Br. J. Pharmac. (1985), **84**, 185–191

Influence of some agents that affect 5-hydroxytryptamine metabolism and receptors on nitrazepam-induced sleep in mice

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1 The effects of 5-hydroxytryptophan (5-HTP), citalopram, *p*-chlorophenylalanine (PCPA), cyproheptadine, lysergic acid diethylamide (LSD-25), metitepine and NSD 1034 on nitrazepam-induced sleep were investigated in mice.

2 Nitrazepam (1.6–25.6 mg kg^{-1}, i.p.) induced a dose-dependent sedative-hypnotic effect.

3 5-HTP (8–128 mg kg^{-1} i.m.) did not induce behavioural sleep but sedated mice and significantly potentiated nitrazepam-induced sleep. Similarly, 5-HTP (4–32 mg kg^{-1}, i.m.) increased pentobarbitone sleeping time.

4 Citalopram (2.5–10 mg kg^{-1}, i.p.) significantly potentiated nitrazepam sleep.

5 PCPA (300–400 mg kg^{-1}, i.p.) completely abolished nitrazepam sleep; 5-HTP (32 mg kg^{-1}, i.m.) reversed this effect.

6 NSD 1034 (75–150 mg kg^{-1}, i.p.) antagonized the potentiating effect of 5-HTP (32 mg kg^{-1}, i.m.) on nitrazepam sleep.

7 Cyproheptadine (5–10 mg kg^{-1}, i.p.) and LSD-25 (2.5–10 $\mu g\,kg^{-1}$, i.p.) partially antagonized nitrazepam sleep. Similarly, 5-HTP-induced potentiation of nitrazepam sleep was antagonized by cyproheptadine and LSD-25.

8 Metitepine (4–8 mg kg^{-1}, i.p.) induced behavioural sleep and significantly potentiated nitrazepam sleep.

9 Ro15–1788 (10 mg kg^{-1}, i.p.) effectively antagonized nitrazepam-induced sleep.

10 These results indicate that enhancement of central 5-HT neurotransmission may underlie nitrazepam-induced sleep in mice.

Introduction

The benzodiazepines are widely used as sedative-hypnotic, muscle relaxant, anticonvulsant and anxiolytic agents (Zbinden & Randall, 1967; Barraclough, 1974; Harvey, 1980). Specific receptor sites for these drugs have now been identified in the vertebrate central nervous system and the functional coupling that appears to occur between these receptors and receptors for γ-aminobutyric acid (GABA) (see Bowery, 1984) is thought to underly the ability of the benzodiazepines to enhance GABA-mediated inhibition (Haefely *et al.*, 1975). However, the remaining links between benzodiazepine receptor activation and the manifestation of the variety of therapeutic actions of these drugs remain unclear.

Previous authors (Wise *et al.*, 1972 Stein *et al.*, 1975) have suggested that benzodiazepines may exert their anxiolytic effects by decreasing central 5-hydroxytryptaminergic transmission, whereas the sedative-hypnotic action of nitrazepam in chicks appear to be associated with an increased availability of 5-hydroxytryptamine (5-HT) at central synapses (Wambebe, 1983b). The present study has therefore been undertaken to evaluate the role of central 5-HT in nitrazepam-induced sleep in the mouse.

Methods

Male albino mice (inbred in our Animal House) weighing between 20–30 g and aged 2–3 months were used. The experiments were performed in a quiet room with an ambient temperature of 22 ± 2 °C.

