

Serotonergic Modulation of Rat Pineal Gland Activity: In Vivo Evidence for a 5-Hydroxytryptamine_{2C} Receptor Involvement

LUCA STEARDO, PALMIERO MONTELEONE, LUGIA TRABACE, CARLA CANNIZZARO, MARIO MAJ, and VINCENZO CUOMO

Department of Pharmacological Sciences, University of Palermo, Palermo, Italy (L.S., C.C.); Institute of Psychiatry, University of Naples SUN, Naples, Italy (P.M., M.M.); and Department of Pharmacology and Human Physiology, University of Bari, Bari, Italy (L.T., V.C.)

Accepted for publication June 30, 2000 This paper is available online at <http://www.jpet.org>

ABSTRACT

There are some suggestions that, in the pineal gland, serotonin acts not only as a precursor of melatonin but also plays a role in the modulation of the pineal biosynthetic activity. To corroborate this possible neuromodulatory role of 5-hydroxytryptamine (serotonin) (5-HT) on the pineal gland, the effects of two 5-HT₂ receptor agonists *meta*-chlorophenylpiperazine (*m*-CPP) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane were assessed in vivo on pineal *N*-acetyltransferase (NAT) activity and melatonin content in rats. *m*-CPP potentiated the enhancement of NAT activity and pineal melatonin content induced by isoproterenol administration during daytime, whereas it did not affect the diurnal basal biosynthetic activity of the gland. At night, *m*-CPP and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane enhanced significantly the physi-

ological increases in both pineal NAT activity and melatonin content. This enhancement was prevented by pretreatment with *N*-(1-methyl-5-indolyl)-*N'*-(3-pyridyl) urea hydrochloride, an antagonist with higher affinity for 5-HT_{2B/C} than for 5-HT_{2A} receptor, as well as by pretreatment with 8-[5-(2,4-dimethoxy-5-(4-trifluoromethyl-phenylsulphonamido)-phenyl-5-oxopentyl]-1,3,8-triazospiro[4,5]decane-2,4-dione, the most specific 5-HT_{2C} receptor now available, but not by pretreatment with ketanserin, an antagonist with higher affinity for 5-HT_{2A} than for 5-HT_{2C} receptor. These results suggest that 5-HT_{2C} receptors are likely involved in the mediation of the serotonergic modulation of pineal biosynthetic activity in rats.

In mammals the pineal gland produces and releases melatonin with a circadian rhythm, with the highest levels occurring during the night (Klein et al., 1983). A central oscillator located in the hypothalamic suprachiasmatic nucleus drives the nocturnally elevated release of norepinephrine (NE) from the sympathetic nerve endings projecting from the superior cervical ganglion to the gland (Borjigin et al., 1999). During the dark phase, the enhanced NE release leads via β_1 -adrenoceptor stimulation to an increase of the intracellular levels of cAMP, which in turn activates 5-hydroxytryptamine (serotonin) (*N*-acetyltransferase (NAT), the rate-limiting enzyme controlling melatonin synthesis (Sugden, 1989). In addition, α_1 -adrenoceptor stimulation, which activates the phosphoinositide pathway and enhances intracellular calcium concentration, is known to be involved in the potentiation of β_1 -adrenoceptor stimulatory effects (Klein et al., 1983; Vacas et al., 1985).

At present, there is growing evidence suggesting that, in addition to the noradrenergic modulation, pineal function is

under a more complicated control because several reports have revealed that multiple receptors for amino acids, peptides, and biogenic amines, other than NE, are located on mammalian pinealocytes (Sarda et al., 1989; Jansen et al., 1990; Schaad and Klein, 1992; Ebadi, 1993; Middendorff et al., 1996; Simonneaux et al., 1998). To date, although the role remains incompletely understood, their presence in the rat pineal gland in significant concentrations raises the hypothesis that they may be importantly involved in the physiological regulation of cellular events underlying melatonin synthesis. Therefore, their functional significance is currently under extensive research.

The finding that 5-HT₂ binding sites have been identified on mammalian pineal gland (Sparks and Little, 1990; Govitrapong et al., 1991) has prompted the idea that these receptors may play a potential role in regulating melatonin synthesis. Even though 5-HT in the pineal has until recently been thought to serve solely as a precursor for the nocturnal synthesis of the hormone, some experimental observations reinforced the suggestion that 5-HT may be implicated in a more complex manner in the physiological control of pineal

Received for publication April 19, 2000.

ABBREVIATIONS: NE, norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); NAT, *N*-acetyl transferase; *m*-CPP, *meta*-chlorophenylpiperazine; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; KET, ketanserin; SB 200646A, *N*-(1-methyl-5-indolyl)-*N'*-(3-pyridyl) urea hydrochloride; RS 102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethyl-phenylsulphonamido)-phenyl-5-oxopentyl]-1,3,8-triazospiro[4,5]decane-2,4-dione; DMSO, dimethyl sulfoxide.

activity. With regard to this, it was of interest that previous studies reported that 5-HT concentrations in the pineal, the highest in the brain, undergo circadian fluctuations, which parallel extracellular 5-HT levels (Young and Anderson, 1982; Azekawa et al., 1991), and it was demonstrated that 5-HT is secreted from the gland in response to noradrenergic stimuli (Aloyo and Walker, 1988).

Furthermore, the finding that 5-HT added to rat pinealocytes in primary culture amplified NAT activation induced by β_1 -adrenoceptor stimulation, whereas it failed to enhance the basal enzymatic activity, has provided additional support to the assumption that extracellular pineal 5-HT may be regarded as one of the physiological factors modulating stimulated melatonin synthesis, possibly through the activation of specific receptor sites (Sugden, 1990).

The present study describes the *in vivo* effects of the 5-HT₂ agonists *meta*-chlorophenylpiperazine (*m*-CPP) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) on NAT activity and melatonin content in rat pineal gland. The results, providing evidence that 5-HT_{2C} receptor is implicated in the physiological regulation of melatonin biosynthesis, contribute to expand current knowledge on the biochemical mechanisms involved in the pineal activity in rodents.

Materials and Methods

Chemicals. *m*-CPP was kindly provided by Angelini A.C.R.A.F. (Rome, Italy) and was dissolved in saline solution. DOI and ketanserin (KET) were purchased from Research Biochemicals International (Natick, MA) and dissolved in saline solution. *N*-(1-Methyl-5-indolyl)-*N'*-(3-pyridyl) urea hydrochloride (SB 200646A) was kindly provided by SmithKline Beecham (The Pinnacles Harlow, Essex, UK); it was ground into 1% methyl cellulose in 0.9% NaCl with a drop of BRIJ 35 (Sigma Chemical Co., St. Louis, MO) dispersant using a mortar and pestle and neutralized to pH 4.5 with HCl. The 8-[5-(2,4-dimethoxy-5-(4-trifluoromethyl-phenylsulphonamido)-phenyl-5-oxopentyl]-1,3,8-triazospiro[4,5]decane-2,4-dione (RS 102221) was purchased from Tocris Cookson Ltd (Bristol, UK) and dissolved in 100 mM dimethyl sulfoxide (DMSO). Isoproterenol hydrochloride was purchased from Sigma Chemical Co. and dissolved in saline solution. The doses of all compounds were chosen according to those reported as effective in the current literature.

