ORIGINAL ARTICLE

# Isoproterenol-stimulated melatonin production by perifused rat pineal glands: Age- and time-related effects

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**Abstract**

**OBJECTIVES**: The purpose of this study was to investigate and compare the effects of the β-adrenergic agonist, isoproterenol, on induced increase in melatonin production in the pineal gland of young and old rats, at different circadian stages.

**MATERIALS AND METHODS**: We report here the effects of $10^{-6}$ M isoproterenol-stimulated melatonin production by perifused pineal glands obtained from young (55 day old) and old (21 month old) male Wistar rats acclimatised to light: dark cycles regimen of 12:12 for 3 weeks. Pineal glands were collected at different circadian stages: 3, 7, 11, 15, 19 and 23 hours after light onset (HALO), and perifused for 510 min.

**RESULTS**: The basal levels of melatonin production in the young rats were approximately twice greater than those of the old rats. Isoproterenol stimulated melatonin production in both young and old rat pineal glands, whatever the circadian stage. The intensity of the response to $10^{-6}$ M isoproterenol infusion was greater in young than in old rat pineal glands ($P<0.001$), with a trend towards an increase during the light phase, at 7 HALO, in both young and old rat pineal glands, although this trend towards increased melatonin response did not reach statistical significance ($P>0.05$).

**CONCLUSIONS**: These results show that isoproterenol is able to stimulate both young and old rat pineal glands whatever the circadian stage. The magnitude of isoproterenol stimulation is greater in young than in old glands. Our results also suggest that the pineal gland response to isoproterenol is not dependent on circadian stage, at least, under our experimental conditions.

## Introduction

Melatonin, the major hormone of the pineal gland, is produced in a circadian manner, with peak production occuring during the dark phase of the light:dark cycle [1]. The day/night rhythm of melatonin depends on the regulation of *N*-acetyltransferase, the key enzyme for melatonin synthesis, by norepinephrine [1]. Norepinephrine produces a rise in *N*-acetyltransferase and subsequently rise of melatonin through the activation of β-adrenergic receptors [1]. The 24 h rhythm of melatonin production observed in young animals including humans is altered during ageing [2–10]. It has been shown that the β-adrenergic receptor density decreases with age [11], and this may casually related to age-associated decline in pineal production of melatonin. We have tried to reassess whether the β-adrenergic-stimulated melatonin production changes with age and circadian stage by studying the effects of the β-adrenergic agonist, isoproterenol, using pineal gland perifusion in rats.

## Materials and methods

*Animals, experimental device and procedure*

The experiments used young and old male Wistar rats (JANVIER, Le Genest Saint-Isle, France), which were housed in a chronobiologic animal facility (Enceinte Autonome d'Animalerie, Ref. A 110-SP-6, ESI Flufrance, Arcueil, France) with food and water available *ad libitum*. The facility was equipped with sound-proof, temperature-controlled (21 ± 1.0°C) compartments, all of the same size and provided with independent light-dark cycles. The rats were synchronised with a lighting regimen with 12 h of light and 12 h of darkness for 3 weeks before the experiments. These experiments were performed in accordance with the principles of Laboratory Animal Care (NIH) and with French laws [12].

Rats were sacrificed by decapitation at the age of 55 days for young rats ($n$ = 30) and 21 months for old rats ($n$ = 30), at six different circadian stages: three during the light phase, i.e. 3, 7, 11 hours after light onset (HALO), and three during the dark phase, i.e.15, 19 and 23 HALO. The animal killed during the dark period were sacrificed under a dim red light to avoid alteration of melatonin production. The pineal glands were quickly removed and kept in 4°C Krebs-Ringer solution at pH 7.4, gassed with 5% $CO_2$ and 95% $O_2$ until transfer into the perifusion chambers.

