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Combined Effects of Diazepam and Melatonin in Two Tests for Anxiolytic Activity in the Mouse

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GUARDIOLA-LEMAÎTRE, B., A. LENÈGRE AND R. D. PORSOLT. *Combined effects of diazepam and melatonin in two tests for anxiolytic activity in the mouse.* PHARMACOL BIOCHEM BEHAV **41**(2) 405–408, 1992. – The effects of behaviorally non-active doses of melatonin and diazepam were investigated in two test models for anxiolytics in mice to see whether mutual enhancement could be observed when the two treatments were combined. The test models used were the four plates test and the tail suspension test. In the former test anxiolytics increase the number of punished crossings and in the latter increase the duration of immobility of mice suspended by the tail. In the four plates test combined treatment with melatonin (128 and 256 mg/kg IP) and diazepam (0.5 mg/kg PO) caused a significant increase in the number of punished crossings, whereas each treatment alone was without effect. Similarly, in the tail suspension test, a clear increase in the duration of immobility was observed after combined treatment (256 mg/kg IP melatonin + 0.5 mg/kg PO diazepam), whereas no effects were observed with the individual treatments alone. These results suggest that melatonin can enhance the anxiolytic actions of diazepam.

Melatonin Benzodiazepine Anxiolytic GABA Four plates test Tail suspension test Drug interaction

THE role of melatonin in sleep has been widely studied in humans as well as in animals. Early studies suggested a role of melatonin in sleep induction (1,4). In a more recent double-blind placebo-controlled study (23) it was shown that melatonin, given at an oral dose of 80 mg to healthy volunteers, exerts a hypnotic effect by accelerating sleep initiation and improving sleep maintenance. In a single case study in a blind man, melatonin was able to suppress daytime naps and to reduce variability in the timing of nighttime sleep onset (6). These results are confirmed by animal studies. Melatonin has been reported to induce sleep in cats (11) and, in rodents, has been shown to potentiate barbiturate-induced sleep (20), to enhance REM sleep (12) and to accelerate reentrainment of circadian rhythm (8,16). It should, however, be noted that these sleep-enhancing effects in man and animals cannot always be reproduced (7, 10, 21), suggesting the importance of procedural parameters.

In more general psychopharmacological studies (7,20), melatonin has been shown to induce mild sedation (Irwin and activity meter tests), motor incoordination (rota-rod test), weak anxiolytic and analgesic activity (four plates, hot plate and writhing tests) and anticonvulsant activity (electrically and chemically induced convulsions).

It is now well established that melatonin interacts with GABA. In rats, pinealectomy diminishes the brain levels of GABA, whereas administration of melatonin enhances GABA in hypothalamus, cerebellum, cortex and pineal gland (17). In gerbils, pinealectomy induces a convulsive state which can be reversed by administration of melatonin (18). In vitro, melatonin enhances the affinity of [^{3}H]-muscimol for GABA receptors (3) but, like diazepam, inhibits the binding of TBPS at the GABA-regulated chloride ionophore (15). Taken together, these results strongly suggest interaction between melatonin and the GABAergic system.

It is possible that melatonin and benzodiazepines exert their tranquillizing effects through common or complementary GABAergic mechanisms. If so, it could be predicted that combined treatment with threshold doses of melatonin and diazepam might induce more marked anxiolytic effects than treatment with either constituent drug alone. The present experiments were undertaken to test this possibility using two simple tests in mice, the four plates test (2) and the tail suspension test (19). In the former test, benzodiazepines increase the level of punished exploration (2), whereas in the latter test, benzodiazepines increase the duration of immobility of mice suspended by the tail (19).

METHOD

Animals

The subjects were male NMRI mice, weighing between 20 and 26 g, supplied by the Centre d'Elevage Roger Janvier (CERJ), 53940 Le Genest Saint Isle, France. They were delivered to the laboratory at least three days before the experiments and on arrival were housed in groups of 10 in transparent macrolon cages (25.5 × 19.5 × 13.5 cm) containing wood shavings supplied by CERJ with free access to food (U.A.R. 113) and tap water. They were kept in an ambient temperature of 21 ± 1°C under artificial lighting (12 hours) between 0800 and 2000.