Animals. Male adult Wistar rats (Morini, S. Polo d'Enza, Italy) were used in the experiments. They were housed four to five per cage in a temperature-controlled environment ($22 \pm 2^\circ\text{C}$) with a constant 12-h light/12-h dark cycle (lights on at 8:00 AM). Animals were allowed free access to food and water. In the experiments performed in darkness, all the procedures were carried out with a red light. This light is of insufficient brightness and of improper wavelength to influence melatonin production (Reiter, 1985). All experimental procedures were carried out in strict compliance with the Guideline of the Italian Ethics Legislation for Animal Experimentation (D.L. 116/92).

Experiment 1. The aim of this experiment was to assess the effects of 5-HT receptor activation by *m*-CPP, a mixed agonist acting prevalently at 5-HT_{2B/C} receptor sites (Barnes and Sharp, 1999), on diurnal basal and isoproterenol-stimulated pineal activity. To this purpose, 60 rats (weighing 300–350 g) were used in this experiment. At 4:00 PM, six rats were sacrificed by decapitation and served as pretreatment controls; the remaining rats were divided in three groups (18 animals each) and treated with 1 mg/kg *m*-CPP, 2.5 mg/kg *m*-CPP, and volume-matched saline *i.p.*, respectively. Fifteen minutes later, in each group, six animals were injected with 0.3 mg/kg isoproterenol, six with 1.0 mg/kg isoproterenol, and six with volume-matched saline *i.p.* At 6:15 PM, all animals were sacrificed by decapitation.

Experiment 2. The aim of this experiment was to assess the effects of 5-HT receptor activation by *m*-CPP on the pineal melatonin production during the night, when the gland is physiologically activated by NE. To this purpose, 54 rats (weighing 200–250 g) were used in this experiment. At 9:00 PM, in dark conditions, six rats were sacrificed by decapitation and served as pretreatment controls; the remaining rats were divided in four groups (12 animals each) and treated with 0.5 mg/kg *m*-CPP, 1.0 mg/kg *m*-CPP, 2.5 mg/kg *m*-CPP, and volume-matched saline *i.p.*, respectively. Sixty, and 120 min later, six animals from each group were sacrificed by decapitation.

Experiment 3. In this experiment we aimed to evaluate whether the effects of *m*-CPP on nocturnal pineal activity were prevented by pretreatment with the serotonergic antagonist KET, which is more selective for 5-HT_{2A} than 5-HT_{2B/C} receptors (Barnes and Sharp, 1999). To this purpose, 45 rats (weighing 250–300 g) were used. At 9:00 PM, in dark conditions, rats were divided in three groups (15 animals each) and injected with 1.5 mg/kg KET, 6.0 mg/kg KET, and volume-matched saline *i.p.*, respectively. At 9:20 PM, in each group, five rats were treated with 1.0 mg/kg *m*-CPP, five with 2.5 mg/kg *m*-CPP, and five with volume-matched saline *i.p.* At 11:20 PM, all the animals were sacrificed by decapitation.

Experiment 4. In this experiment we aimed to evaluate whether the effects of *m*-CPP on nocturnal pineal activity were prevented by pretreatment with SB 200646A, a 5-HT receptor antagonist with higher affinity for the 5-HT_{2B/C} than for 5-HT_{2A} receptor (Kennett et al., 1994). To this purpose, 36 rats (weighing 200–250 g) were used. At 9:15 PM, in dark conditions, rats were divided in three groups (12 animals each): one group received orally 20 mg/kg SB 200646A in a 2-ml/kg volume, one group received orally 40 mg/kg SB 200646A in a 2-ml/kg volume, and the remaining one received orally volume-matched vehicle (1% methyl cellulose in 0.9% NaCl with a drop of BRIJ 35, acidified to pH 4.5 with HCl). At 9:20 PM, in each group, four rats were treated with 1.0 mg/kg *m*-CPP, four with 2.5 mg/kg *m*-CPP, and four with volume-matched saline *i.p.* At 11:20 PM, all the animals were sacrificed by decapitation.

Experiment 5. In this experiment we aimed to evaluate whether the effects of *m*-CPP on nocturnal pineal activity were prevented by pretreatment with the most selective 5-HT_{2C} receptor antagonist now available, RS 102221 (Bonhaus et al., 1997). To this purpose, 36 rats (weighing 200–250 g) were used. At 9:00 PM, in dark conditions, rats were divided in three groups (12 animals each) and injected with 0.5 mg/kg RS 102221, 2.5 mg/kg RS 102221, or volume-matched vehicle (100 mM DMSO) *i.p.*, respectively. At 9:20 PM, in each group, four rats were treated with 1.0 mg/kg *m*-CPP, four with 2.5 mg/kg *m*-CPP 2.5, and four with volume-matched saline *i.p.* At 11:20 PM, all the animals were sacrificed by decapitation.

Experiment 6. In this experiment we aimed to better characterize the type of 5-HT receptor involved in the serotonergic potentiation of pineal biosynthetic activity. Therefore, we investigated the effects of DOI, a potent and partial agonist at 5-HT_{2A/C} receptors (Barnes and Sharp, 1999), on pineal melatonin production and assessed whether these effects were prevented by KET, SB 200646A, or RS 102221. On the basis of the results of previous experiments, only the highest doses of the 5-HT₂ antagonists were tested.

At 8:15 PM, in dark conditions, 40 rats (weighing 300–350 g) were divided in two groups (20 animals each): one group received orally 40 mg/kg SB 200646A in a 2-ml/kg volume and the other one received orally volume-matched vehicle (1% methyl cellulose in 0.9% NaCl with a drop of BRIJ 35, acidified to pH 4.5 with HCl). At 9:15 PM, in each group, five rats were treated with 0.125 mg/kg DOI, five with 0.25 mg/kg DOI, five with 1 mg/kg DOI, and five with volume-matched saline *i.p.* Two hours later, all the animals were sacrificed by decapitation.

At 9:00 PM, in dark conditions, 80 rats (weighing 300–350 g) were divided in four groups (20 animals each) and injected with 6.0 mg/kg KET, RS 102221 mg/kg, volume-matched vehicle (100 mM DMSO), or volume-matched saline *i.p.*, respectively. Fifteen minutes later, in each group, five rats were treated with 0.125 mg/kg DOI, five with

0.25 mg/kg DOI, five with 1 mg/kg DOI, and five with volume-matched saline i.p. Two hours later, all the animals were sacrificed by decapitation.

NAT and Melatonin Assays. After sacrificing the rats, the pineal glands were rapidly dissected, frozen on solid CO₂, and stored at -80°C until processed for melatonin and NAT assay. Pineal glands were homogenized in 0.05 M phosphate buffer, pH 7.4. Aliquots (10 μl) were used to measure NAT activity by the radioenzymatic method of Champney et al. (1984). Each sample was incubated for 20 min at 37°C in the presence of tryptamine-HCl (Sigma Chemical Co.), acetyl coenzyme A (Sigma Chemical Co.), and acetyl-[1-¹⁴C]coenzyme A (Amersham International plc, Bucks, UK; specific activity = 60 mCi/mmol). Results were expressed as nanomoles of NAT formed per gland per hour.

