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Effects of Melatonin on Neophobic Responses in Different Strains of Mice

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KOPP, C., E. VOGEL, M.-C. RETTORI, P. DELAGRANGE, B. GUARDIOLA-LEMAÎTRE AND R. MISSLIN. *Effects of melatonin on neophobic responses in different strains of mice.* PHARMACOL BIOCHEM BEHAV **63**(4) 521–526, 1999.—Anxiolytic properties of melatonin in rodents had usually been examined in behavioral tests based on stressful situations, i.e., in animal models of "state" anxiety. However, no study reports effects of melatonin on emotionality of rodents submitted to situations devoid of stressful components as in the free-exploratory test, which gives to animals the opportunity to choose freely between familiar and unfamiliar places. This procedure has been proposed as a method for measuring an endogenous form of anxiety called "trait" anxiety. The present study first investigated the effects of melatonin on neophobic responses of male C57BL/6, C_3H/He, and BALB/c mice submitted to a free-exploratory test. Results demonstrated that melatonin had no effect in C57BL/6 mice that presented very low neophobic responses, whereas it was effective in reducing neophobia of BALB/c and C_3H/He mice that presented, respectively, strong and intermediate avoidance responses towards unfamiliarity. Indeed, mice of both latter strains treated with melatonin made fewer attempts to enter into the unfamiliar compartment, exhibited a lower latency of the first entry into the unfamiliar places, and spent more time in them. Thus, melatonin appeared to be equally effective in reducing "trait" anxiety in both BALB/c and C_3H/He mice. Moreover, flumazenil was able to counteract, in a dose-dependent manner, the anxiolytic activity of melatonin in BALB/c, suggesting involvement of central GABAergic system in the pharmacological effects of melatonin. © 1999 Elsevier Science Inc.

Free-exploratory paradigm   Inbred strains   Melatonin   Mice   Neophobia   "Trait" anxiety

IN mammals, synthesis of pineal melatonin is controlled by a circadian system and entrained by the light/dark cycle. Suprachiasmatic nuclei, which contain circadian pacemakers, are stimulated by retinal afferences via the retinohypothalamic tract in respect to light/dark cycle, thus modulating pineal activity via a multisynaptic pathway. Melatonin is secreted rhythmically, and presents a low or undetectable level during the day and a peak during the night (3,35).

Melatonin is considered as a neuroendocrine mediator of darkness (4,33), and it is implicated in the regulation of seasonal and circadian rhythmicities (2,27,33,34). In addition to its neuroendocrine functions, melatonin has been shown to possess several psychopharmacological properties in rodents such as sedation, analgesia, anxiolysis, and anticonvulsant activity (1,14,17,38). Furthermore, several studies demonstrated that an administration of melatonin decreased the level of emotionality in a passive avoidance test and in the open-field

paradigm, i.e., when animals were confronted by force to an unfamiliar environment (8–10,19). Thus, melatonin seems to be active in aversive situations that are considered to induce "state" anxiety (28). However, there is no evidence of melatonin effects on an endogenous type of anxiety termed as "trait" anxiety (28), which can be revealed by situations devoid of constraining components. Recently, the free-exploratory paradigm has been proposed as a model of "trait" anxiety (22). This experimental situation consists in giving to animals the opportunity to move freely in simultaneously presented familiar and unfamiliar environments (25), and fails to induce the usual physiological and endocrine signs of fear (31). Literature reports behavior of different inbred strains of mice confronted with this test: BALB/c and C_3H/He mice exhibited a preference for familiar places and strong avoidance responses towards the unfamiliar compartment, in contrast to C57BL/6 mice, which displayed developed exploratory activities (6,22).

Furthermore, meprobamate, ethanol, benzodiazepine receptor full and partial agonists, as well as the NMDA receptor antagonist, MK 801, have been shown to abolish neophobia in BALB/c mice (5,22). By contrast, compounds such as 8-OH-DPAT and sulpiride, which elicited anxiolytic-like effects in mice submitted to "state" anxiety models (30,36), were not effective in reducing neophobia in BALB/c mice (5,22).