Drugs

The following drugs were used: melatonin (R.B.I., France),

Batch No. SB 888 A; diazepam (C.P.F., France), Batch No. F 07898. Both compounds were dispersed in an aqueous suspension of acacia gum (5%) and were administered in a volume of 12.5 ml/kg which served as the vehicle for control administrations. All experiments were performed blind using coded solutions.

Procedure

Four plates test. The procedure followed that described by Aron et al. (2). Mice were individually placed in a white plastic enclosure (25 × 18 × 16 cm) with a floor consisting of four rectangular metal plates (8 × 11 cm). The animal was left to explore freely for 15 seconds and then, for the next 60 seconds, it received a brief electric shock (2 mA; maximum 0.5 seconds) every time it crossed from one plate to another. The number of punished crossings during this period was counted. Ten animals were studied per group.

The experiment contained the following 6 treatment groups: control (vehicle PO −60 min + vehicle IP −30 min); diazepam alone (0.5 mg/kg diazepam PO −60 min + vehicle IP −30 min); two doses of melatonin alone (vehicle PO −60 min + 64 or 128 mg/kg melatonin IP −30 min); two diazepam/melatonin interactions (diazepam 0.5 mg/kg PO −60 min + 64 or 128 mg/kg melatonin IP −30 min).

Thus all animals received two administrations (PO and IP) at the times indicated before the test. The doses were selected on the basis of previous experiments (not shown) as being just below those exerting intrinsic effects in the test.

Tail suspension test. The procedure followed that described by Stéru et al. (19). Mice were suspended by the tail for 6 minutes in a computerized device (ITEMATIC-TST) which measures two parameters, the duration of immobility and the power of the movements (calculated in arbitrary units from the total energy expended by the animal as detected by a strain gauge). The apparatus measures the behavior of 6 mice simultaneously. Ten animals were studied per group.

The experiment contained the following 6 treatment groups: control (vehicle PO −60 min + vehicle IP −30 min); diazepam alone (0.5 mg/kg diazepam PO −60 min + vehicle IP −30 min); two doses of melatonin alone (vehicle PO −60 min + 128 or 256 mg/kg melatonin IP −30 min); two diazepam/melatonin interactions (diazepam 0.5 mg/kg PO −60 min + 128 or 256 mg/kg melatonin IP −30 min).

Thus all animals received two administrations (PO and IP) at the times indicated before the test. The doses were selected on the basis of previous experiments (not shown) as being just below those exerting intrinsic effects in the test.

Statistical Analysis

Results were analyzed for statistical significance using analysis of variance (single factor) followed by individual *t*-tests (two-tailed) between the appropriate groups using the pooled error terms of the analyses of variance.

RESULTS

The results obtained in the four plates test are shown in Fig. 1. Overall analysis of variance indicated the presence of significant differences between the groups, $F(5,54)=2.775$, $p<0.05$. Neither diazepam (0.5 mg/kg PO) nor melatonin (64 and 128 mg/kg IP) administered alone had any effect on the number of punished crossings, $t(54)=0$, 1.043 and 0, respectively, NS. When administered together, the combination of both the low and the high doses of melatonin with diazepam significantly increased the number of punished crossings as compared with the group treated with diazepam alone, $t(54)=2.296$ and 2.504, respectively, $p<0.05$.

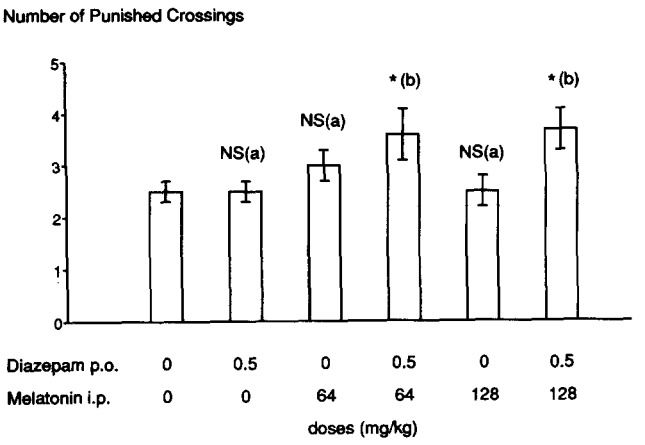

FIG. 1. Effects of separate and combined treatment with melatonin and diazepam on the mean (±s.e.m.) number of punished crossings in the four plates test (N = 10 per group). Diazepam was administered PO 60 minutes before the test and melatonin was administered IP 30 minutes before the test. Results were analyzed for statistical significance using ANOVA followed by individual *t*-tests. a = Compared with vehicle control; b = compared with diazepam alone. NS = not significant, $*p<0.05$ (two-tailed test).