For melatonin assays, homogenate aliquots of 50 μl were extracted by diethyl ether and pineal melatonin levels were determined by radioimmunoassay (Arendt, 1978) using a sheep melatonin antiserum from Guildhay Antiserum (University of Surrey, UK), [³H]melatonin tracer (Amersham International plc; specific activity = 85 Ci/mmol), and unlabeled melatonin (Sigma Chemical Co.). We assayed 500-μl duplicate aliquots, comparing results directly against a standard curve prepared in assay buffer (0.1 mol/l tricine, pH 7.4, containing 9 g of NaCl and 1 g of gelatin per liter). The reaction mixtures were incubated for 18 h at 4°C, and free and antibody-bound fractions of [³H]melatonin were separated using a dextran-coated charcoal solution (2%:0.2%); the lower and the upper detection limits of the assay were 6 and 200 pg/ml, respectively. Intra- and interassay coefficients of variation were 5.1 and 9.8%, respectively.

Statistical Analysis. Results were expressed as mean ± S.D. Data obtained from experiments 1, 3, 4, 5, and 6 were statistically analyzed by two-way ANOVA; when significant group differences emerged, statistical significances between groups were evaluated by the post hoc Tukey's test. Data obtained from experiment 2, comparing pineal NAT activity and melatonin levels from saline- and drug-treated animals over time, were analyzed by two-way ANOVA with repeated measures. Group differences at each time point were evaluated by the post hoc Tukey's test.

Results

Experiment 1. As expected, isoproterenol administration resulted in a clear-cut increase in pineal NAT activity, but this effect was different in rats pretreated with saline compared with those pretreated with *m*-CPP. Two-way ANOVA disclosed significant effects for drug pretreatment ($F_{2,45} = 60.917$, $P < .0001$) and for drug treatment ($F_{2,45} = 105.66$, $P < .0001$), and a significant pretreatment × treatment interaction ($F_{4,45} = 18.731$, $P < .0001$). As shown in Fig. 1, in animals preinjected with saline, isoproterenol was able to induce a significant increase in the pineal NAT activity at the dose of 1 mg/kg ($P < .03$), but not at the dose of 0.3 mg/kg. The isoproterenol-induced increases in NAT activity were clearly potentiated by *m*-CPP pretreatment with a clear-cut dose-dependent effect. Moreover, *m*-CPP was not able by itself to affect basal pineal NAT activity.

Melatonin results paralleled NAT results. Two-way ANOVA disclosed significant effects for drug pretreatment ($F_{2,45} = 66.761$, $P < .0001$) and for drug treatment ($F_{2,45} = 133.404$, $P < .0001$), and a significant pretreatment × treatment interaction ($F_{4,45} = 17.796$, $P < .0001$). In animals preinjected with saline, isoproterenol was able to induce a significant increase in the pineal melatonin content at the dose of 1 mg/kg ($P < .01$), but not at the dose of 0.3 mg/kg (Fig. 1). *m*-CPP was not able by itself to affect basal pineal melatonin levels, whereas it dose dependently potentiated

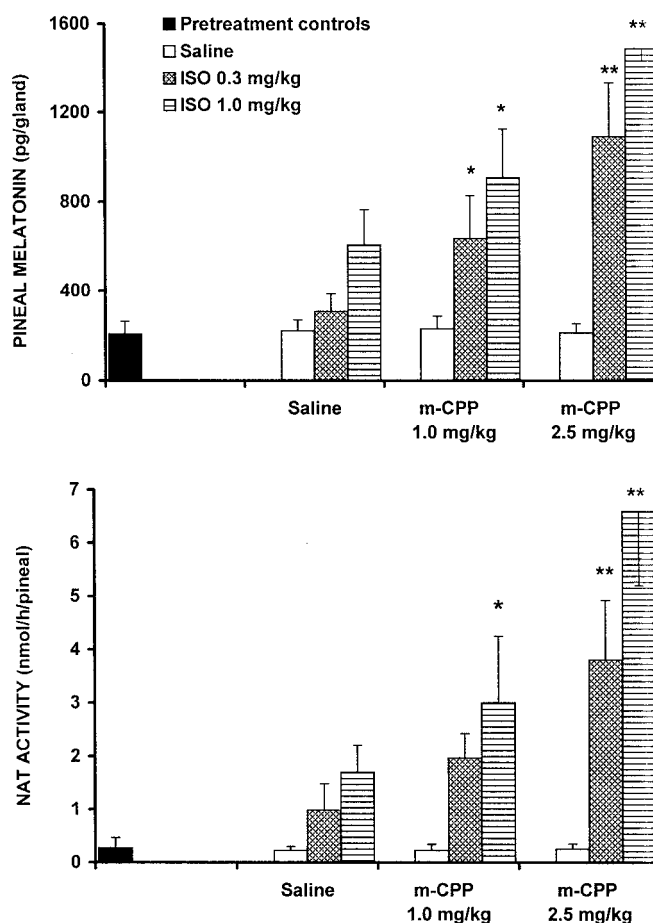


Fig. 1. Effects of different doses of *m*-CPP on basal and isoproterenol (ISO)-stimulated pineal NAT activity (bottom) and melatonin levels (top) during the day in rats. Data represent mean ± S.D. averaged from six animals. * $P < .05$, ** $P < .001$ versus correspondent saline-pretreated animals.

the isoproterenol-induced increase in pineal melatonin content (Fig. 1).

Experiment 2. In the darkness, as expected, pineal NAT activity and melatonin levels progressively rose from 9:00 PM to 11:00 PM in saline-injected animals (Fig. 2). Compared with saline, the administration of *m*-CPP induced greater increases in the NAT activity and melatonin concentrations with a dose-dependent effect (Fig. 2). Two-way ANOVA with repeated measures disclosed significant effects for treatment ($F_{3,16} = 4.676$, $P < .01$) and for time ($F_{2,32} = 35.528$, $P < .0001$). Analogously, two-way ANOVA with repeated measures performed on melatonin data showed significant effects for treatment ($F_{3,16} = 3.235$, $P < .04$) and for time ($F_{2,32} = 31.868$, $P < .0001$).

