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The $GABA_A$ receptor mediates the hypnotic activity of melatonin in rats

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Abstract

The present investigation assessed whether hypnotic activity of melatonin was mediated by the $GABA_A$ receptor in rats. Electroencephalography (EEG) was measured in this experiment. Melatonin, at a dose of 10 mg/kg ip, showed a significant sleep-promoting effect in rats. Flumazenil (3.5 and 7 mg/kg), a specific antagonist of the benzodiazepine (BZP) recognition site on the $GABA_A$ receptor, and picrotoxin (2 and 4 mg/kg), the ligand of the picrotoxin site on the $GABA_A$ receptor, seemed to be devoid of intrinsic influence on each sleep parameter when used alone, but they significantly antagonized the melatonin-induced increase in total sleep time (TS), slow-wave sleep time (SWS) and paradoxical sleep time (PS), and the decrease in time to sleep onset (TSO) and wakefulness time (W). A significant interaction was shown between melatonin and flumazenil or picrotoxin. When bicuculline methiodide (2 and 4 mg/kg), a specific antagonist of the GABA binding site on the $GABA_A$ receptor, was used together with melatonin, the melatonin-induced increase in TS, SWS and PS, and the decrease in W were abolished. However, there was no interaction between melatonin and bicuculline methiodide on sleep parameters except PS. These results indicate that the hypnotic activity of melatonin may be linked to the $GABA_A$ receptor and mediated through the BZP recognition site, the picrotoxin site on the $GABA_A$ receptor and partially through the GABA binding site on the $GABA_A$ receptor.

Keywords: Melatonin; Sleep; $GABA_A$ receptor; Flumazenil; Picrotoxin; Bicuculline methiodide

1. Introduction

In recent years, a number of neuropharmacological effects of melatonin (including decrease in locomotor activity, hypnotic activity, analgesia, anticonvulsion, anti-anxiety, etc.) have been observed. Melatonin exerts a depressive influence on the central nervous system (CNS) (Acuna-Castroviejo et al., 1995). The well-described effect of melatonin on the CNS is sedation and hypnotic activity. Although melatonin, as a sedative, has been shown to induce sleep in both animals (Marczynski et al., 1964; Sugden, 1983; Mirmiran and Pevet, 1986) and humans (Cramer et al., 1974; Lieberman et al., 1984; James et al., 1990), the underlying mechanism(s) or receptor(s) through which melatonin induces sleep remain to be established.

γ-Aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the CNS. Many positive or negative modulators for the $GABA_A$ receptor, such as benzodiazepines (BZPs), barbiturates, steroids, polyvalent cations and ethanol, can regulate the $GABA_A$ receptor to induce GABA responses (Rabow et al., 1995) which have profound meaning for brain function. It is known that some neuropsychiatric disorders, such as anxiety, epilepsy, sleep disorders and convulsive disorders, have been effectively treated with therapeutic agents which increase the concentration of GABA or enhance the action of GABA at the $GABA_A$ receptor in nervous tissue.

Accumulating evidence indicates that there is a relationship between melatonin and the GABAergic system in the CNS. For example, melatonin increases concentrations of GABA in the hypothalamus (Xu et al., 1995), augments GABA turnover in several brain regions (Rosenstein and Cardinali, 1986), increases GABA-induced chloride influx in the hypothalamus (Rosenstein et al., 1989), potentiates $GABA_A$ receptor-mediated current (Wu et al., 1999) and causes an enhancement of [^{3}H] GABA binding (Coloma and Niles, 1988). Electrophysiological experiments in anaesthetized animals show that melatonin exhibits GABA-like effects and potentiates the effect of GABA in neuronal

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activity (Stankov et al., 1992). In addition, it also is reported that the melatonin-induced decrease in locomotor (Golombek et al., 1991), anxiolytic (Pierrefiche et al., 1993), anticonvulsive (Green et al., 1982) and analgesic (Golombek et al., 1992) activity can be blocked by pretreatment with flumazenil, an antagonist of the BZP recognition site on the $GABA_A$ receptor. However, there is no direct evidence to indicate whether the $GABA_A$ receptor is involved in the hypnotic activity of melatonin. Therefore, the objective of this research is to investigate the relationship between the $GABA_A$ receptor and the hypnotic activity of melatonin to clarify whether hypnotic activity of melatonin is mediated by the GABAergic system. In this experiment, electroencephalogram recording was used to investigate whether hypnotic activity of melatonin was antagonized by antagonists and ligand of different recognition sites of the $GABA_A$ receptor in rats.

