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Antagonistic Effects of S 22153, a New mt1 and MT2 Receptor Ligand, on the Neophobia-Reducing Properties of Melatonin in BALB/c Mice

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KOPP C., E. VOGEL, M.-C. RETTORI, P. DELAGRANGE, P. RENARD, D. LESIEUR AND R. MISSLIN. *Antagonistic effects of S 22153, a new mt1 and MT2 receptor ligand, on the neophobia-reducing properties of melatonin in BALB/c mice.* PHARMACOL BIOCHEM BEHAV **64**(1) 131–136, 1999.—When exposed to a free-exploratory situation, BALB/c mice are well known to exhibit strong avoidance responses toward unfamiliar places (neophobia). Because melatonin was found to significantly reduce neophobia in BALB/c mice, it seemed interesting to examine potential antagonistic effects of S 22153, a new melatonin mt1 and MT2 receptor ligand, on the neophobia-reducing properties of melatonin in BALB/c mice confronted with the free-exploratory paradigm. S 22153 was able to block, in a dose-dependent manner, the anxiolytic-like properties of melatonin when it was administered 5 min before melatonin. The antagonistic effects of S 22153 persisted when the drug was administered 2 or 4 h before melatonin, and were almost abolished when it was administered 6 h before melatonin. These results suggest that the anxiolytic-like effects of melatonin on the neophobic responses in BALB/c mice are mediated by mt1 and/or MT2 receptors. © 1999 Elsevier Science Inc.

Anxiety Free-exploratory paradigm Melatonin Mice Neophobia

IN mammals, synthesis of pineal melatonin is controlled by the circadian system and entrained by the light/dark cycle. The suprachiasmatic nuclei (SCN), the site of the circadian clock in mammals, are stimulated by retinal afferents via the retinohypothalamic tract in respect of the light/dark cycle and modulate, via a multisynaptic pathway, the activity of the pineal gland, so that melatonin synthesis is high at night and low during the day (3,26). Melatonin is considered to be a neuroendocrine mediator of darkness information (4), and its implication in the regulation of seasonal and circadian rhythmicities is well documented (2,16,20,24).

In addition to its neuroendocrine functions, several pharmacological effects of melatonin have been described in rodents such as sedation, analgesia, anticonvulsant activity, and anxiolytic properties (1,7–9,11,13,28). Melatonin has been usually reported to exert anxiolytic-like activity in experimental situations such as passive avoidance test and the open field (7–9,13), which are constraining situations inducing "state" anxiety (18). More recently, melatonin has also been shown to counteract avoidance responses to an unfamiliar environment (neophobia) in BALB/c mice confronted with the free-exploratory paradigm (17), an experimental situation that consists of giving to animals the opportunity to move freely in simultaneously presented familiar and unfamiliar places (15). This paradigm, which fails to induce the usual physiological and endocrine signs of fear (22), has been proposed as a valid method for measuring the so-called "trait" anxiety in BALB/c mice (6,14). In this model, only drugs interacting, directly or

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indirectly, with the GABA–benzodiazepine (BZD) complex, have been shown to abolish neophobia in BALB/c mice (6,14). By contrast, compounds such as 8-OH-DPAT and sulpuride, which elicited anxiolytic-like effects in mice submitted to "state" anxiety models (23,27), were not effective in reducing neophobia in BALB/c mice (6,14). Consistently, the ability of melatonin to abolish BALB/c neophobic reactions was found to be counteracted by the central BZD receptor antagonist flumazenil (17). This is in good agreement with the well-established interactions between melatonin and GABA–BZD system in the central nervous system [see (12)for a review]. However, because melatonin does not act as a BZD receptor agonist, the "trait" anxiety-reducing properties of melatonin cannot be related to a direct action of melatonin on BZD receptors. Therefore, the question of the involvement of melatonin-specific receptors in the mediation of melatonin effects on BALB/c neophobia may be raised.