Experiment 3. The *m*-CPP-induced potentiation of the nocturnal increase in pineal NAT activity was not prevented by pretreatment with both 1.5 mg/kg KET and 6.0 mg/kg KET (Fig. 3). Two-way ANOVA showed no significant effect for drug pretreatment ($F_{2,36} = 0.211$, NS) and no significant pretreatment × treatment interaction ($F_{4,36} = 0.900$, NS), but a significant effect for *m*-CPP treatment ($F_{2,36} = 27.645$, $P < .0001$). Melatonin results paralleled NAT results (Fig. 3). Two-way ANOVA showed no significant effect for drug pretreatment ($F_{2,36} = 0.018$, NS) and no significant pretreatment × treatment interaction ($F_{4,36} = 0.256$, NS), but a

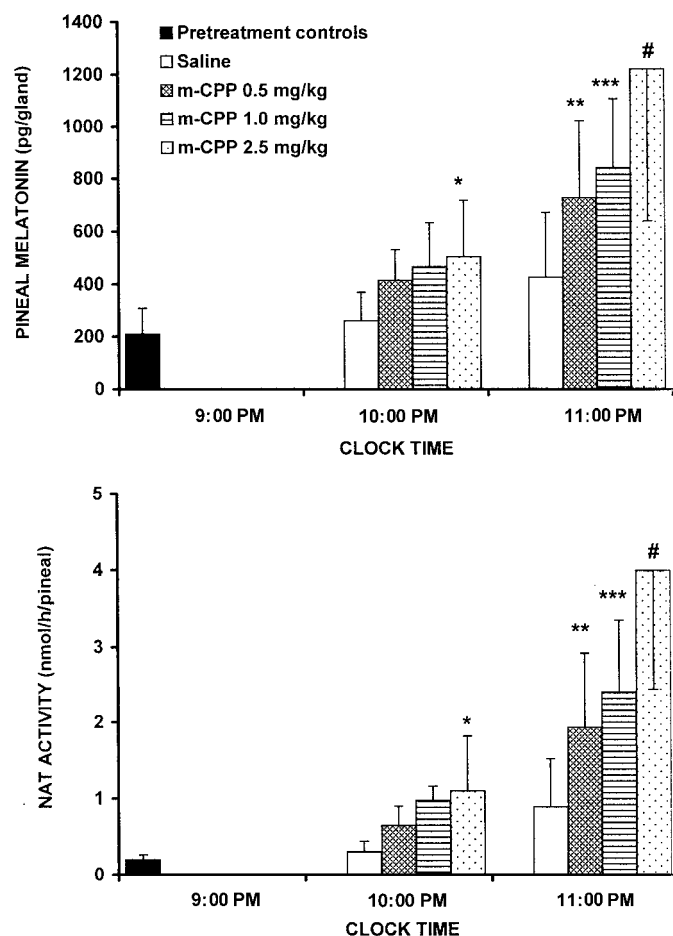


Fig. 2. Effects of different doses of *m*-CPP on nocturnal pineal NAT activity (bottom) and melatonin levels (top) in rats. Data represent mean \pm S.D. averaged from six animals. * $P < .05$, ** $P < .02$, *** $P < .005$; # $P < .001$ versus correspondent saline-injected animals.

significant effect for *m*-CPP treatment ($F_{2,36} = 69.099$, $P < .0001$).

Experiment 4. The *m*-CPP-induced potentiation of the nocturnal increase in both pineal NAT activity and melatonin content was prevented or significantly blunted by pretreatment with 40 mg/kg SB 200646A, but not by pretreatment with the lower dose of this compound (Fig. 4). Two-way ANOVA performed on NAT data showed significant effects for drug pretreatment ($F_{2,27} = 13.650$, $P < .0001$) and for drug treatment ($F_{2,27} = 64.616$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{4,27} = 5.175$, $P < .005$). As concerns melatonin, two-way ANOVA showed significant effects for drug pretreatment ($F_{2,27} = 17.718$, $P < .0001$) and for drug treatment ($F_{2,27} = 70.950$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{4,27} = 3.984$, $P < .02$).

Experiment 5. The *m*-CPP-induced potentiation of the nocturnal increase in both pineal NAT activity and melatonin content was prevented by pretreatment with 2.5 mg/kg RS 102221, but not by pretreatment with the lower dose of this compound (Fig. 5). Two-way ANOVA performed on NAT data showed significant effects for drug pretreatment ($F_{2,27} = 5.199$, $P < .02$) and for drug treatment ($F_{2,27} = 8.375$, $P < .002$), and a significant pretreatment \times treatment interaction ($F_{4,27} = 5.175$, $P < .005$). As concerns melatonin, two-

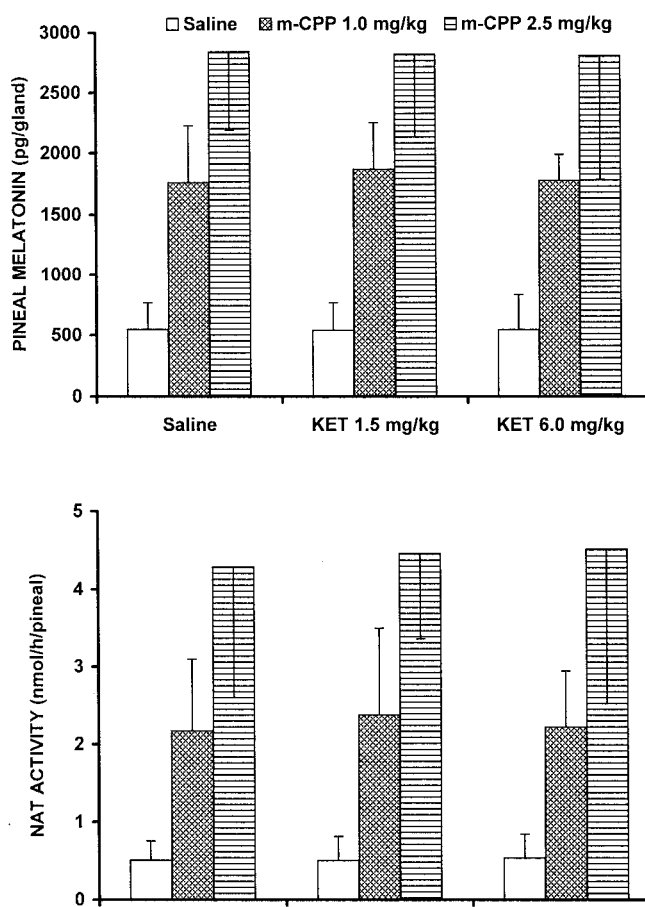


Fig. 3. Effects of KET on *m*-CPP-induced increase in nocturnal pineal NAT activity (bottom) and melatonin content (top) in rats. Data represents mean \pm S.D. averaged from five animals.

way ANOVA showed significant effects for drug pretreatment ($F_{2,27} = 7.901$, $P < .002$) and for drug treatment ($F_{2,27} = 29.052$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{4,27} = 5.340$, $P < .003$).

Experiment 6. Compared with saline, the administration of DOI induced greater increases in nocturnal pineal NAT activity and melatonin content with a dose-dependent effect (Fig. 6). This action was clearly prevented or significantly blunted by pretreatment with SB 200646A. Two-way ANOVA showed significant effects for drug pretreatment ($F_{1,32} = 15.007$, $P < .0006$) and for drug treatment ($F_{3,32} = 13.435$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{3,32} = 4.099$, $P < .02$). Melatonin results paralleled NAT results (Fig. 6). Two-way ANOVA showed significant effects for drug pretreatment ($F_{1,32} = 19.099$, $P < .0001$) and for drug treatment ($F_{3,32} = 22.723$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{3,32} = 3.677$, $P < .03$).