The aim of the present study was first to compare potential anxiolytic effects of melatonin on neophobia of C57BL/6, BALB/c, and C_3H/He mice submitted to a free-exploratory test (Experiment 1). It seemed interesting to involve C_3H/He mice in this study because they exhibit a normal melatonin secretion pattern, in contrast to C57BL/6 and BALB/c mice, which are known to present a total or partial deficiency of melatonin secretion (7,11,12,20,21,29). Furthermore, because several previous studies suggest a possible link between melatonin and the activity of the central GABAergic neurons (14–18,24), a second experiment was conducted to examine whether the central-type benzodiazepine receptor antagonist, flumazenil (26), could prevent anxiolytic effects of melatonin in BALB/c mice confronted with the free-exploratory paradigm (Experiment 2).

<div align="center">METHOD</div>

Subjects

Male BALB/c, C57BL/6, and C_3H/He mice from the Breeding Center Iffa Credo (France), 10 weeks of age at the 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 lights on at 0100 h so that we could observe animals, under dim red light, in their active period, between 1400 and 1700 h. 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, previously described (32), 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 floor level. It can 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 latency of the first entry of animals into the unfamiliar compartment (latency), 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). Mice were considered as presenting preference for unfamiliar places when they spent more than half testing time in the unfamiliar compartment.

Experiment 1

Melatonin (Sigma-Aldrich Chimie, St. Quentin Fallavier, France) and vehicle were administered orally by intubation 40 min before testing. BALB/c, C57BL/6, and C_3H/He mice were randomly allocated to five groups (*n* = 14 for each group) receiving vehicle (saline with 1.6% ethanol–0.4% distilled water) or melatonin at doses of 0.01–0.1–1–10 mg/kg, in an injection volume of 10 ml/kg body weight.

Experiment 2

BALB/c mice were randomly allocated to the following groups (*n* = 10 for each group): vehicle (saline with 1% of Tween 80), melatonin (1 mg/kg) alone, melatonin (1 mg/kg) in combination with flumazenil (0.75–1.5–3 mg/kg; a gift from Hoffmann-La Roche, Basle, Switzerland). Drugs were administered as above.

Statistical Analysis

Data obtained in Experiment 1 were treated with an analysis of variance (ANOVA) with two factors (strains × groups). The contribution of each factor was then analyzed according to the classes of the other factor using either an analysis of variance (ANOVA) followed by a Newman–Keuls a posteriori *t*-test, if groups came from population with homogeneous standard deviations, or with a Kruskal–Wallis nonparametric ANOVA test followed by a Mann–Whitney test, if groups came from population with heterogeneous standard deviations. A Fisher exact test was used to determine whether two experimental groups differed significantly in the proportion of mice that presented preference for the unfamiliar places.

Data obtained in Experiment 2 were treated with a Kruskal–Wallis nonparametric ANOVA test followed by a Mann–Whitney test, because groups did not come from population with homogeneous standard deviations.