S 22153 (N-[2-(5-ethyl-benzo[b]thien-3-yl) ethyl] acetamide) has recently been presented as a new selective ligand of melatonin receptors, with high affinity for mt1 (pKi: 8.7 for S 22153 and 9.9 for melatonin) and MT2 (pKi: 8.4 for S 22153 and 8.9 for melatonin) receptors expressed in HEK 293 cells and with antagonistic properties in rodents (10): in vitro, S 22153 antagonized the melatonin-induced potentiation of electrically evoked contractions of isolated rat tail arteries and, in vivo, this compound was able to block the melatonin-induced phase advance of locomotor activity rhythm in mice under free-running conditions. Therefore, this new pharmacological tool was used to better characterize the neophobic-reducing properties of melatonin: we examined the potential ability of S 22153 to counteract the effects of melatonin in BALB/c mice confronted with the free-exploratory paradigm (Experiment 1), and we determined the duration of S 22153 activity (Experiment 2). In both experiments, melatonin was dosed at 1 mg/kg because maximal antineophobic effects were previously obtained with this dose in the same experimental conditions (17).

METHOD

Subjects

Male BALB/c mice from the Breeding Center Iffa Credo (France), 10 weeks of age at time of testing, were used. Mice were housed by five in a standard cage, with food pellets and water available ad lib under controlled conditions of temperature (23 ± 1°C). Animals were kept on a 12 L:12 D cycle, with light on a 0100 h so that we could observe animals in their active period, under dim red light.

The experimental procedures carried out in this study were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Apparatus and Procedure

The apparatus consisted of a rectangular polyvinylchloride box (30 × 20 × 20 cm), covered with Plexiglas and subdivided into six equal square units (10 × 10 × 20 cm), which were all interconnected by small holes located at the floor level. It could be divided in half lengthwise by closing a temporary partition. The apparatus was kept on a stand in the mouse room. The experimenter always stood next to the box on the same place. Approximately 24 h before testing each subject was randomly placed in one-half of the apparatus with the temporary partition in place, to be familiarized with it. The floor of this half only was covered with fresh sawdust, and the animal had unlimited access to food and water during the familiarization period. The duration of this period was 24 h. At the end of the familiarization period, the temporary partition between familiar and unfamiliar compartment was removed, and the subject was then observed for 10 min. Measures were taken of the number of approach responses followed by avoidance reactions towards the unfamiliar places (attempts), the time spent in the unfamiliar compartment (time), the total number of square units entered (locomotion), and the total number of rears made by the animals (rears).

Drugs

Melatonin (Sigma–Aldrich Chimie, St Quentin Fallavier, France) and S 22153 (Institut de Recherches Internationales Servier, Courbevoie, France) were prepared for injection by dispersion in 0.9% saline with Tween 80 (1%) immediately before use, and were administered orally by intubation at different concentrations in an injection volume of 10 ml/kg body weight.

Experiment 1

BALB/c mice were randomly allocated to the following groups ($n = 10$): melatonin (0 or 1 mg/kg) in combination with S 22153 (0, 1, 5, or 25 mg/kg). S 22153 was administered 1 to 2 h after dark onset, 5 min before melatonin; melatonin was administered 40 min before testing. Animals were submitted to the free-exploratory paradigm, as described above.

Experiment 2

BALB/c mice were randomly allocated to the following groups ($n = 10$): melatonin (0 or 1 mg/kg) in combination with S 22153 (0 or 10 mg/kg). S 22153 was administered 1 to 2 h after dark onset, 5 min, 2 h, 4 h, or 6 h before melatonin; melatonin was administered 40 min before testing. Animals were submitted to the free-exploratory paradigm, as described above.

Statistical Analysis

Data were treated with either an analysis of variance (ANOVA) followed by a Newman–Keuls a posteriori *t*-test, if groups came from a population with homogeneous standard deviations, or with a Kruskal–Wallis nonparametric ANOVA test followed by Mann–Whitney tests, if groups came from a population with heterogeneous standard deviations.

RESULTS

Experiment 1 (Fig. 1)

ANOVA revealed significant differences between groups for attempts (KW = 39.07; $p < 0.0001$), time (KW = 28.44; $p < 0.0001$), locomotion (KW = 27.81; $p < 0.001$) and rears (KW = 29.93; $p < 0.0001$). Melatonin alone or combined with 1 mg/kg of S 22153 significantly decreased the number of attempts ($p < 0.01$); melatonin also increased time spent in the unfamiliar compartment ($p < 0.05$), locomotion ($p < 0.01$), as well as the number of rears ($p < 0.01$). Mice treated with S 22153 alone (1–5–25 mg/kg) as well as those treated with melatonin combined with S 22153 (5–25 mg/kg) did not significantly differ from controls. In addition, mice treated with melatonin combined with S 22153 (5–25 mg/kg) and mice treated with S 22153 alone (1–5–25 mg/kg) exhibited significantly more attempts ($p < 0.01$), spent significantly less time in the unfamiliar compartment ($p < 0.05$) and displayed significantly