The results obtained with the duration of immobility parameter in the tail suspension test are shown in Fig. 2A. Overall analysis of variance indicated the presence of significant differences between the groups, $F(5,54)=7.867$, $p<0.001$. Inspection of the figure suggests that both diazepam (0.5 mg/kg PO) and the higher dose of melatonin (256 mg/kg IP) administered alone tended to increase the duration of immobility but neither tendency was statistically significant, $t(54)=1.642$ and 1.295, respectively, NS. The lower dose of melatonin (128 mg/kg IP) alone was totally without effect, $t(54)=0.066$, NS. When administered together with diazepam, the higher dose of melatonin (256 mg/kg IP) clearly increased the duration of immobility as compared with diazepam alone, $t(54)=3.699$, $p<0.001$, whereas no effect was observed with the combination of the lower dose of melatonin (128 mg/kg IP) with diazepam, $t(54)=0.555$, NS. The mean increase in immobility observed with the high dose of melatonin + diazepam (+139 s) was greater than the sum of the mean increases observed with either drug alone (diazepam: +43 s; melatonin: +34 s).

The results obtained with the power of movements parameter in the tail suspension test are shown in Fig. 2B. Overall analysis of variance indicated the presence of significant differences between the groups, $F(5,54)=2.611$, $p<0.05$. Inspection of the figure suggests that diazepam (0.5 mg/kg PO) and both doses of melatonin (128 and 256 mg/kg IP) tended to decrease the power of the movements and that more marked effects were observed after combined treatment with the two drugs. The tendencies observed with the drugs alone, $t(54)=1.464$, 0.855 and 0.092, respectively, NS, or with the combination of the low dose of melatonin + diazepam when compared with diazepam alone, $t(54)=0.732$, NS, were, however, far from statistical significance. On the other hand, the decrease in the power of movements observed when the high dose of melatonin was combined with diazepam fell just short of statistical significance when compared with diazepam alone, $t(54)=1.891$, $p<0.10$. In con-

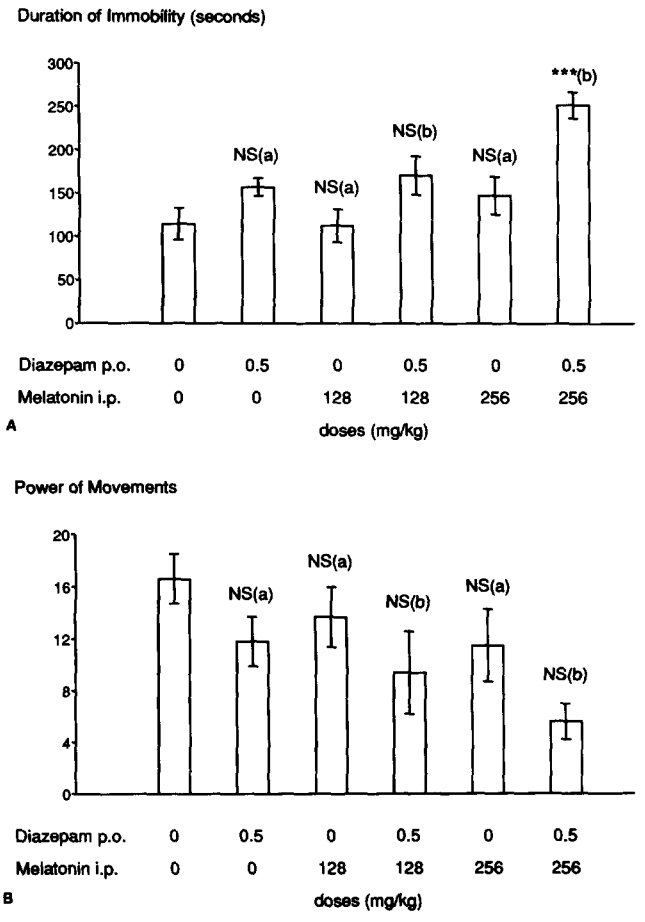

FIG. 2. Effects of separate and combined treatment with melatonin and diazepam on the mean (±s.e.m.) duration of immobility (A) and mean (±s.e.m.) power of movements (B) in the tail suspension test (N = 10 per group). Diazepam was administered PO 60 minutes before the test and melatonin was administered IP 30 minutes before the test. Results were analyzed for statistical significance using ANOVA followed by individual *t*-tests. a = compared with vehicle control; b = compared with diazepam alone. NS = not significant, ***$p<0.001$ (two-tailed test).

trast to what was observed with the immobility parameter, the mean decrease in the power of the movements observed after combined treatment with the high dose of melatonin and diazepam (−11.0) appeared to represent no more than the sum of the mean decreases observed with either drug alone (diazepam: −4.8; melatonin: −5.1).