The perifusion system has been described previously [13]. Briefly, this consisted of a plastic column closed with two pistons, a thermostatic bath, and a peristaltic pump. The Krebs-Ringer medium contained (mM) NaCl 120, KCl 5, $KH_2PO_4$ 1.2, $CaCl_2$ 2.6, $MgSO_4$ 0.67, $NaHCO_3$ 22.5, and glucose 10. The pineal glands (one gland per chamber) were perifused with oxygenated Krebs-Ringer solution at a constant flow rate of 0.1 ml/min. The pineal glands and medium were maintained at 37°C by the thermostatic bath (Techne TE-8J). We have previously shown that melatonin production by perifused rat pineal glands stabilises 3 to 4 h after the beginning of the perifusion and remains stable up to 8 h [14]. For this study we therefore collected perifusates into plastic tubes every 10 min from 3 h 30 min (210 min) to 8 h 30 min (510 min) of perifusion with an automatic fraction collector. The perifusates were stored at –20°C until assayed for melatonin.

Isoproterenol (Sigma, Saint-Quentin, France) was dissolved in Krebs-Ringer medium at a concentration of $10^{-6}$ M and was infused for 30 min (250–280 min of the perifusion). The (–) enantiomer of isoproterenol was used, as we have previously shown that the effect of the β-adrenergic agonist on pineal melatonin production varies according to its enantiomeric forms [15].

*Melatonin radioimmunoassay*

Melatonin was measured directly in the perifusate fractions, by radioimmunoassay with a specific rabbit antiserum (R 19540) provided by INRA (Nouzilly, France) and $^{125}$I labeled melatonin tracer (NEN-Dupont, Boston, MA., USA), according to the method described by Fraser et al. [16].

The detection limit of the melatonin assay was 5 pg/tube. The intra-assay and inter-assay coefficients of variation were 4 and 10% respectively for a concentration of 50 pg/ml of melatonin, 7 and 9% for a concentration of 200 pg/ml, and 10 and 15% for 1000 pg/ml.

*Presentation of data and statistical analysis*

Concentrations of melatonin released during the perifusion were expressed as pg/min/gland. Results were expressed as means ± standard errors of the mean (S.E.M.) of data obtained in the five chambers. In order to evaluate the effect of isoproterenol infusion on melatonin release, the intensity of the response was established as the peak over the basal values. The peak area was calculated as the sum of the increased melatonin release relative to the basal one for each perifusate fraction (in % x min). ANOVA (analysis of variance) and Student's unpaired $t$-test were used to analyse the experimental data.

## Results

As expected, the initial levels of melatonin released by the perifused rat pineal glands were higher in the dark phase than in the light phase in both age groups ($P$<0.01) (*Figure 1*). The mean values ($n$ = 5 chambers for each circadian stage) of the initial melatonin levels (at the beginning of the perifusion) in the young animals, during the light phase at 3, 7, and 11 HALO were 26 ± 4 pg/min/gland, 27 ± 1 pg/min/gland, and 28 ± 2 pg/min/gland respectively, whereas those found in the dark phase at 15, 19 and 23 HALO were 224 ± 15 pg/min/gland, 248 ± 15 pg/min/gland, and 196 ± 15 pg/min/gland respectively. The initial pineal melatonin levels in the old rats were lower than those in young rats ($P$<0.05). Mean values during the light phase at 3, 7, and 11 HALO were 16 ± 3 pg/min/gland, 15 ± 1

**Figure 1.** Comparison of initial levels of the melatonin release by young (55 day old) and old (21 month old) rat pineal glands obtained at different circadian stages. Each value is the mean ± S.E.M. of data obtained in five perifusion chambers (one gland per chamber). The initial melatonin levels are significantly greater in the dark phase (15 to 23 HALO) than in the light phase (3 to 11 HALO) (***P*<0.01). These levels are greater in young than in old rat pineal glands (**P*<0.05).