2. Methods

2.1. Animals and drugs

Male Wistar rats (250–300 g) were supplied by the Experimental Animal Center of Shenyang Pharmaceutical University. Sixty-one rats were housed on a 12-h light/12-h dark cycle (lights on from 0800 to 2000 h). Food and water were available ad libitum and room temperature was maintained at 22 ± 2 °C. All animals were used in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China on November 14, 1988.

Melatonin (Changzhou Medical Technique, China) was dissolved in corn oil. Picrotoxin and bicuculline methiodide (Sigma, USA) were dissolved in saline. Flumazenil, synthesized by the Department of Pharmaceutics of Shenyang Pharmaceutical University (China), was dissolved in corn oil. All drugs were administered intraperitoneally. Each injection was given in a volume of 0.5 ml/100 g body weight.

2.2. Implantation of electrodes and polygraphic recording

Electrodes for polygraphic recording of the electroencephalogram (EEG) and electromyogram (EMG) were implanted in rats as described by Timo-Iaria et al. (1970). Male rats were anesthetized with chloral hydrate (400 mg/kg ip). Four stainless steel screw electrodes were inserted through the skull into the surface of the parietal cortices for subsequent bipolar EEG recordings. Two electrodes were placed over the left hemisphere and the other two at the corresponding position over the right hemisphere (limb area, occipital cortex area). To record EMG, two silver wire electrodes were inserted bilaterally into the dorsal neck muscles. The leads from all electrodes were then fixed to the skull with dental cement. Before suturing the animal, an antibiotic ointment was applied to the incision to prevent infection.

Following surgery, each rat was housed singly and was allowed at least 1 week to recover before being used in the experiment. The rat was recorded in its home cage where it could move freely to some extent on the day of experiment. Recordings began at 8 a.m. and lasted about 4 h. At the start of the recording session, each rat was weighed, injected intraperitoneally, connected to the recording cable and returned to its cage. In order to minimize the stress involved in experimental procedures, all rats were habituated to a separate recording chamber and the recording conditions for several days before the actual experiment. One rat was tested at a time and all rats in a specific drug group were tested once in the experiment. Waves of EEG were classified into three types: slow-wave sleep (SWS), paradoxical sleep (PS) and wakefulness (W). Definitions of SWS, PS and W are described by Mirmiran and Pevet (1986). The total time of SWS and PS was defined as total sleep time (TS). Time to sleep onset (TSO) was defined as the time from injection to the occurrence of the first SWS episode.

Recording was made by an eight-channel physiological recorder (Nihon Kohden, Japan) at a chart speed of 25 mm/s. The half-amplitude frequency response was set at 1–35 Hz for the EEG and at 30–75 Hz for the EMG.

Table 1
Effect of flumazenil on the hypnotic activity of melatonin

Group	No. of rats	Dose (mg/kg)	TSO (min)	TS (min)	SWS (min)	PS (min)	W (min)
Control	7	–	82.10 ± 10.66	61.35 ± 7.14	59.02 ± 5.96	1.05 ± 0.52	178.65 ± 7.14
MT	5	10	25.41 ± 1.73**	117.73 ± 6.83**	110.89 ± 7.90**	6.84 ± 1.36**	122.27 ± 6.83**
FLU	4	3.5	67.10 ± 6.46	60.80 ± 7.60	59.00 ± 6.81	1.80 ± 0.98	179.20 ± 7.60
FLU	4	7	69.62 ± 10.67	52.95 ± 6.05	51.78 ± 5.66	1.18 ± 0.42	187.05 ± 6.05
MT + FLU	4	10 + 3.5	108.23 ± 7.50‡	50.97 ± 3.96‡	50.70 ± 3.92‡	0.27 ± 0.22‡	189.03 ± 3.96‡
MT + FLU	4	10 + 7	47.68 ± 5.78	67.92 ± 14.19‡	67.08 ± 13.76‡	0.85 ± 0.85‡	172.08 ± 14.19‡

Data are expressed as mean ± S.E.M. EEG recording lasted for 4 h.
MT = melatonin; FLU = flumazenil.
** $P < .01$, compared with control.
‡ $P < .01$, compared with MT.