DISCUSSION

The present experiments examined the effects of combined treatment with diazepam and melatonin in two simple models for anxiolytic activity in the mouse. The four plates test is a classical screening test for benzodiazepine-like anxiolytics (2) and shows less clear activity with atypical compounds such as buspirone or ipsapirone (unpublished findings). The tail suspension test, on the other hand, shows a clear behavioral effect (increase in immobility) with a variety of tranquillizing compounds including both benzodiazepines (19) and buspirone-like compounds (unpublished findings) and thus may represent a more general screening test for anxiolytic activity. Furthermore, the second parameter measured in the tail suspension test, the power of movements, is clearly reduced by benzodiazepines (19) but not by buspirone-like compounds (unpublished findings) and thus would appear to reflect the muscle relaxant activity of the benzodiazepines.

The aim of the present experiments was to establish whether a behaviourally inactive dose of melatonin which, like benzodiazepines, is known to interact with central GABA activity (3, 15, 17) can enhance the anxiolytic effects of a nonactive dose of diazepam in the two models. The results obtained suggested that this was indeed the case. In the four plates test melatonin, in the dose-range tested (64 and 128 mg/kg IP), was devoid of any intrinsic activity but clearly caused an increase in punished exploration at both doses when administered together with a subactive oral dose of diazepam. Similar results were obtained in the tail suspension test where melatonin, at a slightly higher dose (256 mg/kg IP), did not markedly affect the duration of immobility when administered alone, but caused a clear increase in immobility when administered in conjunction with a behaviorally inactive dose of diazepam. Furthermore, the effects observed in both tests appeared to be more than just the summation of the effects of the two drugs alone. The results obtained in the tail suspension test suggest that the enhancement might be specific to anxiolytic activity. Whereas mutual enhancement of the immobility-reducing effects of the two compounds was observed, the effects on the power of movements were less clearcut. The greater decrease observed after combined treatment was not statistically significant and, in contrast to the effects on immobility, appeared to represent merely the addition of the tendencies observed with the two drugs alone.

Melatonin is an endogenous molecule which, in addition to its mild anxiolytic and sedative activity (7, 10, 20), accelerates reentrainment of circadian rhythms (8,16). Two questions arise from these properties. Firstly, what is the link between these activities, in particular between the anxiolytic activity and circadian reentrainment? Is the circadian reentrainment a consequence of the anxiolytic/sedative activity or does it result from other mechanisms? A second but related question concerns the neurochemical mechanism of melatonin action. Melatonin binding sites have been reported in several brain areas in various species, including the suprachiasmatic nucleus (14) which has been suggested to be the pacemaker of the circadian system (13). However, to our knowledge, no direct relationship between the stimulation of these sites and a physiological activity has yet been reported. Nonetheless, decreased melatonin synthesis is known to occur at two stages of human life, puberty and old age (9, 13, 22), which are associated with a reduction in the quality of sleep (24). There is thus some evidence that melatonin directly contributes to the generation of circadian rhythms. On the other hand, there is a demonstrated link between melatonin and GABA (3, 15, 17, 18), a system which is clearly implicated in the anxiolytic effects of the benzodiazepines (5). It is thus tempting to speculate that melatonin may induce its sleep-enhancing/anxiolytic effects via two independent but additive mechanisms. Support for a dual mechanism can be derived from the fact that circadian reentrainment is observed in a dose range about 1 mg/kg (8,16), whereas sedative/anxiolytic activity is observed at doses about 100 fold higher [(7,20), present experiments].

The above reasoning would suggest that melatonin, or derivatives thereof, might usefully supplement traditional pharmacological treatments for sleep disturbance or anxiety (7). The present procedures could usefully serve as a simple test for screening such effects.

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