase) varies with changes in light intensity in a circadian pattern[3,8]. As the activity of dopamine containing amacrine neurones of the retina is influenced by changes in illumination[9–12] it was of interest to determine the effect of melatonin and its precursors, serotonin and *N*-acetylserotonin, on the release of ^{3}H-dopamine from rabbit retina. I report here that picomolar concentrations of melatonin (IC_{50} 9 pM) selectively inhibited the calcium-dependent release of ^{3}H-dopamine from rabbit retina, but not from striatum. Melatonin, was 1,000 times more potent than its precursor *N*-acetylserotonin in inhibiting the release of ^{3}H-dopamine in retina, while the putative neurotransmitter serotonin[13], was inactive. It is suggested that the light-dependent production of melatonin could play a physiological role in modulating the activity of dopamine-containing neurones in the retina.

Albino rabbits (2–3 kg) maintained on 14–10 h light–dark cycle, were killed by decapitation about the middle of the light cycle. Retinas were dissected free of pigmented epithelium and prepared for superfusion as described previously[14,15]. All experimental procedures were carried out in conditions of illumination. The pieces of rabbit retina or striatal slices (0.4 mm) were labelled *in vitro* with 0.1 μM ^{3}H-dopamine (specific activity 28.4 Ci mmol^{-1}) for 20 min in Kreb's solution at 37 °C. Thereafter, the retina pieces or striatal slices were superfused with Krebs' solution (calcium concentration 1.3 mM) until the spontaneous outflow of radioactivity had levelled off. Tritium release was elicited twice in each experiment either by field stimulation at 3 Hz for 1 min (20 mA, 2 ms duration) or by exposure to 1 μM tyramine for 2 min. The first (S_1) and second (S_2) periods of stimulation were applied 60 min and 100 min after the end of the incubation with ^{3}H-dopamine respectively. Four-minute samples of the superfusate were collected before, during and after the period of stimulation and the tritium released determined. Results were calculated as the percentage of the total tissue radioactivity released in each sample[14,15].

Field stimulation releases ^{3}H-dopamine previously taken up by rabbit retina pieces through a calcium-dependent process[14,15]. In the controls, the spontaneous outflow expressed as the percentage of total tissue radioactivity released during the 4-min preceding the first period of stimulation (Sp_1) was 1.65 ± 0.13% ($n = 8$). The increase above the spontaneous levels, of the total tissue radioactivity released by the first 1-min period of stimulation (S_1) at 3 Hz was 1.49 ± 0.21% ($n = 8$) and after the second period of stimulation (S_2) was 1.56 ± 0.20% ($n = 8$). The ratio obtained between the two consecutive periods of stimulation, in the absence of drugs (S_2/S_1) was 1.06 ± 0.04 ($n = 8$), (Fig. 1*A*). The putative neurotransmitter, serotonin (0.1 nM–1 μM), did not affect either the spontaneous or field stimulation-evoked release of ^{3}H-dopamine from rabbit retina (Fig. 1). Concentrations of serotonin higher than 1 μM significantly increased the spontaneous outflow of radioactivity, through a calcium-independent process (unpublished observations). Figure 1*A* shows that the active metabolites of serotonin, *N*-acetylserotonin (1 nM–1 μM) and melatonin (1 pM–1 μM)