<div align="center">RESULTS</div>

Experiment 1

ANOVA revealed a significant interaction between strains and groups for attempts ($F(8, 195)$ = 3.21, $p < 0.01$), latency ($F(8, 195)$ = 4.63, $p < 0.001$), time, ($F(8, 195)$ = 3.65, $p < 0.001$), locomotion ($F(8, 195)$ = 2.47, $p < 0.02$) and rears ($F(8, 195)$ = 2.82, $p < 0.01$). When considering mice receiving vehicle injections (control mice), significant differences were found between strains in attempts (KW = 28.69, $p < 0.001$; Fig. 1A), latency (KW = 29.813, $p < 0.001$; Fig. 1B), time (KW = 23.48, $p < 0.001$; Fig. 1C), locomotion (KW = 13.04, $p < 0.002$; Fig. 1D), and rears (KW = 17.4, $p < 0.0002$; Fig. 1E). BALB/c control mice made significantly more attempts ($p < 0.001$), presented a significant higher latency of entry into unfamiliar places ($p < 0.001$), spent significantly less time in them ($p < 0.001$), and exhibited significantly less locomotion ($p < 0.01$) and less rears ($p < 0.01$) than C_3H/He and C57BL/6 controls. Furthermore, C_3H/He controls made significantly more attempts ($p < 0.001$) and presented a significant higher latency ($p < 0.001$) than C57BL/6 controls. Moreover, the proportion of mice that exhibited a preference for unfamiliar places was lower in BALB/c than in C57BL/6 controls during the first 5 min of testing (Fisher exact test; $p < 0.01$) and than in C_3H/He and C57BL/6 controls during the last 5 min of testing (Fisher