Moreover, the DOI-induced potentiation of nocturnal pineal biosynthetic activity was clearly prevented by pretreatment with 2.5 mg/kg RS 102221, but not by pretreatment with 6.0 mg/kg KET (Fig. 7). As concerns NAT activity, two-way ANOVA showed significant effects for drug pretreatment ($F_{3,64} = 19.934$, $P < .0001$) and for drug treatment ($F_{3,64} = 75.534$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{9,64} = 4.673$, $P < .001$). Melatonin results paralleled NAT results (Fig. 7). Two-way ANOVA

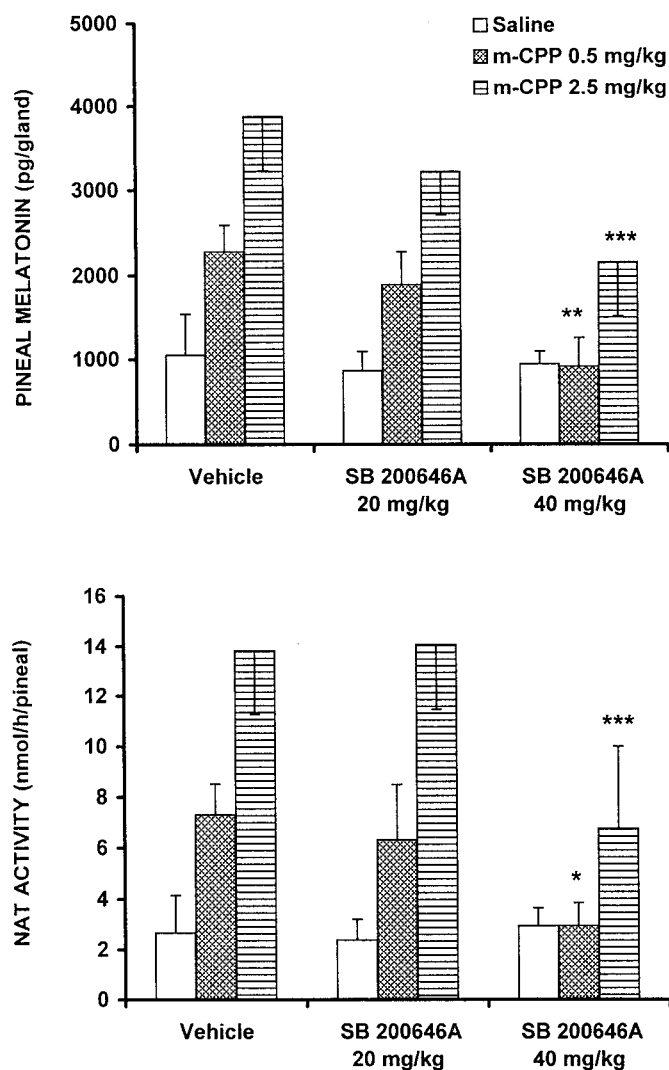


Fig. 4. Effects of different doses of SB 200646A on *m*-CPP-induced increase in nocturnal pineal NAT activity (bottom) and melatonin content (top) in rats. Data represents mean \pm S.D. averaged from four animals * P < .05, ** P < .005, *** P < .001 versus correspondent vehicle-pretreated animals.

showed significant effects for drug pretreatment ($F_{3,64} = 14.303$, $P < .0001$) and for drug treatment ($F_{3,64} = 59.504$, $P < .0001$), and a significant pretreatment \times treatment interaction ($F_{9,64} = 2.513$, $P < .02$).

Discussion

The major finding of the present study was that acute i.p. administration of serotonergic agents acting as agonists at 5-HT₂ receptors enhanced NAT activity and melatonin synthesis in rats, giving grounds for a functional involvement of such receptors in the biochemical events modulating the physiological activity of rat pineal gland. The present data are of relevance in that they extend knowledge about the multifactorial control of melatonin production and confirm serotonergic mechanisms exert a marked and varied influence on the activity of the gland. Indeed, in addition to its role as precursor in the biosynthetic pathway leading to melatonin formation, 5-HT was also reported to be secreted by rat pinealocytes in culture (Aloyo and Walker, 1988), and 5-HT-

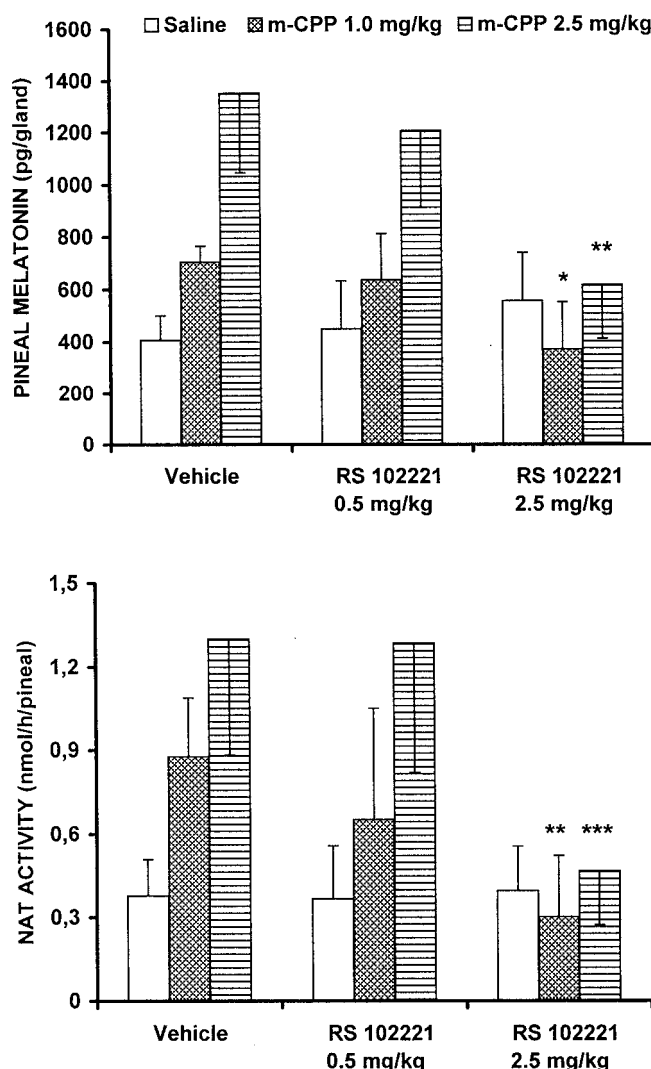


Fig. 5. Effects of different doses of RS 102221 on *m*-CPP-induced increase in nocturnal pineal NAT activity (bottom) and melatonin content (top) in rats. Data represents mean \pm S.D. averaged from four animals * P < .05, ** P < .02, *** P < .001 versus correspondent vehicle-pretreated animals.

containing fibers were identified within mammalian pineal (Matsurra and Sano, 1983), although the functional role for the extracellular pineal 5-HT remained for the most part speculative. The current results may suggest that in rat pineal gland extracellular 5-HT, interacting with 5-HT₂ receptor subtypes, phasically reinforces the nocturnal synthesis of melatonin and potentiates that induced by the adrenergic challenge during the day.