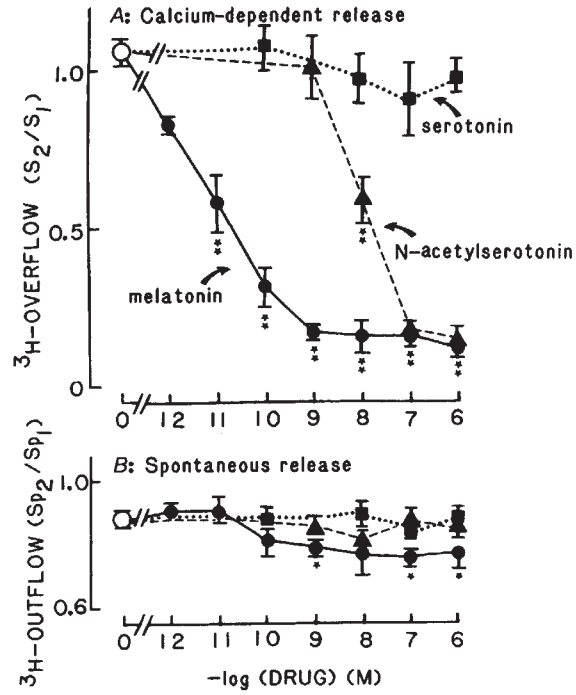

Fig. 1 Effect of serotonin, *N*-acetylserotonin and melatonin on the spontaneous and calcium-dependent release of ^{3}H-dopamine from rabbit retina. Ordinates: *A*, ^{3}H-overflow, is the percentage of total tissue radioactivity released by field stimulation above the spontaneous levels of release. Results are expressed as the ratio obtained between the second (S_2) and the first (S_1) stimulation periods within the same experiment. The calcium-dependent release of ^{3}H-dopamine was elicited by a 1-min period of field stimulation at 3 Hz (20 mA, 2 ms). In the controls, the percentage of the total tissue radioactivity released after the first 1-min period of stimulation (S_1) was 1.49 ± 0.21% ($n = 8$). The radioactivity retained by the control tissue after 120 min superfusion was: 47.3 ± 4.8, nCi per chamber ($n = 8$). *B*, ^{3}H-outflow, is the percentage of total tissue radioactivity release in the four-min sample preceding the second (Sp_2) and the first (Sp_1) period of field stimulation, expressed as the ratio Sp_2/Sp_1. In the controls, the spontaneous outflow calculated as the percentage of total tissue radioactivity released during the 4-min preceding the first period of stimulation (Sp_1) was: 1.65 ± 0.13% ($n = 8$) and the second stimulation (Sp_2) was 1.17 ± 0.14% ($n = 8$). The ratio Sp_2/Sp_1 was: 0.88 ± 0.02. Abscissae: Molar concentrations of the drugs (logarithmic scale). Drugs were added 20 min before S_2. Only one concentration of each drug was tested per experiment. ○, control; ●, melatonin; ▲, *N*-acetylserotonin; ■, serotonin. Shown are mean values ±s.e.m. of 3 to 8 experiments per group. $^*P < 0.05$; $^{**}P < 0.001$ when compared with the corresponding control, (Student's two-tailed *t*-test).

inhibited in a concentration-dependent manner the calcium-dependent release of ^{3}H-dopamine from the retina. The concentration of melatonin inhibiting the release of ^{3}H-dopamine by 50% (IC_{50}) was 9 pM, being 1,000 times more potent than its precursor *N*-acetylserotonin (IC_{50} 8.6 nM). *N*-Acetylserotonin at 0.1 μM and melatonin at 1 nM maximally inhibited the calcium-dependent release of ^{3}H-dopamine by 80% of the control value (Fig. 1*A*). This inhibitory effect of melatonin was almost fully reversed by superfusing the retina with Krebs' solution free of melatonin for 20 min before a third period of stimulation (data not shown). It is of interest that the concentrations of the most potent dopamine receptor agonist[16] (pergolide, IC_{50} 5 nM), opiate receptor agonist[15] (D-Ala^2,Met^5-enkephalinamide, IC_{50} 30 nM) or α-receptor agonist[17] (clonidine, IC_{50} 56 nM), necessary to inhibit the release of ^{3}H-dopamine from retina by 50% are 500–6,000 times higher than the concentration of melatonin required to produce the same effect.

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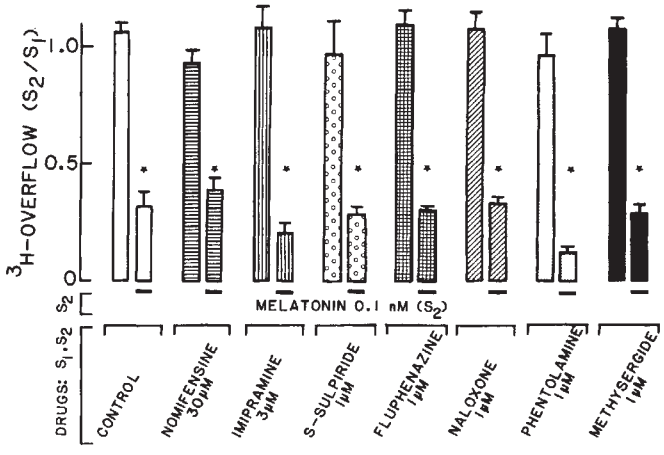