exact test; $p < 0.001$). In addition, the proportion of mice preferring unfamiliar places was lower in C_3H/He than in C57BL/6 controls during the first 5 min of testing (Fisher exact test; $p < 0.03$). Finally, in control mice of each strain,
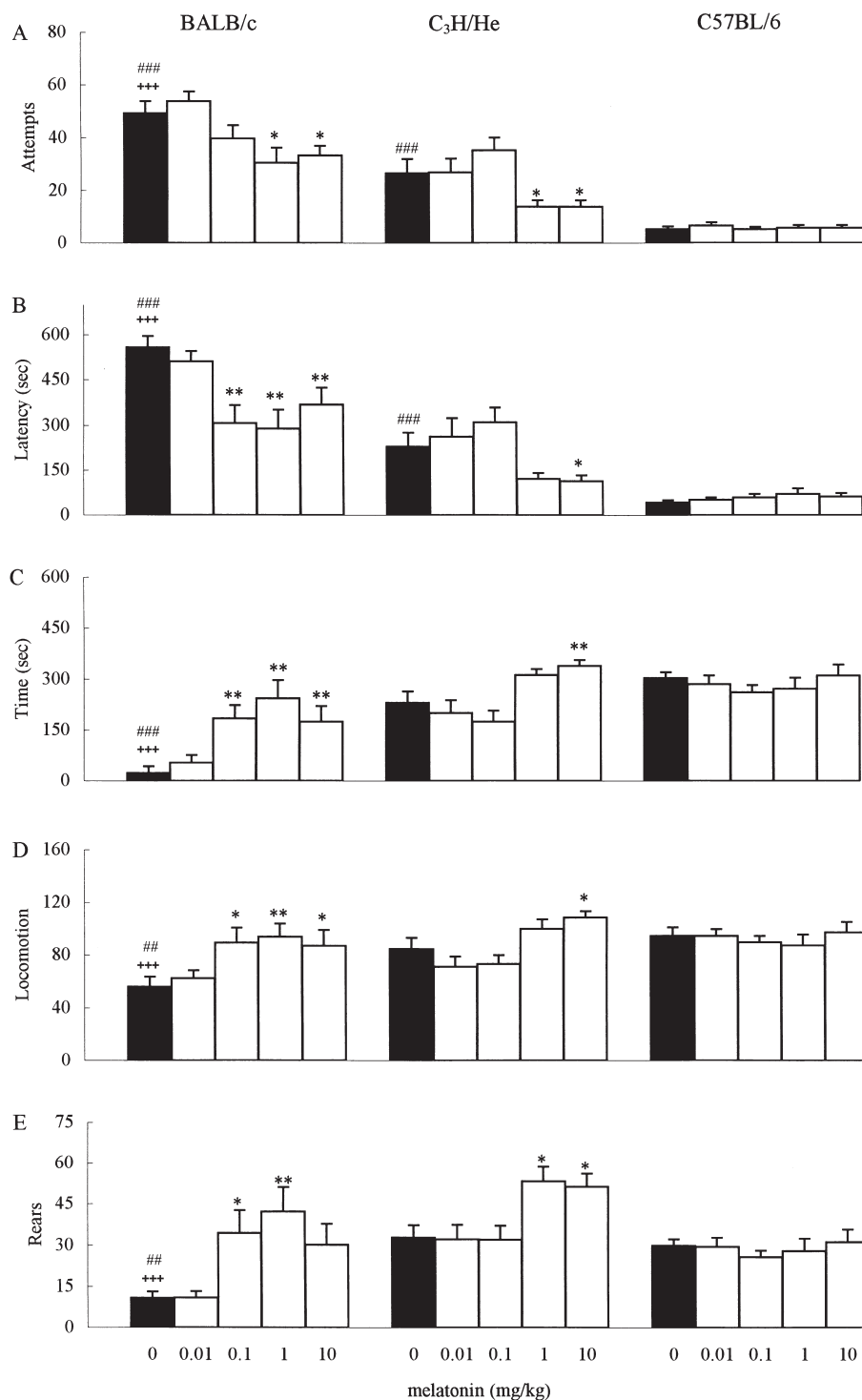
FIG. 1. Effects of melatonin on behavior of BALB/c, C_3H/He, and C57BL/6 mice confronted with the free-exploratory test. The panels show (A) the number of attempts at entry into the unfamiliar compartment followed by avoidance responses (attempts), (B) latency of the first entry of animals into the unfamiliar place (latency), (C) the time spent in the unfamiliar compartment (time), (D) locomotion, and (E) the number of rears. Values are means + SEM. *$p < 0.05$ and **$p < 0.01$ relative to control animals of the considered strain (black bars). ##$p < 0.01$, ###$p < 0.001$ relative to C57BL/6 control mice. +++$p < 0.001$ relative to C_3H/He control mice.
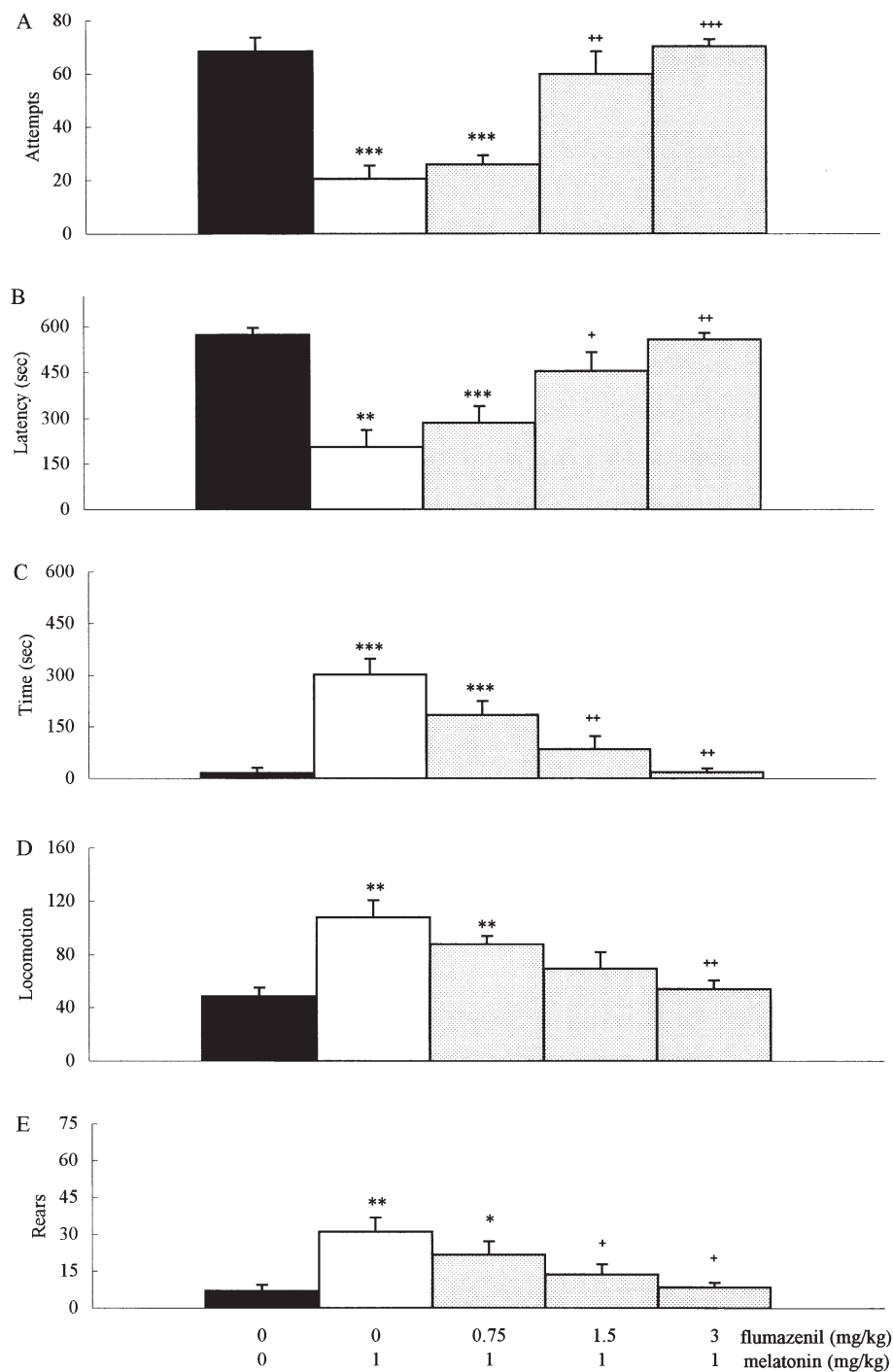
FIG. 2. Antagonism of melatonin effects on behavior of BALB/c mice in the free-exploratory test by flumazenil. The panels show (A) the number of attempts at entry into the unfamiliar compartment followed by avoidance responses (attempts), (B) latency of the first entry of animals into the unfamiliar place (latency), (C) the time spent in the unfamiliar compartment (time), (D) locomotion, and (E) 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).