The findings of the first set of experiments, designed to explore the effects of the phenylpiperazine *m*-CPP, a mixed agonist acting prevalently at 5-HT_{2B/C} receptor sites (Barnes and Sharp, 1999), on the diurnal activity of the gland indicated that the drug, by itself, failed to influence melatonin synthesis at any doses. Conversely, it yielded a dose-dependent increase of the hormonal production stimulated by the β_1 -adrenoceptor activation with the selective agonist isoproterenol. In fact, during the day, the combination of *m*-CPP and isoproterenol resulted in a more pronounced effect compared with that provoked by the β_1 -agonist alone, suggesting a 5-HT_{2B/C} receptor-mediated potentiation of the β_1 -adrener-

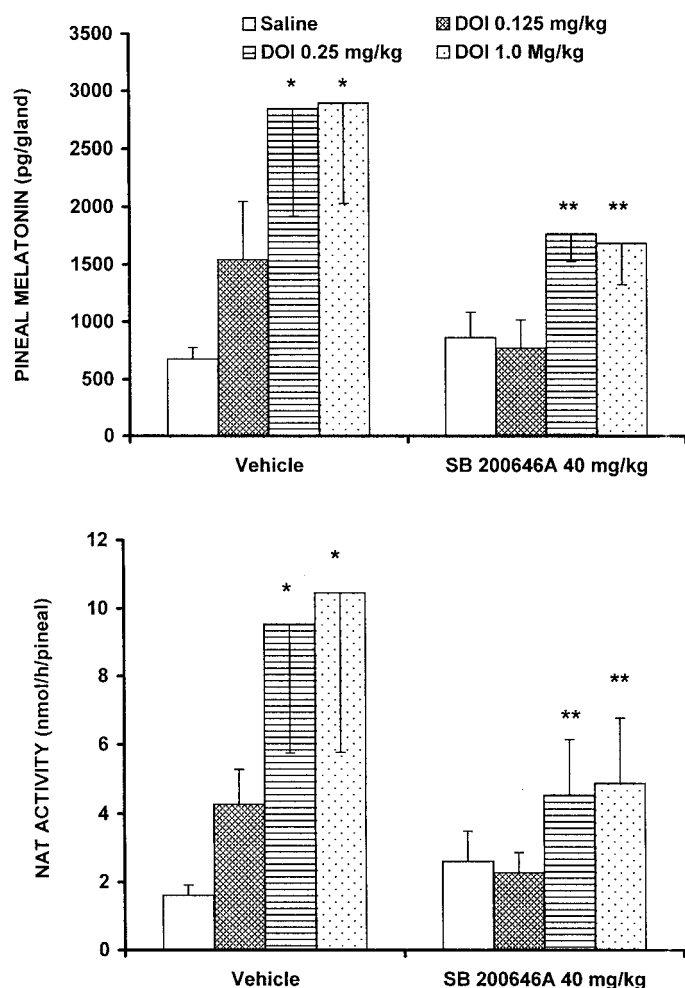


Fig. 6. Effects of different doses of DOI on nocturnal pineal NAT activity (bottom) and melatonin levels (top) in rats pretreated with vehicle or SB 200646A. Data represent mean \pm S.D. averaged from five animals. * P < .001 versus saline-treated animals; ** P < .02 versus correspondent vehicle-pretreated rats.

gic stimulation of the pineal function. This assumption was further supported by the findings of the second set of experiments, aimed at examining the effect of *m*-CPP on rat pineal gland during the night, when the hormonal production was already physiologically activated by the enhanced release of NE from the sympathetic terminals. Thus, *m*-CPP, administered during darkness, dose dependently increased NAT activity and amplified the physiological rise in melatonin synthesis provoked by the nocturnal adrenergic stimulation. Taken together, these data may be interpreted as support to the hypothesis that *m*-CPP, unable per se to influence NAT activity, affected pineal function by reinforcing NE-induced melatonin synthesis through a mechanism that, consistently with its pharmacological profile, might be related with a 5-HT_{2B/C} receptor mediation. However, because binding studies have revealed that *m*-CPP displays a more complex pharmacology than originally considered, it cannot be completely excluded that at least part of its effects might well result from the interaction with non-5-HT_{2B/C} receptor sites. In fact *m*-CPP, although predominantly serotonergic, is not selective at one receptor subtype. To clarify this issue, because selective antagonists are now available, which greatly

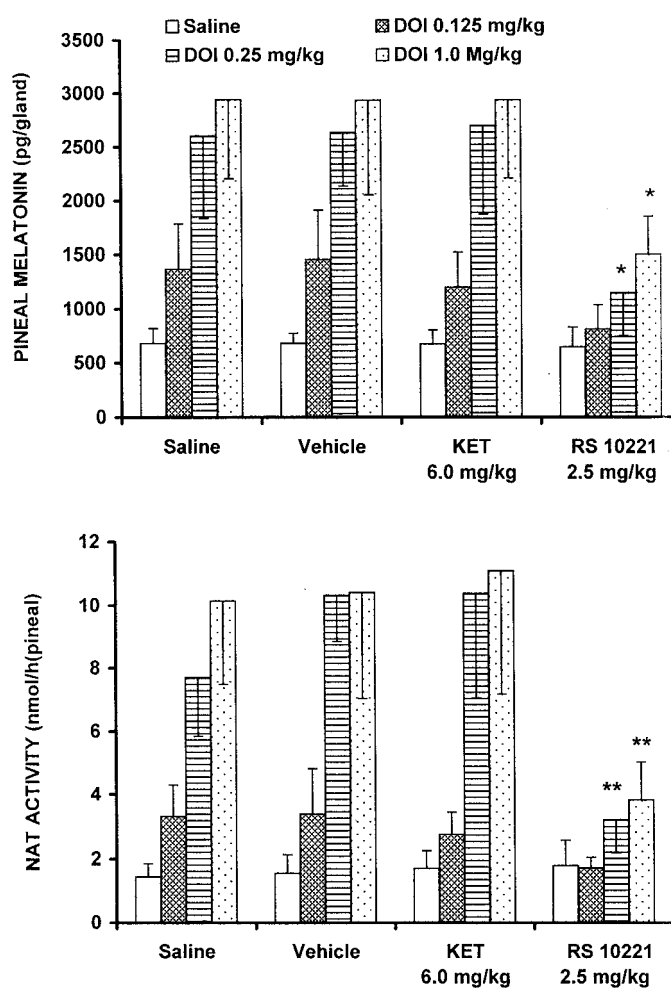


Fig. 7. Effects of different doses of DOI on nocturnal pineal NAT activity (bottom) and melatonin levels (top) in rats pretreated with saline, vehicle, KET or RS 102221. Data represent mean \pm S.D. averaged from five animals. * P < .005 and ** P < .001 versus correspondent vehicle-pretreated rats.

aid the delineation of 5-HT receptor subtype, compounds with high affinity for different 5-HT receptors were investigated for their potential ability to attenuate *m*-CPP-induced enhancement of pineal activity. According to a large body of converging evidence that implicates agonist interactions with 5-HT_{2C} receptors as the main mediators of pharmacological effects of *m*-CPP (Callahan and Cunningham, 1994; Fiorella et al., 1995; Gommans et al., 1998), antagonists with different selectivity for such receptor subtype were tested.