The animal house was illuminated between 06 h 00 min and 18 h 00 min daily. Eight mice were observed together at the same time; four served as controls (injected with 10 ml kg^{-1} i.p. of either physiological saline or 3% v/v Tween 80 diluted appropriately with physiological saline) for each drug interaction study. Additional control experiments were done at the beginning and end of the project. The results obtained with the control mice were pooled. The remaining four mice were injected with the test drug. Each experiment was repeated twice. The mice were placed in a specially-designed cage, 1 h before the start of the experiment so as to acclimatize them to the environment. The experiments were performed between 12 h 00 min–18 h 00 min each day to avoid behavioural changes resulting from circadian rhythm. After injection, the mice were observed until they awoke from nitrazepam-induced sleep. The criterion for sleep in mice was loss of righting reflex. The mice were regarded as sedated when they were calm, immobile and all behavioural activities abolished while retaining their righting reflexes for at least 5 min. A stop-clock was used for timing the onset and duration of sleep. The technical assistants who recorded the behavioural observations were unaware of the drugs injected to the mice. Weighed quantities of nitrazepam (Mogadon, Roche Product) and Ro15–1788 (ethyl 8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo [1, 5-a] [1, 4] benzodiazepine-3-carboxylate; Roche Products), *p*-chlorophenylalanine (PCPA; AB Hassle, Goteborg) were separately suspended in 3% v/v Tween 80 after which they were appropriately reconstituted with physiological saline before their injection into mice intraperitoneally. 5-Hydroxytryptophan (5-HTP; Sigma Chemical Co.) was dissolved in physiological saline and administered intramuscularly. Citalopram (LU-10-171 B HB, Hillimbech & Co.) was dissolved in physiological saline and injected subcutaneously. LSD-25 (lysergic acid diethylamide, Sandoz Ltd., Basle) injection was diluted with physiological saline and injected intraperitoneally. Weighed quantities of cyproheptadine (Merck, Sharpe and Dohme), NSD 1034 (*n*–(3-hydoxybenzyl)-N-methyl hydrazine dihydrogen phosphate, Smith and Nephew Research Ltd., Essex) and metitepine maleate (Roche Products) were also dissolved in physiological saline before intraperitoneal injection. Pentobarbitone sodium solution (Nembutal, May & Baker) was diluted with physiological saline before administration intraperitoneally. All the drugs were injected in the form of their salts as mentioned above. A fresh solution of each drug was prepared on the day of the experiment to avoid any chemical deterioration of the drug solutions. The drug pretreatment times before nitrazepam administration were 30 min (5-HTP, NSD 1034, Ro15–1788., citalopram and metitepine), 1 h (LSD-25, cyproheptadine) and 24 h (PCPA). The pretreatment periods were established from preliminary studies in our laboratory. The results were evaluated using either Student's *t* test (onset and duration of sleep) or Chi-squared test (number asleep) by comparison of the test results with the appropriate controls. A *P* value of 5% or less was regarded as statistically significant.

Results

Sedative-hypnotic effect of nitrazepam

Low doses (1.6–3.2 mg kg^{-1}, i.p.) of nitrazepam did not induce behavioural sleep while higher doses (4.8–25.6 mg kg^{-1}, i.p.) hypnotized the mice dose-dependently (Table 1). The solvent vehicle (i.e. 3% v/v Tween 80 diluted appropriately with physiological saline) had no observable effect on the gross behaviour of mice.

Sedative effect of 5-hydroxytryptophan

Apparently, 8 mg kg^{-1} i.m. of 5-HTP had no sedative effect on mice while 32–64 mg kg^{-1}, i.m. sedated all the mice observed (Table 2).

Table 1 Hypnotic effect of nitrazepam in mice

Nitrazepam (mg kg^{-1} i.p.)	*No. asleep/ No. Used*	*Onset of sleep* (min) Mean ± s.e. mean	*Duration of sleep* (min) Mean ± s.e. mean
1.6	0/8	– –	– –
3.2	0/8	– –	– –
4.8	4/8	72.4 ± 2.2	49.8 ± 1.6
6.4	6/8	68.2 ± 2.8	53.1 ± 1.5
12.8	8/8	46.5 ± 2.2	62.0 ± 1.8
25.6	8/8	32.5 ± 3.0	70.6 ± 1.5

Table 2 Sedative effect of 5-hydroxytryptophan (5-HTP) in mice.

5-HTP ($mg\,kg^{-1}$ i.m.)	*No. sedated/ No. used*	*Onset of sedation* (min) Mean ± s.e. mean	*Duration of sedation* (min) Mean ± s.e. mean
8	0/8	– –	– –
16	4/8	31.7 ± 1.2	92.9 ± 1.7
32	8/8	29.5 ± 2.4	96.8 ± 1.1
64	8/8	29.0 ± 1.9	101.3 ± 5.5

Influence of 5-HTP, PCPA and NSD 1034 on nitrazepam sleep

5-HTP (32–64 $mg\,kg^{-1}$, i.m.) significantly shortened the onset and prolonged the duration of nitrazepam sleep. On the other hand, PCPA (300–400 $mg\,kg^{-1}$, i.p.) completely abolished nitrazepam sleep; 5-HTP (32 $mg\,kg^{-1}$, i.m.) reversed this effect. NSD 1034 (75–150 $mg\,kg^{-1}$, i.p.) reduced the number of mice that slept and significantly shortened 5-HTP-nitrazepam sleeping time (Table 3).