Table 2
Effect of picrotoxin on the hypnotic activity of melatonin

Group	No. of rats	Dose (mg/kg)	TSO (min)	TS (min)	SWS (min)	PS (min)	W (min)
Control	7	–	82.10 ± 10.66	61.35 ± 7.14	59.02 ± 5.96	1.05 ± 0.52	178.65 ± 7.14
MT	5	10	25.41 ± 1.73**	117.73 ± 6.83**	110.89 ± 7.90**	6.84 ± 1.36**	122.27 ± 6.83**
PT	5	2	64.34 ± 5.76	77.66 ± 10.39	76.16 ± 9.94	1.50 ± 0.82	162.34 ± 10.39
PT	4	4	68.35 ± 6.44	77.05 ± 10.52	77.05 ± 10.52	0.00 ± 0.00	162.95 ± 10.52
MT + PT	4	10 + 2	87.25 ± 18.66‡	46.68 ± 2.62‡	46.48 ± 2.51‡	0.20 ± 0.20‡	193.32 ± 2.62‡
MT + PT	4	10 + 4	92.78 ± 14.37‡	62.42 ± 8.60‡	61.58 ± 8.13‡	0.85 ± 0.50‡	177.58 ± 8.60‡

Data are expressed as mean ± S.E.M. EEG recording lasted for 4 h.
MT = melatonin; PT = picrotoxin.
**$P < .01$, compared with control.
‡ $P < .01$, compared with MT.

2.3. Statistics

The results were expressed as the mean ± S.E.M. The summed effects of drugs were analyzed by multifactor analysis of variance (ANOVA) followed by Duncan's test for significant differences between groups. Two-way ANOVA was used to evaluate the interaction between drug treatment and melatonin. Differences with $P < .05$ were considered statistically significant. All statistical analyses were carried out by SAS software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Influence of flumazenil on hypnotic activity of melatonin

Melatonin (10 mg/kg) was administered intraperitoneally immediately after flumazenil (3.5, 7 mg/kg ip) at 8 a.m., the start of EEG recording. The results showed that melatonin alone, at a dose of 10 mg/kg, produced a significant reduction in TSO and W, and an increase in TS, SWS and PS. Flumazenil did not affect all the sleep parameters, but it significantly inhibited the melatonin-induced decrease in TSO and W, and inhibited the increase in TS, SWS and PS when it was used in combination with melatonin (Table 1). There was a significant interaction between melatonin and flumazenil [Melatonin (10 mg/kg) × Flumazenil (3.5 mg/kg): TSO: $F(1,15) = 27.32$, $P < .01$; TS: $F(1,15) = 18.38$, $P < .01$; SWS: $F(1,15) = 17.29$, $P < .01$; PS: $F(1,14) = 13.89$, $P < .01$; W: $F(1,15) = 17.91$: $P < .01$]; [Melatonin (10 mg/kg) × Flumazenil (7 mg/kg): TS: $F(1,16) = 5.49$, $P < .05$; SWS: $F(1,16) = 4.72$, $P < .05$; W: $F(1,16) = 5.12$, $P < .05$; PS: $F(1,15) = 11.60$, $P < .01$].

3.2. Influence of picrotoxin on hypnotic activity of melatonin

Melatonin (10 mg/kg) was administered intraperitoneally immediately after picrotoxin (2, 4 mg/kg ip) at 8 a.m., the start of EEG recording. Although a convulsive state (head and body twitching, spasmodic activity) arose in one third of rats 15 min after picrotoxin injection (2 mg/kg) and two thirds of rats 15 min after picrotoxin injection (4 mg/kg), all sleep parameters were not influenced. Picrotoxin (2 and 4 mg/kg) completely antagonized the melatonin-induced decrease in TSO and W, and the increase in TS, SWS and PS when it was used in combination with melatonin (Table 2). A significant interaction was observed between melatonin and picrotoxin on all sleep parameters [Melatonin (10

Table 3
Effect of bicuculline methiodide on the hypnotic activity of melatonin

Group	No. of rats	Dose (mg/kg)	TSO (min)	TS (min)	SWS (min)	PS (min)	W (min)
Control	7	–	82.10 ± 10.66	61.35 ± 7.14	59.02 ± 5.96	1.05 ± 0.52	178.65 ± 7.14
MT	5	10	25.41 ± 1.73**	117.73 ± 6.83**	110.89 ± 7.90**	6.84 ± 1.36**	122.27 ± 6.83**
BIC	4	2	86.60 ± 24.08	44.42 ± 9.80	44.42 ± 9.80	0.00 ± 0.00	195.58 ± 9.80
BIC	4	4	80.32 ± 10.25	44.20 ± 11.60	43.18 ± 10.88	1.02 ± 0.73	195.80 ± 11.60
MT + BIC	4	10 + 2	35.27 ± 11.74	84.73 ± 2.17‡	82.73 ± 0.98†	2.00 ± 2.00‡	155.27 ± 2.17†
MT + BIC	4	10 + 4	57.13 ± 17.09	73.03 ± 7.74‡	71.70 ± 7.18‡	1.33 ± 0.78‡	166.97 ± 7.74‡