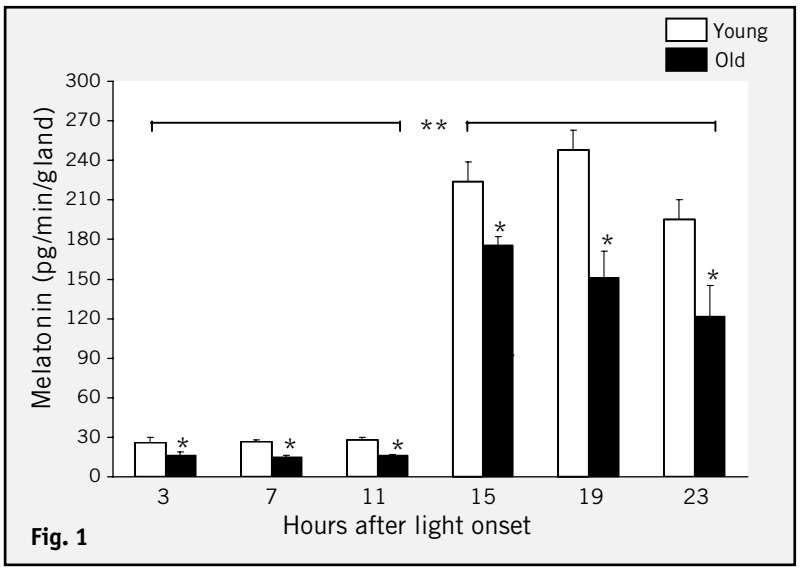


Fig. 1

**Figure 2.** Profile of $10^{-6}$ M isoproterenol-stimulated melatonin production from perifused rat pineal glands of young (55 day old) and old (21 month old) rats obtained at different circadian stages. Each value is the mean ± S.E.M. of data obtained in five perifusion chambers (one gland per chamber). Isoproterenol (arrows) was infused for 30 min, 250 min after the beginning of the perifusion. 3 to 11 HALO: light phase; 15 to 23 HALO: dark phase.