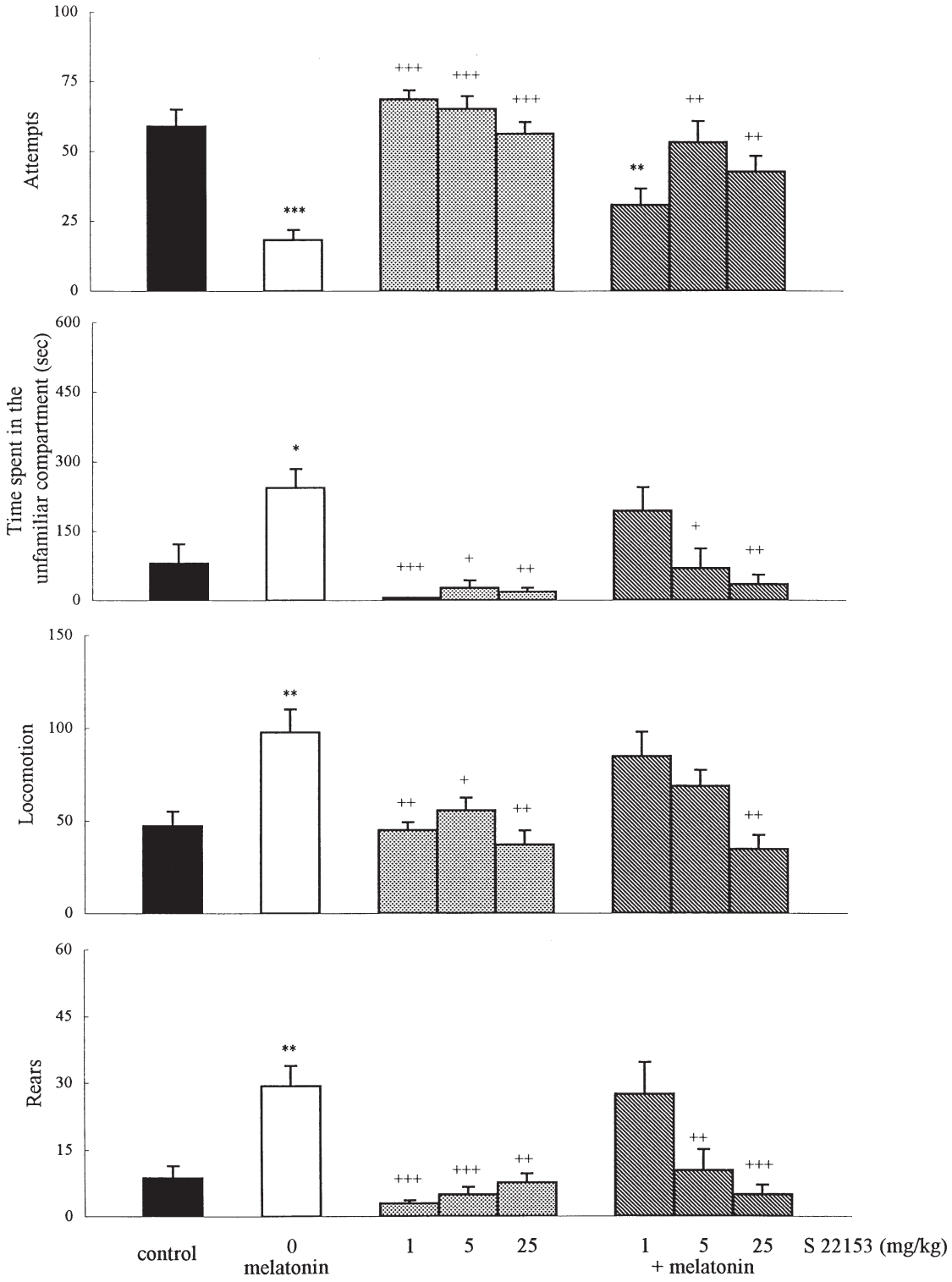

FIG. 1. Dose-dependent effects of S 22153 on the neophobia-reducing properties of 1 mg/kg of melatonin in BALB/c mice confronted with the free-exploratory test. The four panels show the number of attempts, the time spent in the unfamiliar compartment, locomotion, and the number of rears. Values are means + SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ relative to control animals (black bars). $+p < 0.05$, $++p < 0.01$ and $+++p < 0.001$ relative to melatonin-treated mice (white bars).