Data are expressed as mean ± S.E.M. EEG recording lasted for 4 h.
MT = melatonin; BIC = bicuculline methiodide.
**$P < .01$, compared with control.
‡ $P < .01$, compared with MT.
† $P < .05$, compared with MT.

mg/kg) × Picrotoxin (2 mg/kg): TSO: $F(1,17) = 14.04$, $P < .01$; TS: $F(1,17) = 30.78$, $P < .01$; SWS: $F(1,17) = 29.75$, $P < .01$; PS: $F(1,16) = 16.68$, $P < .01$; W: $F(1,17) = 30.23$, $P < .01$]; [Melatonin (10 mg/kg) × Picrotoxin (4 mg/kg); TSO: $F(1,16) = 15.99$, $P < .01$; TS: $F(1,16) = 18.08$, $P < .01$; SWS: $F(1,16) = 17.70$, $P < .01$; PS: $F(1,15) = 8.79$, $P < .01$; W: $F(1,16) = 17.83$, $P < .01$].

3.3. Influence of bicuculline methiodide on hypnotic activity of melatonin

Melatonin (10 mg/kg) was administered intraperitoneally immediately after bicuculline methiodide (2, 4 mg/kg ip) was given at 8 a.m., the start of EEG recording. Bicuculline methiodide seemed to be devoid of pharmacological activity on sleep parameters. However, the melatonin-induced increase in TS, SWS and PS, and the decrease in W were abolished when bicuculline methiodide was administered together with melatonin. It had no influence on TSO when administered alone or used in combination with melatonin (Table 3). A significant interaction was obtained only between melatonin (10 mg/kg) and bicuculline methiodide (4 mg/kg) on PS [$F(1,14) = 8.05$, $P < .05$].

4. Discussion

The hypnotic properties of melatonin in previous studies did not draw a complete correlation because the effect of melatonin was affected by many factors, such as photoperiod, the status of the pineal gland, administration time, dosage, species differences and so on. Most studies are in agreement with the view, however, that melatonin is a potent sleep-promoting agent. Holmes and Sugden (1982) reported that melatonin (10 mg/kg) reduced TSO and W but increased both SWS and PS. Qualitatively similar but smaller effects were produced by a dose of 2.5 mg/kg. However, Tobler et al. (1994) did not find that melatonin (3 mg/kg ip) affected the vigilance states and brain temperature in the rat. Additionally, there is no evidence for a sleep-inducing effect of 3–5 mg/kg of melatonin in the hamster or rat in another experiment (Huber et al., 1998). A higher dose of 10 mg/kg, therefore, was chosen in the present research to study the hypnotic activity of melatonin. The present study indicated that melatonin, at a dose of 10 mg/kg ip, indeed had an obvious sleep-inducing effect in Wistar rats by using EEG analysis. Melatonin significantly reduced W and TSO, and increased TS, SWS and PS. The results are consistent with the studies reported by Holmes and Sugden (1982).

GABA is one of the important neurotransmitters mediating inhibitory postsynaptic potentials (Krnjevic and Schwartz, 1967). Up to 30–50% of all synapses are GABAergic in the CNS (Paredes and Agmo, 1992). Increasing evidence points to an important role for GABA in regulating sleep. It has been reported that GABA levels in the posterior hypothalamus are increased during sleep (Nitz and Siegel, 1996). Microinjection of muscimol, a potent GABA agonist, in the middle and anterior parts of the posterior hypothalamus, induces long-lasting behavioral and electroencephalographic signs of sleep with short latency (Lin et al., 1989). We have shown that the hypnotic activity of melatonin was blunted by pretreatment with semicarbazide hydrochloride, the inhibitor of the synthetic enzyme for GABA (Wang et al., 2002). In addition to the synthetic enzyme for GABA, the $GABA_A$ receptor may be another important target for melatonin. Accumulating evidence indicates that pineal activity can affect BZP receptor function in the brain. Pinealectomy significantly decreases and melatonin injections restore BZP receptor density in the cerebral cortex of the rat (Lowenstein et al., 1985; Acuna-Castroviejo et al., 1986). It is likely that melatonin does not bind to GABA or BZP binding sites themselves, because in vitro binding data show that melatonin is a weak competitor of BZP binding in brain membranes at concentrations greater than 10^{-5} M (Acuna-Castroviejo et al., 1995). However, more experiments support the view that melatonin activity is linked to allosteric modulation of some of the components of the $GABA_A$ receptor supramolecular complex in brain. Under certain circumstances, melatonin affects GABA binding of brain membranes in a direct way. Melatonin is capable of enhancing the binding of the GABA agonist [^{3}H]muscimol in vitro (Coloma and Niles, 1984). Moreover, melatonin augments GABA and BZP binding to brain membranes (Niles et al., 1987; Gomar et al., 1993). Taken together, these studies indicate that melatonin affects the function of the $GABA_A$ receptor, and some of the neuropharmacological actions of melatonin (including hypnotic activity) may be mediated through the $GABA_A$ receptor.