Fig. 2 Effects of neuronal uptake inhibitors and various receptor antagonists on melatonin-induced inhibition of ^{3}H-dopamine release. Ordinate: ^{3}H-overflow, is the percentage of total tissue radioactivity released by field stimulation above the spontaneous levels of release. Results are expressed as the ratio obtained between the second (S_2) and the first (S_1) stimulation periods within the same experiment. Release was elicited by field stimulation for 1 min at 3 Hz (20 mA, 2 ms). The neuronal uptake inhibitors or the receptor antagonists were added to the medium 40 min before S_1 and maintained until the end of the experiment. The percentage of total tissue radioactivity released during the first period of field stimulation (S_1) was 1.49 ± 0.21%, (n = 8), in controls; 4.41 ± 0.62% (n = 4), with 30 μM nomifensine; 1.78 ± 0.09% (n = 3), with 3 μM imipramine; 2.86 ± 0.3% (n = 6), with 1 μM *S*-sulpiride; 2.10 ± 0.50% (n = 4), with 1 μM fluphenazine; 1.71 ± 0.29% (n = 4), with 1 μM (−)naloxone; 2.06 ± 0.24% (n = 4), with 1 μM phentolamine; and 2.61 ± 0.46% (n = 4), with 1 μM methysergide. Melatonin 0.1 nM, was added 20 min before the second period of stimulation (S_2) only in the groups indicated with a black bar (—). Only one concentration of melatonin was tested per experiment. Shown are mean values ±s.e.m. of 3 to 8 experiments per group. *P < 0.001 when compared with the corresponding control, (Student's two-tailed t-test).

The effect of melatonin on the release of radioactivity elicited by the indirect amine tyramine was also investigated. The total tissue radioactivity released above the spontaneous levels by the first 2-min period of exposure to 1 μM tyramine (S_1) was 3.62 ± 0.22% (n = 4), and after the second period of stimulation with tyramine (S_2) was 3.65 ± 0.40%, n = 4. The release of radioactivity elicited by tyramine from the retina is mediated through a calcium-independent process since omission of calcium did not affect this release. A concentration of melatonin (0.1 nM), which inhibited the calcium-dependent release of ^{3}H-dopamine by 70% (Fig. 1*A*), failed to modify the increase in radioactivity elicited by tyramine [S_2 = 4.17 ± 0.36% (n = 3), obtained in the presence of 0.1 nM melatonin]. The spontaneous outflow of radioactivity, which is also a calcium-independent process[14], was only slightly inhibited by concentrations of melatonin higher than 1 nM (Fig. 1*B*). These results suggest that melatonin inhibits only the calcium-dependent release of ^{3}H-dopamine from the rabbit retina.

The inhibitory effect of melatonin on the depolarization-evoked ^{3}H-dopamine release appears to be selective for the retina, since this hormone failed to modify the calcium-dependent release of ^{3}H-dopamine from other structures with dense dopaminergic innervation such as the striatum (Table 1) and olfactory tubercle (unpublished observations). Similarly, Zisapel and colleagues[18,19] reported that melatonin inhibited the calcium-dependent release of ^{3}H-catecholamines from slices of rat preoptic and hypothalamic areas previously labelled with ^{3}H-dopamine, but not from striatal slices. Table 1 also shows no inhibitory effect of *N*-acetylserotonin (0.1 μM) or serotonin (0.1 μM) on ^{3}H-dopamine release from the striatum. On the contrary, under identical experimental conditions, the dopamine receptor antagonist apomorphine (0.1 μM) induced a significant

Table 1 Lack of effect of serotonin, *N*-acetylserotonin and melatonin on the calcium-dependent release of ^{3}H-dopamine from rabbit striatum

Drugs in S_2	n	^{3}H-Transmitter overflow: % Total tissue radioactivity* S_1	S_2	Ratio† S_2/S_1
Control	6	2.09 ± 0.26	2.17 ± 0.25	1.05 ± 0.03
Serotonin 0.1 μM	7	2.80 ± 0.26	3.01 ± 0.15	1.13 ± 0.11
N-acetylserotonin 0.1 μM	7	2.84 ± 0.43	3.10 ± 0.31	1.16 ± 0.09
Melatonin 0.1 μM	4	2.55 ± 0.52	2.69 ± 0.45	1.07 ± 0.05
Apomorphine 0.1 μM	4	2.19 ± 0.87	0.86 ± 0.46‡	0.35 ± 0.07§

Drugs in the concentrations indicated were added to the medium 20 min before S_2. In the controls, the spontaneous outflow of radioactivity calculated as the percentage of total tissue radioactivity released during the 4-min preceding the first period of stimulation (Sp_1) was: 0.92 ± 0.06% (n = 6) and the second stimulation (Sp_2) was: 0.96 ± 0.04% (n = 6). The ratio Sp_2/Sp_1 was: 0.95 ± 0.03 (n = 6). Neither serotonin, *N*-acetylserotonin, melatonin nor apomorphine modified the spontaneous outflow of radioactivity at the concentration indicated when added 20 min before the second stimulation (S_2). The radioactivity retained by the control tissue after 120 min of superfusion was: 88.05 ± 7.52 nCi per slice, n = 6. Shown are mean values ±s.e.m.; n = no of experiments per group.