the number of attempts was significantly correlated (Pearson correlation coefficient) positively with latency (BALB/c: $r = 0.77$, $p < 0.001$; C$_3$H/He: $r = 0.90$, $p < 0.001$; C57BL/6: $r = 0.69$, $p < 0.001$) and negatively with the time spent in the unfamiliar compartment (BALB/c: $r = -0.77$, $p < 0.001$; C$_3$H/He: $r = -0.87$, $p < 0.001$; C57BL/6: $r = -0.56$, $p < 0.001$), locomotion (BALB/c: $r = -0.60$, $p < 0.001$; C$_3$H/He: $r = -0.71$, $p < 0.001$; C57BL/6: $r = -0.44$, $p < 0.001$), and rears (BALB/c: $r = -0.64$, $p < 0.001$; C$_3$H/He: $r = -0.75$, $p < 0.001$; C57BL/6: $r = -0.42$, $p < 0.001$).

When comparing melatonin-treated mice in each strain with the respective control animals, it appears that melatonin was able to significantly modify the responses towards unfamiliarity of BALB/c and C$_3$H/He mice without significantly affecting those of C57BL/6 mice.

In BALB/c mice, ANOVA revealed significant differences among groups for attempts, $F(4, 65) = 4.72$, $p < 0.02$; Fig. 1A], latency (KW = 17.92, $p < 0.01$; Fig. 1B), time (KW = 18.87, $p < 0.001$; Fig. 1C), locomotion (KW = 12.79, $p < 0.02$; Fig. 1D), and rears (KW = 14.20, $p < 0.007$; Fig. 1E). Melatonin significantly decreased the number of attempts (1–10 mg/kg; $p < 0.05$) and latency (0.1–1–10 mg/kg; $p < 0.01$) while it significantly increased time (0.1–1–10 mg/kg; $p < 0.01$), locomotion (0.1–1–10 mg/kg; $p < 0.05$) and rears (0.1–1 mg/kg; $p < 0.05$). Moreover, the proportion of mice which preferred unfamiliar places was significantly increased in BALB/c mice treated with 0.1-1-10 mg/kg of melatonin in comparison with controls (Fisher test; $p < 0.05$, $p < 0.01$, $p < 0.02$, respectively).

In C$_3$H/He mice, ANOVA revealed significant differences among groups for attempts (KW = 17.62, $p < 0.001$; Fig. 1A), latency (KW = 16.97, $p < 0.001$; Fig. 1B), time (KW = 19.39, $p < 0.001$; Fig. 1C), locomotion (KW = 17.24, $p < 0.01$; Fig. 1D), and rears, $F(4, 65) = 4.87$, $p < 0.002$; Fig. 1E. Melatonin significantly decreased the number of attempts (1–10 mg/kg; $p < 0.05$) and latency (10 mg/kg; $p < 0.05$), while it significantly increased time (10 mg/kg; $p < 0.01$), locomotion (10 mg/kg; $p < 0.05$), and rears (1–10 mg/kg; $p < 0.05$). The proportion of mice that presented preference for unfamiliar places was significantly increased in C$_3$H/He mice treated with 10 mg/kg of melatonin in comparison with controls (Fisher exact test; $p < 0.03$).

In C57BL/6 mice, ANOVA did not reveal any significant differences among groups for attempts, $F(4, 65) = 0.33$, $p < 0.85$ (Fig. 1A), latency (KW = 1.92, $p < 0.75$; Fig. 1B), time, $F(4, 65) = 0.62$, $p < 0.65$ (Fig. 1C), locomotion, $F(4, 65) = 0.35$, $p < 0.84$ (Fig. 1D), and rears, $F(4, 65) = 0.33$, $p < 0.86$ (Fig. 1E).