It is well known that the $GABA_A$ receptor is a supramolecular complex together with the central-type BZP receptor, comprising several recognition sites (such as the BZP, picrotoxin and GABA sites) (Mohler, 1992). Flumazenil, the specific antagonist of the BZP ligand site on the $GABA_A$ receptor, is a valuable in vivo tool because it can indicate whether a drug is acting at the BZP site to produce a pharmacological response. The present study indicated that flumazenil (3.5 and 7 mg/kg) seemed to be devoid of any significant intrinsic influence on all sleep parameters in rats by EEG recording. However, it significantly antagonized the melatonin-induced increase in TS, SWS and PS, and antagonized the decrease in TSO and W. Furthermore, there was a significant interaction between melatonin and flumazenil on all sleep parameters ($P < .05$ or $P < .01$). The data indicate that the BZP recognition site is one of the targets for melatonin hypnotic action, and melatonin may enhance the postsynaptic actions of GABA through binding to the BZP site. Our present results and previous studies show that some neuropharmacological effects exerted by melatonin, such as hypnotic, sedative and anticonvulsive actions, are similar to those induced by BZPs. Both melatonin and BZPs may share an action on the brain BZP site on the $GABA_A$ receptor.

The picrotoxin recognition site on the $GABA_A$ receptor is another important site where the action of GABA can be noncompetitively inhibited by several convulsants directly by inhibiting the chloride channel (Mohler, 1992). Although a convulsive state (head and body twitching, spasmodic activity) arose in one third of rats 15 min after picrotoxin injection (2 mg/kg) and two thirds of rats 15 min after picrotoxin injection (4 mg/kg), all sleep parameters were not influenced. However, when picrotoxin was used in combination with melatonin, it completely prevented the melatonin-induced increase in TS, SWS and PS, and prevented the decrease in TSO and W. Similar to the antagonistic effect of flumazenil on the hypnotic activity of melatonin, the results suggest that melatonin may be bound to the picrotoxin site on the $GABA_A$ receptor, and the picrotoxin recognition site, therefore, is another target for the hypnotic action of melatonin.

Activation of the GABA binding site on the $GABA_A$ receptor by GABA or its conformationally restricted analogues triggers a conformational change of the receptor, which allows the Cl^- channel to open. In this experiment, bicuculline methiodide (2 and 4 mg/kg), the specific antagonist acting at the GABA binding site on the $GABA_A$ receptor, did not influence any sleep parameter in rats when it was used alone. Although the melatonin-induced increase in TS, SWS and PS, and the decrease in W disappeared when it was used in combination with melatonin, no significant interaction was observed on sleep parameters except PS ($P < .05$). The result indicates that the melatonin-induced increase in SWS and PS, and the decrease in W may not be mediated through a unique pathway. Possibly, the GABA binding site only mediates the melatonin-induced increase in PS. However, a dose–response relationship was not found when flumazenil (3.5 and 7 mg/kg), picrotoxin (2 and 4 mg/kg) or bicuculline methiodide (2 and 4 mg/kg) was used alone or in combination with melatonin (10 mg/kg). In order to fully analyze the relationship between the mechanism of action of melatonin and the $GABA_A$ receptor, lower or higher doses of flumazenil or picrotoxin should be used in future studies.

In summary, the present study showed that melatonin-induced hypnotic activity was significantly antagonized by flumazenil and picrotoxin, but was not totally antagonized by bicuculline methiodide. These results indicate that the hypnotic activity of melatonin may not be mediated by only one specific binding site, but may possibly be mediated through the BZP site and the picrotoxin site together on the $GABA_A$ receptor. The GABA binding site on the $GABA_A$ receptor may partially participate in melatonin-induced sleep.

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