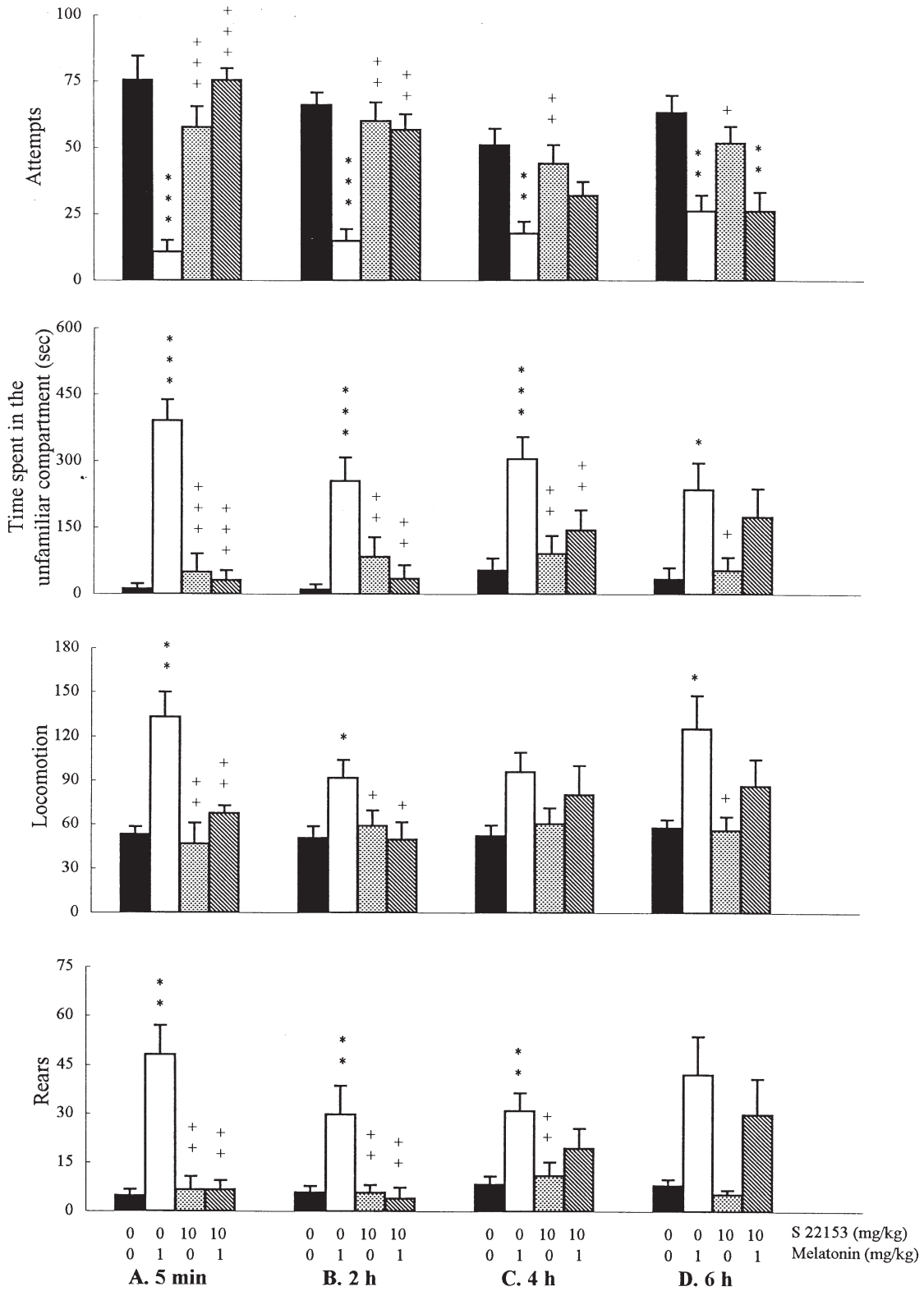

FIG. 2. Time-dependent effects of S 22153 on the neophobia-reducing properties of melatonin in BALB/c mice confronted with the free-exploratory test. The four panels show the number of attempts, the time spent in the unfamiliar compartment, locomotion, and the number of rears. S 22153 was administered 5 min (A), 2 h (B), 4 h (C), and 6 h (D) before melatonin. Values are means + SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ relative to control animals (black bars). $+p < 0.05$, $++p < 0.01$, $+++p < 0.001$ relative to melatonin-treated mice (white bars).

less locomotion ($p < 0.05$) and rearing behavior ($p < 0.01$) than mice treated with melatonin alone.