* Percentage of the total tissue radioactivity released above the spontaneous levels by 1-min period field stimulation at 3 Hz (20 mA, 2 ms). The interval between two consecutive periods of stimulation (S_1, S_2) was 40 min.

† Ratio of the percentage of total tissue radioactivity released during the second stimulation (S_2) to that released during the first stimulation period (S_1).

‡ P < 0.05; § P < 0.001, when compared with control (Student's two-tailed t-test).

decrease in ^{3}H-dopamine release (Table 1), probably through activation of inhibitory dopamine autoreceptors[14].

The inhibition of ^{3}H-dopamine release by 0.1 nM melatonin was not modified in the presence of inhibitors of dopamine uptake (nomifensine, 30 μM) and serotonin uptake (imipramine, 3 μM) (Fig. 2). These results suggest that the decrease in ^{3}H-dopamine release elicited by melatonin is not due to an increase in the neuronal uptake of monoamines. Furthermore it is unlikely that melatonin is interacting with retinal D-2 dopamine autoreceptors[14–16], presynaptic α_2 and opiate receptors[15,17] or serotonin receptors[13], as a concentration of 1 μM of various antagonists for these receptors, (*S*-sulpiride, fluphenazine, phentolamine, naloxone, methysergide) did not significantly antagonize the inhibitory effect of melatonin (Fig. 2).

It is not yet known whether the rabbit retina contains melatonin, but the potent effect of this hormone in inhibiting the calcium-dependent release of ^{3}H-dopamine may have important physiological consequences in the modulation of dopaminergic activity in retina. For example it has been shown that exposure of dark-adapted animals to light significantly increases the activity of tyrosine hydroxylase[9,12] and the rates of dopamine release[10], synthesis[11] and turnover[11,12] in rat, rabbit and cat retina. The mechanism(s) involved in the light-induced increase in activity of retinal dopamine neurones is not known[11]. The results of this publication lead to the suggestion that, if melatonin is found in the retina of the rabbit as reported in other species[2–8], light might modulate the activity of the dopamine-containing amacrine cells of the retina indirectly, through melatonin, since its synthesis via *N*-acetyltransferase is regulated by environmental light[3,8]. For example in darkness, depolarized photoreceptors produce and secrete melatonin[20] while exposure to light, by rapidly inactivating *N*-acetyltransferase[3,8], leads to a decrease both in melatonin synthesis and secretion from the hyperpolarized photoreceptors[20]. It might be suggested that during the dark-period the elevated concentration of melatonin in the retina[3,8], exerts an inhibitory action on the activity of retinal dopamine neurons. On the contrary the low levels of melatonin during the light period might disinhibit the dopamine-containing amacrine cells of the retina, leading to an increase in tyrosine hydroxylase activity and dopamine release, synthesis and turnover[9–12].

The light-dependent production of melatonin may modulate not only eye pigmentation[20] and photoreceptor metabolism[21], but also the activity of dopamine-containing amacrine cells in retina in physiological conditions. Finally, if the dopamine

neurones are involved in adaptation of the retina to light, as suggested by Ehinger[22], it might be speculated that melatonin by inhibiting and disinhibiting the dopamine amacrine cells in dark and light respectively, might be a physiological mediator of light adaptation.

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Patch- and voltage-clamp analysis of cyclic AMP-stimulated inward current underlying neurone bursting

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The second messenger cyclic AMP has been variously reported to affect the electrical activity of different neurones by decreasing outward potassium current[1–3], increasing outward current[4] and increasing inward current[5–8]. The recently developed patch clamp method of recording single ionic channels[9] allows direct measurement of the action of cyclic AMP on membrane conductances. Using the patch clamp, the closure of potassium channels by cyclic AMP has previously been documented on the single channel level[10]. We report here that in a bursting molluscan neurone, intracellular iontophoresis of cyclic AMP under voltage clamp elicits an inward current of maximal amplitude in the pacemaker voltage region. Patch-clamp analysis reveals inward channels whose opening frequency is augmented by cyclic AMP stimulation and whose activity accompanies burst episodes. Channel opening frequency is significantly increased by depolarization of the whole soma, but not by focal depolarization of the patch; this may reflect the action of another second messenger that acts in concert with cyclic AMP to confer voltage sensitivity.