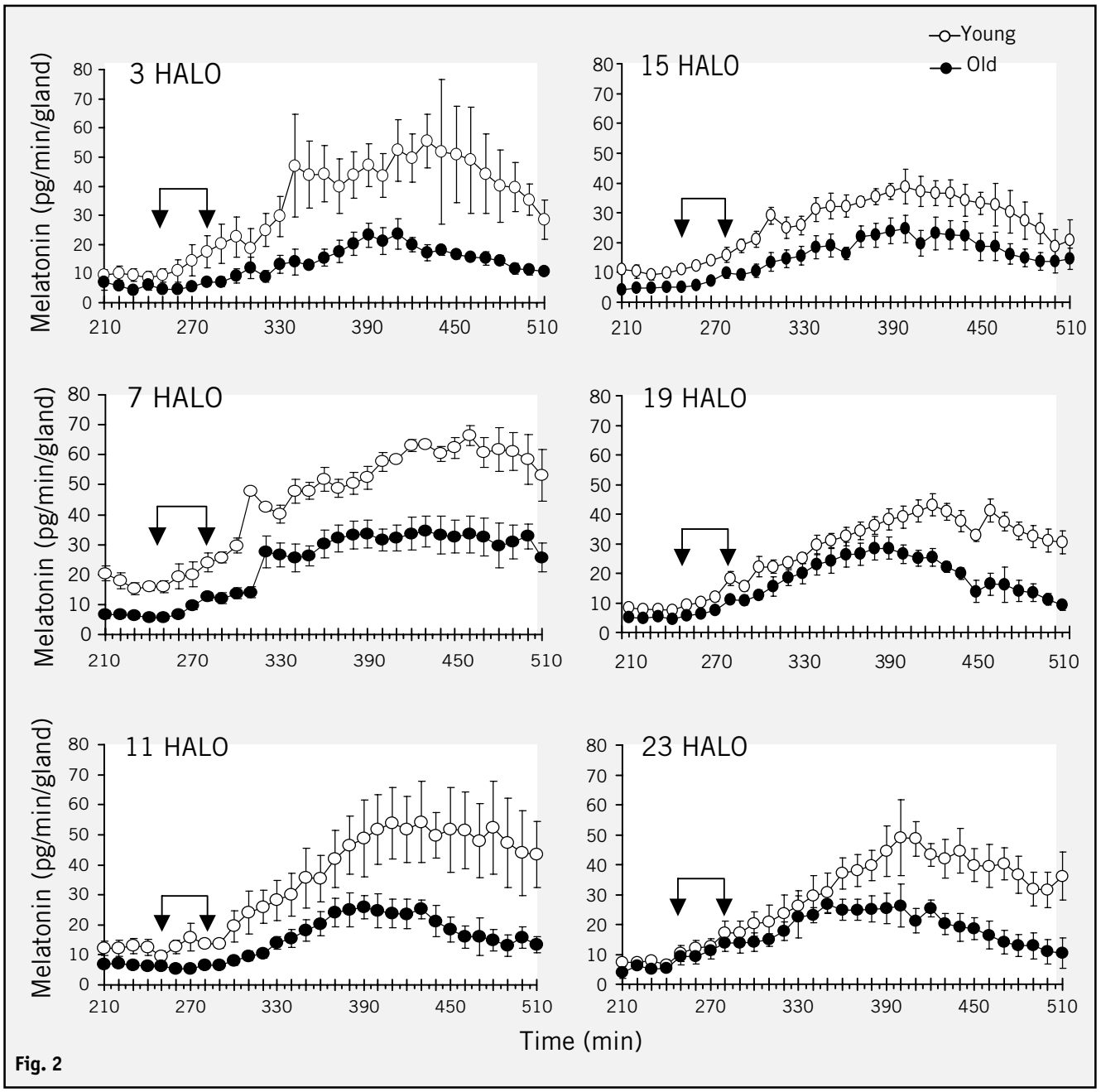


Fig. 2

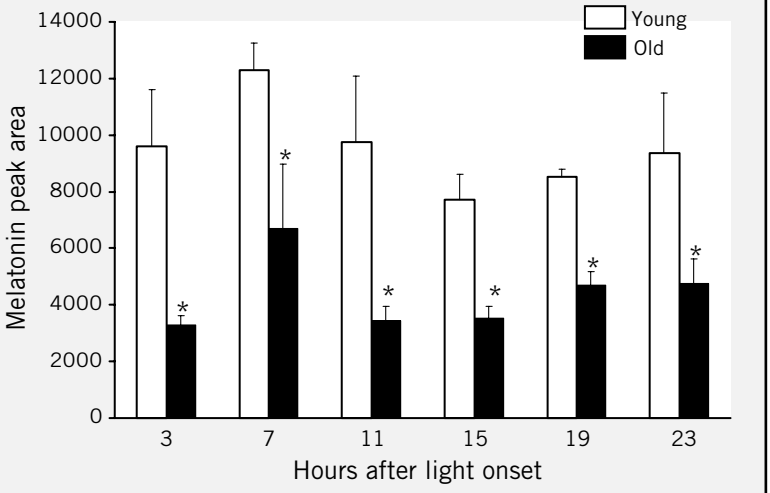


Fig. 3

**Figure 3.** Comparison of the increased melatonin release in response to a $10^{-6}$ M isoproterenol infusion by young (55 day old) and old (21 month old) rat pineal glands obtained at different circadian stages. Each value is the mean ± S.E.M. of data obtained in five perifusion chambers (one gland per chamber). The melatonin peak area is significantly greater in young than in old rat pineal glands (*$P$<0.001). 3 to 11 HALO: light phase; 15 to 23 HALO: dark phase.

pg/min/gland, and 16 ± 1 pg/min/gland respectively, while those reported in the dark phase at 15, 19 and 23 HALO were 176 ± 6 pg/min/gland, 151 ± 20 pg/min/gland, and 122 ± 23 pg/min/gland respectively (*Figure 1*).