Experiment 2 (Fig. 2)

Effects of testing time on the neophobic behavior of vehicle- and melatonin-treated mice. There were no significant time-dependent variations in the behavior of mice treated with vehicle only (attempts: KW = 5.56, $p < 0.14$; time spent in the unfamiliar compartment: KW = 3.02, $p < 0.39$; locomotion: KW = 1.58, $p < 0.66$; rears: KW = 3.83, $p < 0.28$), and in the behavior of mice treated with melatonin only (attempts: KW = 4.54, $p < 0.21$; time spent in the unfamiliar compartment: KW = 6.49, $p < 0.09$; locomotion: KW = 4.58, $p < 0.21$; rears: KW = 2.09, $p < 0.55$).

Behavioral effects of S 22153 when administered 5 min before melatonin. ANOVA revealed significant differences between groups for attempts, $F(3, 36) = 20.76$, $p < 0.0001$, time, $F(3, 36) = 29.50$, $p < 0.0001$, locomotion (KW = 18.10, $p < 0.001$), and rears (KW = 14.55, $p < 0.01$). Melatonin alone significantly decreased the number of attempts ($p < 0.001$) and significantly increased time spent in the unfamiliar compartment ($p < 0.001$), locomotion ($p < 0.01$), as well as the number of rears ($p < 0.01$). Mice treated with S 22153 alone as well as those treated with melatonin combined with S 22153 did not significantly differ from controls. In addition, mice treated with S 22153 alone as well as those treated with melatonin combined with S 22153 exhibited significantly more attempts ($p < 0.001$), spent significantly less time in the unfamiliar compartment ($p < 0.001$), and displayed significantly less locomotion ($p < 0.01$) and rearing behavior ($p < 0.01$) than mice treated with melatonin alone.

Behavioral effects of S 22153 when administered 2 h before melatonin. ANOVA revealed significant differences between groups for attempts, $F(3, 36) = 17.61$, $p < 0.0001$, time (KW = 20.12, $p < 0.001$), locomotion, $F(3, 36) = 3.41$, $p < 0.05$, and rears (KW = 14.15, $p < 0.01$). Melatonin alone significantly decreased the number of attempts ($p < 0.001$) and significantly increased time spent in the unfamiliar compartment ($p < 0.001$), locomotion ($p < 0.05$), as well as the number of rears ($p < 0.01$). Mice treated with S 22153 alone as well as those treated with melatonin combined with S 22153 did not significantly differ from controls. In addition, mice treated with S 22153 alone as well as those treated with melatonin combined with S 22153 exhibited significantly more attempts ($p < 0.01$), spent significantly less time in the unfamiliar compartment ($p < 0.01$), and displayed significantly less locomotion ($p < 0.05$) and rearing behavior ($p < 0.01$) than mice treated with melatonin alone.

Behavioral effects of S 22153 when administered 4 h before melatonin. ANOVA revealed significant differences between groups for attempts, $F(3, 36) = 6.48$, $p < 0.01$, time, $F(3, 36) = 7.12$, $p < 0.001$, and rears (KW = 9.02, $p < 0.05$), but not for locomotion, $F(3, 36) = 2.09$; $p < 0.12$. Melatonin alone significantly decreased the number of attempts ($p < 0.01$) and significantly increased time spent in the unfamiliar compartment ($p < 0.001$) as well as the number of rears ($p < 0.01$). Mice treated with S 22153 alone as well as those treated with melatonin combined with S 22153 did not significantly differ from controls. Mice treated with S 22153 alone exhibited significantly more attempts ($p < 0.01$), spent significantly less time in the unfamiliar compartment ($p < 0.01$), and displayed significantly less rearing behavior ($p < 0.01$) than mice treated with melatonin alone. Mice treated with melatonin combined with S 22153 did not significantly differed from those treated with melatonin alone, except for the time spent in the unfamiliar compartment ($p < 0.01$).