The bursting cells that we studied were the paired ventral white cells (VWCs) of the marine mollusc *Pleurobranchea*[11]. Buccal ganglia were dissected from *Pleurobranchea* (20–100 g) and pinned to a layer of Sylgard on the bottom of the recording chamber. Saline composition was (in mM): 420 NaCl, 25 $MgCl_2$, 25 $MgSO_4$, 10 KCl, 10 $CaCl_2$ and 5 MOPS, adjusted to *p*H 7.5 at 13 °C. For voltage clamping, VWCs were axotomized and stabilized against insect pins[12]. A single-barrelled voltage electrode and a double-barrelled current electrode were inserted into the VWC. One barrel of the current electrode was filled with 0.2 M cyclic AMP adjusted to *p*H 7.4 with KOH. Current was passed between the two barrels to iontophorese cyclic AMP. Permanent records were made on a Gould 2400 chart recorder.

To clean the membrane surface for patch clamping, the cells were bathed in 0.25% trypsin for 90 min. All VWCs survived trypsin treatment with normal resting potentials (−50 to −60 mV) and overshooting spikes. The somata were voltage clamped with a single electrode voltage clamp (Dagan 8100) and single channels were recorded using a Dagan 8900 patch-clamp apparatus[9]. Patch pipettes were filled with saline having the same composition as the bath saline. Seal resistances were 5 to 20 GΩ. The signal was recorded on a digital storage oscilloscope and on an FM tape recorder for storage. The tape recorder was played back at a slower speed onto the chart recorder for single channel frequency analysis. After an inward channel was isolated, and baseline activity was established, the bath was perfused with a membrane soluble, cyclic AMP analogue *p*-chlorophenylthio adenosine 3′5′monophosphate (CPT-cAMP; ICN Biochemicals)[13] at 10^{-4} M.

Under voltage clamp, cyclic AMP iontophoresis elicited an inward current. Peak current responses of over 10 nA could be elicited, but injection current was purposely kept low to avoid jamming of the current electrode. Injection of the non-cyclic 5′ AMP was without noticeable effect. Figure 1*A* shows a typical current response to intracellular cyclic AMP injection. The amplitude of the current response to injected cyclic AMP varied with the holding potential, as is shown in Fig. 1*A* and *B*. Cyclic AMP induced inward current could be recorded at holding potentials as low as −90 mV. The potassium equilibrium potential has been estimated by us and other researchers[10,14] to lie between −65 to −75 mV, suggesting potassium is not a major charge carrier of this current. The voltage region (−20 to −30 mV) that supports the largest cyclic AMP current coincides with the negative slope region in *I–V* curves for the VWC and other bursting neurones that is indicative of slow inward current[12,15–17]. This correlation of effective voltage ranges suggests the cyclic AMP current contributes to slow inward current.

Of all the different types of inward and outward current channels recorded in the patch clamp experiments, only inward channels were cyclic AMP-responsive while the soma was voltage clamped. Inward current channels were recorded in ~35% of all successful patches. Inward channel open times ranged from 1 to 20 ms, and the frequency of occurrence ranged from 0 to 10 openings per s. Conductance through the inward channels was approximately ohmic, with conductances around 20 pS at 13 °C. Voltage clamping of the patch pipette revealed a reversal potential for these channels of +40 to +50 mV. In four experiments where the cell was voltage clamped to −50 mV, and in two at −60 mV, CPT-cAMP increased the average opening frequency from 1.45 ± 0.35 to 3.1 ± 0.48 openings per second. The opening frequency was sampled for a minimum of 3 min before and after addition of CPT-cAMP. In two cells, opening frequency was sampled for up to 15 min, but the frequency did not differ significantly from a 3-min sample. In a typical experiment, bath addition of CPT-cAMP significantly increased the opening frequency by 140% from 1.25 ± 0.19 to 2.98 ± 0.32 openings per second. Mean open time of the channel in CPT-cAMP (8.00 ± 0.87 ms) did not change significantly from baseline (7.21 ± 0.87 ms).

In the presence of CPT-cAMP, channel opening frequency increased before changes in whole cell electrical activity, and while the VWCs were voltage clamped (Fig. 2), indicating the frequency was modulated by CPT-cAMP, and not ionic changes induced by action potentials. Single channel opening frequency increased 10–20 min after bath addition of the cyclic AMP analogue[18], and was temporally correlated with a more negative holding current (increased inward current) under voltage clamp. In unclamped cells, spontaneous burst episodes were initiated within 1–5 min after clear increases in channel opening frequency were detected. During a CPT-cAMP initiated burst, action potentials progressively broadened concurrent with an

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