KET, a 5-HT_{2A/C} antagonist with almost two orders of magnitude more selectivity for 5-HT_{2A} versus 5-HT_{2B/C} receptors (Janssen, 1983; Barnes and Sharp, 1999), failed to affect *m*-CPP-provoked potentiation of pineal activity at any doses tested, promoting the idea that 5-HT_{2A} receptors were not involved in mediating the hormonal response observed. This finding was consistent with previous results showing that 5-HT_{2A} receptor antagonists generally were inadequately effective against most *m*-CPP-induced responses (Gommans et al., 1998). In addition, although the high affinity of KET for α_1 -adrenergic receptors (Fozard, 1982), its failure to reverse *m*-CPP effects on NAT activity and melatonin levels argued against interactions with such receptors,

because their activation was proved to potentiate the β_1 -adrenergic receptor stimulation of the pineal biosynthetic activity (Klein et al., 1983). In contrast, SB 200646A, an antagonist with higher affinity for the 5-HT_{2B/C} than for the 5-HT_{2A} receptor (Forbes et al., 1993; Kennett et al., 1994a,b), was able to reverse *m*-CPP rising effect on melatonin synthesis, providing evidence that *m*-CPP induced increase of pineal activity through a mechanism involving 5-HT_{2B/C} receptor stimulation. To further characterize the receptor subtype responsible for the rise in the hormonal production observed herein, the effect of the novel high-affinity and selective 5-HT_{2C} receptor antagonist RS 102221 was investigated (Weinhardt et al., 1996; Bonhaus et al., 1997). The efficacy of RS 102221 to completely antagonize in a dose-related manner *m*-CPP-induced enhancement of NAT activity and melatonin formation strongly implicated agonist interactions at 5-HT_{2C} receptors as mediators for the effect reported in the present study.

Additional support to this view stemmed from the results of the experiments in which, instead of *m*-CPP, DOI, a potent and partial agonist at 5-HT_{2A/C} receptors (Barnes and Sharp, 1999), was used with the same experimental procedure. Interestingly, acute DOI administration resulted in findings similar to those obtained in consequence of the exposure to *m*-CPP. Also in this case, the ability of SB 200646A, but not of KET, to reverse increases in NAT activity and melatonin synthesis induced by DOI strengthened further the likelihood of 5-HT_{2B/C} receptors being involved in the pineal response to DOI. Finally, the evidence that RS 102221 completely counteracted the effect of DOI on melatonin production confirmed that 5-HT_{2C} receptors were involved in the enhancement of melatonin production by serotonergic compounds studied in the present investigation. The findings of this study, therefore, provide persuasive evidence that 5-HT_{2C} agonists provoke a marked increase of pineal NAT activity and melatonin production, when administered to rats during the night and intensify the pineal hormonal production induced by β_1 -adrenoceptor pharmacological stimulation during the day. These results are consistent with previous results showing that such an action exists in pinealocytes in that activation of σ -receptors (Steardo et al., 1995), vasoactive intestinal peptide receptors (Simonneaux et al., 1998), and α_1 -adrenoceptors (Klein et al., 1983) per se does not alter NAT activity but does potentiate the β -adrenergic induction of this enzyme.

Based on the evidence provided by the current investigation, the indoleaminergic potentiation of melatonin production appears likely to be phasic because the failure of 5-HT₂ antagonists to inhibit the physiological activation of the hormone synthesis occurring at night argues against a tonic stimulation of NAT activity.

Currently, the precise site at which *m*-CPP and DOI interact with 5-HT_{2C} receptors to increase the NE-induced melatonin production remains largely indefinite. Using immunohistochemical preparations, the presence of 5-HT-containing axons entering the pineal anteriorly, that were not affected by sympathectomy (Matsurra and Sano, 1983), was well documented in many species including rodents, implying a postsynaptic location for such receptors sites. Conversely, recent studies have clearly demonstrated that rat pineal gland in acute organ culture releases 5-HT after α_1 -adrenergic stimulation, suggesting 5-HT as a possible autocrine fac-

tor in the physiological regulation of the hormonal formation, by interacting at a receptor site not necessarily associated with synapses (Aloyo and Walker, 1988). Along these lines, biochemical studies reported that NAT activity was enhanced by adding exogenous 5-HT to suspension cultures of rat pinealocytes stimulated with isoproterenol (Sugden, 1990), whereas ritanserin, a 5-HT₂ antagonist, reduced cAMP accumulation in pinealocytes activated by isoproterenol in vitro (Olcese and Munker, 1994). All these findings support the hypothesis that 5-HT_{2c} receptors might be located on the pineal cells to regulate melatonin synthesis, although other possibilities along the multisynaptic pathway from the retina to the pineal gland, of course, might exist concurrently (Kennaway and Moyer, 1998).

Finally, the present experiments, founded on pharmacological evidence, indicate a possible function for 5-HT₂ sites on the mammalian pineal gland. Because increasing evidence suggests an involvement of melatonin in the pathogenesis of several neuropsychiatric illnesses, including affective disorders and psychosis (Reiter, 1991), it is relevant that pineal activity appears to be also under the functional control of 5-HT_{2C} receptors, which have been recently implicated in the pathophysiology of such disorders (Fone et al., 1996; Moreau et al., 1996; Jenck et al., 1998). If the hypothesis provided by the present investigation is correct, then additional innovative perspectives may be suggested for a better understanding of pathological mechanisms responsible for the aforementioned diseases because dysregulated 5-HT₂ receptors in the pineal might result in dysfunctional melatonin synthesis and release.

In conclusion, it is interesting to speculate that 5-HT, released by noradrenaline from pinealocytes and/or released from presynaptic nerve endings, activating 5-HT_{2C} receptor sites, phasically modulates adrenergic induction of NAT activity and hence melatonin synthesis, allowing a subtle regulation of the nocturnal hormonal peak that is a crucial circadian message for the synchronization of physiological function.

Acknowledgments

We thank Drs. Antonio Attanasio and Michele Mazzoccoli, and Michele Persichella for skilful assistance in various aspects of this study. We thank Drs. I. Forbes (SmithKline Beecham) and I. Cane (Angelini A.C.R.A.F.) for the generous gift of SB 200646A and *m*-CPP, respectively. In addition, we thank the staff of the "Stazione Zoologica A. Dohrn", Naples, for help with the present manuscript.

References

- Aloyo VJ and Walker RF (1988) Alpha-adrenergic control of secretion of serotonin release from rat pineal glands. *Neuroendocrinology* **48**:61–66.
- Arendt J (1978) Melatonin assay in body fluid. *J Neural Transm* **21 (Suppl)**:265–278.
- Azekawa T, Sano A, Sei H and Morita Y (1991) Diurnal changes in pineal extracellular indoles of freely moving rats. *Neurosci Lett* **132**:93–96.
- Barnes NM and Sharp T (1999) A review of central 5-HT receptors and their function. *Neuropharmacology* **38**:1083–1152.
- Bonhaus DW, Weinhardt KK, Taylor M, Desouza A, McNeeley PM, Szczepanski K, Fontana DJ, Trinh J, Rocha CL, Dawson MW, Flippin LA and Eglen RM (1997) RS 102221: A novel high affinity and selective 5-HT_{2C} receptor antagonist. *Neuropharmacology* **36**:621–629.
- Borjigin J, Li X and Snyder SH (1999) The pineal gland and melatonin: Molecular and pharmacological regulation. *Annu Rev Pharmacol Toxicol* **39**:53–65.
- Callahan PM and Cunningham KA (1994) Involvement of central 5-HT_{2C} receptors in mediating the discriminative stimulus properties of *m*-chlorophenylpiperazine (*m*-CPP). *Eur J Pharmacol* **257**:27–38.
- Champney TH, Holtorf AP, Steger RP and Reiter RJ (1984) Concurrent determination of enzymatic activity and substrate concentrations in the melatonin synthetic pathway within the same pineal gland. *J Neurosci Res* **11**:59–65.
- Ebadi M (1993) Multiple pineal receptors in regulating melatonin synthesis, in