Experiment 2

ANOVA revealed significant differences between groups for attempts (KW = 31.46; $p < 0.0001$; Fig. 2A), latency (KW = 25.71; $p < 0.0001$; Fig. 2B), time (KW = 26.13; $p < 0.0001$; Fig. 2C), locomotion (KW = 17.27; $p < 0.002$; Fig. 2D), and rears (KW = 12.98; $p < 0.02$; Fig. 2E).

Mice treated with 1 mg/kg of melatonin alone or in combination with 0.75 mg/kg of flumazenil presented more attempts ($p < 0.001$) and a lower latency ($p < 0.01$), spent more time in the unfamiliar compartment ($p < 0.001$), and exhibited more locomotion ($p < 0.01$) and rears ($p < 0.05$) than controls. Mice treated with 1 mg/kg of melatonin in combination with 1.5 and 3 mg/kg of flumazenil did not significantly differ from controls. In addition, when compared with melatonin-treated mice, they presented a significant increase in the number of attempts ($p < 0.01$) and in latency ($p < 0.05$), and a signifi-

cant decrease in time ($p < 0.01$) and in rears ($p < 0.05$), the decrease in locomotion ($p < 0.01$) being significant only at the dose of 3 mg/kg of flumazenil.

DISCUSSION

The aim of the present experiment was to compare the potential anxiolytic properties of melatonin in C57BL/6, BALB/c, and C$_3$H/He mice submitted to the free-exploratory paradigm presented as a method for measuring "trait" anxiety (22). Results show that when exposed to an unfamiliar compartment, BALB/c as well as C$_3$H/He control mice exhibited strong neophobic responses when compared to the C57BL/6 mice, confirming previous data (6,22): BALB/c mice displayed a greater number of neophobic responses, such as attempts, and a higher latency of entry into unfamiliar places than C$_3$H/He, which in turn, emitted a greater number of neophobic responses than C57BL/6. Moreover, it must be noted that the number of attempts was negatively correlated with time, locomotion, and rears: when confronted with the unfamiliar compartment, the more mice exhibited avoidance responses towards this compartment, the less they spent time in it and the less they displayed locomotion and rears. Thus, it appeared evident that the emotional responses of mice towards unfamiliarity were inversely proportional to their exploratory activities.

Melatonin was able to reduce the level of neophobic responses in BALB/c and C$_3$H/He mice, while it did not significantly modify the behavior of the C57BL/6. Thus, melatonin promoted exploration in treated BALB/c and C$_3$H/He mice by reducing avoidance responses towards unfamiliar places. These effects were observed in BALB/c mice at 0.1, 1, and 10 mg/kg, and in C$_3$H/He mice at only 1 and 10 mg/kg doses. This is perhaps due to higher endogenous melatonin levels in C$_3$H/He mice than in the BALB/c (11,12).

Although it has been established that C57BL/6 mice present a wider distribution of melatonin binding sites in brain than C$_3$H/He mice (37), it is unlikely that this fact can explain why melatonin was unable here to modify C57BL/6 mice behavior. Actually, we observed in a previous experiment (unpublished data) a behavioral sedation induced by a dose of 40 mg/kg of melatonin in C57BL/6 mice submitted to the same experimental paradigm. Therefore, it seems more likely to consider that the differential action of melatonin on the different strains of mice must be related to the fact that BALB/c and C$_3$H/He mice presented strong neophobic reactions, in contrast to C57BL/6 mice. Besides, it has been shown that benzodiazepine receptor agonists were able to reduce neophobia in BALB/c mice without affecting responses of C57BL/6 mice towards unfamiliarity (22).

In conclusion, the present results can be taken as indications that melatonin is able not only to reduce the so-called "state" anxiety (8–10,17,19) but also the type of anxiety termed as " trait" anxiety. Melatonin presents a profile similar to benzodiazepines, in contrast, for instance, to 5-HT interacting drugs that effectively modulated "state" anxiety (13,23,30), but not "trait" anxiety (22). Because the well-known benzodiazepine receptor antagonist, flumazenil, was able to counteract, in a dose-dependent manner, the anxiolytic properties of melatonin in BALB/c mice, our present results support a link between melatonin and the activity of central GABAergic neurons. This hypothesis has been already suggested by previous studies (14–18,24). Thus, melatonin appears to be a promising anxiolytic agent, all the more so because, with a large range of effective doses, this neurohormone seems to be devoid of sedative side effects.

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