Behavioral effects of S 22153 when administered 6 h before melatonin. ANOVA revealed significant differences between groups for attempts, $F(3, 36) = 8.39$, $p < 0.01$, time (KW = 8.33; $p < 0.05$), and locomotion (KW = 7.91, $p < 0.05$), but not for the number of rears (KW = 6.93, $p < 0.07$). Melatonin alone significantly decreased the number of attempts ($p < 0.01$) and significantly increased time spent in the unfamiliar compartment ($p < 0.05$) as well as locomotion ($p < 0.05$). Mice treated with S 22153 alone did not significantly differ from controls; they also exhibited significantly more attempts ($p < 0.05$), spent significantly less time in the unfamiliar compartment ($p < 0.05$) and displayed significantly less locomotion ($p < 0.05$) than mice treated with melatonin alone. Mice treated with melatonin combined with S 22153 made significantly less attempts than controls ($p < 0.01$), and did not significantly differ from mice treated with melatonin alone.

DISCUSSION

The purpose of the present study was to investigate the potential antagonistic activity of S 22153, a new melatonin mt1 and MT2 receptor ligand, on the anxiolytic-like effects of melatonin in BALB/c mice, known to display strong neophobic reactions in the free-exploratory paradigm (14). Above all, it must be noted that BALB/c neophobia, which is usually recorded at the beginning of nighttime (6,14), did not present any time-dependent variations throughout the nocturnal period in the present study. Results showed that, whatever the testing time throughout the night period, melatonin significantly reduced the number of attempts, well known to reflect avoidance reactions toward unfamiliar places (5,14), and thus promoted their exploratory activities, such as locomotion and/or rearing behavior, indicating antineophobic properties of melatonin. Thus, the anxiolytic-like effects of melatonin on the BALB/c neophobia closely resemble those of BZD receptor agonists, such as chlordiazepoxide and diazepam (6,14), and can be opposed to the lack of effects of psychostimulant compounds like α_2-adrenoceptor antagonists (yohimbine and idazoxan) in the same experimental situation (6). Pretreatment with S 22153, 5 min before melatonin, was able to block, in a dose-dependent manner, the neophobia-reducing activity of melatonin in BALB/c mice. These antagonistic effects of S 22153 persisted when the drug was administered 2 h before melatonin; they weakened when it was administered 4 h before melatonin, and were almost abolished when it was administered 6 h before melatonin. S 22153 had no intrinsic effects. Overall, these results showed that S 22153 possesses robust, and long lasting, antagonistic melatoninergic properties.

Because S 22153 is selective ligand of melatonin receptors, with high affinity for mt1 and MT2 receptors, it can be proposed that the anxiolytic-like effects of melatonin on BALB/c neophobia may be mediated through the activation of these melatoninergic receptor subtypes, both coupled to G proteins belonging to the $G_{i/O}$ family [see (29)]. In mice, mt1 receptor was reported to account for all detectable high affinity ^{125}iodo-melatonin binding in the brain: high binding level was particularly found in the SCN, and was functionally related to the acute inhibitory action of melatonin on this structure (19). Interestingly, the same study showed that mt1 receptors are also present in limbic areas, such as central amygdala nucleus (19). Because amygdala is directly implicated in the regulation of novelty-induced responses in mice (21), it can be pro-

posed that mt1 receptor activation may be involved in the anxiolytic-like effects of melatonin observed in the present study. Concerning MT2 receptor, it has been usually reported as mainly expressed in mammalian retina, mediating the reported physiological effects of melatonin in this structure [see (25)]. However, a recent study provided evidence for the presence of an MT2 receptor in mice SCN, and showed that this receptor participates in circadian phase-shift activities of melatonin in this species (19). Thus, a functional role of the MT2 receptor in central melatonin activities cannot be excluded in mice.

In conclusion, the present findings support the hypothesis that melatonin may exert its neophobia-reducing action through mt1 and/or MT2 specific receptors. Further investigations using subtype-selective drugs for melatonin receptors are required to evaluate the contribution of each of the two receptor subtypes to the regulation of mice emotional responsiveness toward unfamiliar places.

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