- Melatonin Biosynthesis Physiological Effects and Clinical Applications* (Yu H-S and Reiter RJ eds) pp 39–71, CRC Press, Boca Raton, FL.
- Fiorella D, Helsley S, Rabin RA and Winter JC (1995) 5-HT_{2C} receptor-mediated phosphoinositide turnover and the stimulus effects of m-chlorophenylpiperazine. *Psychopharmacology* **122**:237–243.
- Fone KCF, Shalders K, Fox ZD, Arthur R and Marsden CA (1996) Increased 5-HT_{2C} receptor responsiveness occurs on rearing rats in social isolation. *Psychopharmacology* **123**:346–352.
- Forbes IT, Kennett GA, Gadre A, Ham P, Hayward CJ, Martin RT, Thompson M, Wood MD, Baxter GS, Glen A, Murphy OE, Stewart BA and Blackburn TP (1993) N-(1-methyl-5-indolyl)-N'-(3-pyridyl)urea hydrochloride: The first selective 5-HT_{1C} receptor antagonist. *J Med Chem* **36**:1104–1107.
- Fozard JR (1982) Mechanism of the hypotensive effect of ketanserin. *J Cardiovasc Pharmacol* **4**:829–838.
- Gommans J, Hijzen TH, Maes RAA and Olivier B (1998) Discriminative stimulus properties of m-CPP: Evidence for a 5-HT_{2C} receptor mode of action. *Pharmacology* **137**:292–302.
- Govitrapong P, Prapapanich V and Ebadi M (1991) Identification of serotonin 5-HT₂ receptors in bovine pineal gland. *J Pineal Res* **11**:182–187.
- Jansen KLR, Dragunow M and Faull RLM (1990) Sigma receptors are highly concentrated in the rat pineal gland. *Brain Res* **507**:158–163.
- Janssen PA (1983) 5-HT₂ receptor blockade to study serotonin-induced pathology. *Trends Neurosci* **4**:198–206.
- Jenck F, Moreau J-L, Berendsen HHG, Boes M, Broekkamp CLE, Martin JR, Wichmann J and van Delft AML (1998) Antiaversive effects of 5-HT_{2C} receptor agonists and fluoxetine in a model of panic-like anxiety in rats. *Eur Neuropsychopharmacol* **8**:161–168.
- Kennaway DJ and Moyer RW (1998) Serotonin 5-HT_{2C} agonists mimic the effects of light pulses on circadian rhythms. *Brain Res* **806**:257–270.
- Kennett GA, Baily F, Piper DC and Blackburn TP (1994b) Effect of SB 200646A, a 5-HT_{2C}/5-HT_{2B} receptor antagonist, in two conflict models of anxiety. *Psychopharmacology* **118**:178–182.
- Kennett GA, Grewal GS, Forbes I, Gadre A and Blackburn TP (1994a) In vivo properties of SB 200646A, a 5-HT_{2C/2B} receptor antagonist. *Br J Pharmacol* **111**:797–802.
- Klein DC, Sugden D and Weller JL (1983) Postsynaptic α -adrenergic receptors potentiate the β -adrenergic stimulation of pineal serotonin N-acetyltransferase. *Proc Natl Acad Sci USA* **80**:599–603.
- Matsurra T and Sano Y (1983) Distribution of monoamine-containing nerve fibers in the pineal organ of untreated and sympathectomized dogs. *Cell Tissue Res* **234**:519–531.
- Middendorff R, Maronde E, Paust H-J, Muller D, Davidoff M and Olcese J (1996) Expression of C-type natriuretic peptide in the bovine pineal gland. *J Neurochem* **67**:517–524.
- Moreau J-L, Boes M, Jenck F, Martin JR, Mortas P and Wichmann J (1996) 5-HT_{2C} receptor agonists exhibit antidepressant-like properties in the anhedonia model of depression in rats. *Eur Neuropsychopharmacol* **6**:169–175.
- Olcese J and Munker M (1994) Extracellular serotonin promotes melatonin release from cultured rat pinealocytes: Evidence for an S₂-type receptor-mediated autocrine feedback. *Brain Res* **643**:150–154.
- Reiter RJ (1985) Action spectra, dose-response relationships, and temporal aspects of light's effects on the pineal gland. *Ann NY Acad Sci* **25**:215–230.
- Reiter RJ (1991) Pineal melatonin: Cell biology of its synthesis and its physiological interactions. *Endocr Rev* **12**:151–159.
- Sarda N, Gharib A, Reynaud D, Ou L and Pacheco H (1989) Identification of adenosine receptor in rat pineal gland: Evidence for A-2 selectivity. *J Neurochem* **53**:733–737.
- Schaad NC and Klein DC (1992) Characterization of alpha2-adrenergic receptors of rat pinealocytes. *Endocrinology* **130**:2804–2810.
- Simonneaux V, Kielen-Campard P, Loeffler JP, Basille M, Gonzalez BJ, Vaudry H, Robberecht P and Pevet P (1998) Pharmacological, molecular and functional characterization of vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating polypeptide receptors in the rat pineal gland. *Neuroscience* **85**:887–896.
- Sparks DL and Little KY (1990) Altered pineal serotonin binding in some suicides. *Psychiatry Res* **32**:19–28.
- Steardo L, Monteleone P, d'Istria M, Serino I, Maj M and Cuomo V (1995) (+)-N-Allylnormetazocine enhances n-acetyltransferase activity and melatonin synthesis: Preliminary evidence for a functional role of sigma receptors in the rat pineal gland. *J Pharmacol Exp Ther* **275**:845–849.
- Sugden D (1989) Melatonin biosynthesis in the mammalian pineal gland. *Experientia* **45**:922–932.
- Sugden D (1990) 5-Hydroxytryptamine amplifies β -adrenergic stimulation of N-acetyltransferase activity in rat pinealocytes. *J Neurochem* **55**:1655–1658.
- Vacas MI, Sarmiento IK and Cardinali DP (1985) Interaction between β - and α -adrenoceptors in rat pineal adenosine cyclic 3', 5'-monophosphate phosphodiesterase activation. *J Neural Trans* **26**:295–304.
- Weinhardt KK, Bonhaus DW and De Souza A (1996) Some benzenesulfonamido-substituted valerophenones that are selective antagonists for the 5-HT_{2C} receptor. *Bioorg Med Chem Lett* **6**:2687–2692.
- Young SN and Anderson GM (1982) Factors influencing melatonin, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, 5-hydroxytryptamine and tryptophan in rat pineal gland. *Neuroendocrinology* **35**:464–468.

Send reprint requests to: Luca Steardo, M.D., Istituto di Farmacologia, Università di Palermo, Via Forlanini 1, 90134 Palermo, Italy. E-mail: monter@tin.it