The basal melatonin levels (levels after stabilisation) in the young rats were approximately twice greater than those of the old rats ($P$<0.05) (*Figure 2*). The mean values ($n$ = 5 chambers for each circadian stage) of melatonin released by perifused pineal glands removed at 3 HALO were 10 ± 2 pg/min/gland in young rats versus 7 ± 3 pg /min/gland in old rats; for glands removed at 7 HALO, 20 ± 2 pg/min/gland in young rats versus 7 ± 1 pg/min/gland in old rats; for glands removed at 11 HALO, 12 ± 2 pg/min/gland in young rats versus 7 ± 1 pg/min/gland in old rats; for glands removed at 15 HALO, 11 ± 2 pg/min/gland in young rats versus 4 ± 1 pg/min/gland in old rats; for glands removed at 19 HALO, 8 ± 1 pg/min/gland in young rats versus 5 ± 1 pg/min/gland in old rats, and for glands removed at 23 HALO, 7 ± 1 pg/min/gland in young rats versus 4 ± 2 pg/min/gland in old rats (*Figure 2*).

Isoproterenol ($10^{-6}$ M) stimulated melatonin production in both the young and old rat pineal glands, regardless of the circadian stage (*Figure 2*). The melatonin release induced by $10^{-6}$ M isoproterenol increased linearly for at least 2 h 30 min after stimulation. By 410–430 min, melatonin concentrations reach levels 3 to 6 times higher than baseline ($P$<0.001) regardless circadian stage in both age groups, and fell gradually after this time. The maximal values of melatonin concentrations released by perifused pineal glands after stimulation by isoproterenol were observed in the middle of the light phase, at 7 HALO, in both age groups, and were 66 ± 3 pg/min/gland in young rats and 34 ± 5 pg/min/gland in old rats respectively (*Figure 2*).

The intensity of the response to $10^{-6}$ M isoproterenol infusion was greater in young than in old rat pineal glands ($P$<0.001) (*Figure 3*), with a trend towards an increase during the light phase, at 7 HALO, in both young and old rat pineal glands, although this trend towards increased melatonin response did not reach statistical significance ($P$>0.05) (*Figure 3*).

## Discussion

This *in vitro* perifusion study shows that the levels of melatonin released by the rat pineal glands are age-dependent, which is consistent with the fact that pineal melatonin concentrations fall with age, in both rodents [2–7] and humans [8–10]. Under our experimental conditions, the basal melatonin levels in old rats were approximately 2 times lower than those of young rats. It has been suggested that the decrease in the rate of pineal melatonin production with ageing in rats may be due to the increasing dilution factor as a consequence of the increase in body size [4,17], and not due to a decrease in sympathetic activity [18], and that the reduction in pineal β-receptor density and the age-associated decline in pineal production of melatonin are inter-related [11].

We have previously shown that rat pineal glands in a perifusion system can be stimulated by the β-adrenergic agonist, isoproterenol [15]. This increase in melatonin has been reported in young adult rats [15,19], but not in old rats. Our study shows that isoproterenol stimulates melatonin production in both young and old rat pineal glands, whatever the circadian stage. The intensity of the response to isoproterenol infusion was greater in young than in old rat pineal glands with a trend towards an increase during the light phase, at 7 HALO, in both young and old rat pineal glands, but this trend towards increased melatonin response did not reach statistical significance ($P$>0.05). Zhao and Touitou [15] have already reported that the response of the melatonin release to isoproterenol stimulation by perifused pineal glands in young rats was not significantly different between the middle of the light phase (7 HALO) and the middle of the dark phase (19 HALO). In contrast to rats, β-ad-

renergic receptor agonists did not stimulate daytime melatonin secretion in humans [20].

In conclusion, our data show that pineal glands removed from aged rats are stimulable by isoproterenol whatever the circadian stage. However, the magnitude of isoproterenol stimulation is lower in old than in young pineal glands. Our data suggest that the pineal gland response to isoproterenol is not circadian stage-dependent, at least, under our experimental conditions.

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