Influence of LSD-25, cyproheptadine and 5-HTP on nitrazepam sleep

LSD-25 (10 $\mu g\,kg^{-1}$, i.p.) significantly ($P < 0.01$) reduced the proportion of mice that slept following injection of nitrazepam and delayed the onset of sleep. LSD-25 (5–10 $\mu g\,kg^{-1}$, i.p.) also delayed the onset but did not significantly prolong the duration of sleep induced by a combination of 5-HTP (32 $mg\,kg^{-1}$ i.m.) and nitrazepam. Similarly, cyproheptadine (5–10 $mg\,kg^{-1}$, i.p.) delayed the onset and shortened the duration of nitrazepam sleeep. Cyproheptadine (5–10 $mg\,kg^{-1}$, i.p.) also antagonized 5-HTP-induced potentiation of nitrazepam sleep. These effects of cyproheptadine were statistically significant ($P < 0.001$; Table 4).

Influence of citalopram, metitepine and Ro15–1788 on nitrazepam sleep

Citalopram (5–10 $mg\,kg^{-1}$, s.c.) significantly prolonged nitrazepam sleeping time. Metitepine (4–8 $mg\,kg^{-1}$, i.p.) induced behavioural sleep in all the mice injected with the drug. In addition, nitrazepam sleeping time was profoundly prolonged by metitepine (4–8 $mg\,kg^{-1}$, i.p.). Ro15–1788

Table 3 Influence of 5-hydroxytryptophan (5-HTP), *p*-chlorophenylanine (PCPA) and NSD 1034 on nitrazepam sleep

Nitrazepam	*Doses* ($mg\,kg^{-1}$) *5-HTP*	*PCPA*	*NSD 1034*	*No. asleep/ No. Used*	*Onset of sleep* (min) Mean ± s.e. mean	*Duration of sleep* (min) Mean ± s.e. mean
12.8	0	0	0	28/28	46.3 ±1.5	62.2 ±2.0
12.8	16	0	0	8/8	43.0 ±2.3	73.0* ±4.0
12.8	32	0	0	8/8	33.0* ±2.1	89.5* ±2.8
12.8	64	0	0	8/8	32.3 ±2.7	89.5* ±3.4
12.8	0	100	0	8/8	52.8* ±3.2	60.1 ±4.6
12.8	0	200	0	4/8	61.3* ±0.7	57.5 4.5
12.8	0	300	0	0/8	– –	– –
12.8	0	400	0	0/8	– –	– –
12.8	32	100	0	8/8	47.3+ ±2.7	64.6 ±4.0
12.8	32	200	0	8/8	53.0 ±1.7	64.9 ±4.9
12.8	32	300	0	8/8	58.5+ ±3.4	62.3 ±5.6
12.8	32	400	0	8/8	62.7 ±7.9	61.3 ±2.4
12.8	32	0	75	8/10	30.4 ±4.2	25.4++ ±2.5
12.8	32	0	150	6/10	28.6 ±5.5	17.4++ ±1.8

* Significantly different from the controls (same dose of nitrazepam, $P < 0.001$, Student's *t* test).
\+ Significantly different from the mice given 5-HTP and nitrazepam but no PCPA ($P < 0.001$, Student's *t* test).
++ Significantly different from the mice given 5-HTP and nitrazepjam but no NSD 1034 ($P < 0.001$, Student's *t* test).

Table 4 Influence of lysergic acid diethylamide (LSD-25), cyproheptadine (Cypro) and 5-hydroxytryptophan (5-HTP) on nitrazepam sleep

Doses					*Onset of sleep* (min)	*Duration of sleep* (min)
Nitrazepam (mg kg^{-1})	*5-HTP* (mg kg^{-1})	*LSD-25* (μg kg^{-1})	*Cypro* (mg kg^{-1})	*No. asleep/ No. used*	Mean ± s.e. mean	Mean ± s.e. mean
12.8	0	0	0	28/28	46.3 ±1.5	62.2 ±2.0
12.8	32	0	0	8/8	33.0* ±2.1	89.3* ±2.8
12.8	–	2.5	0	8/8	43.9 ±1.6	64.4 ±3.8
12.8	–	5.0	0	6/8	46.1 ±1.6	63.7 ±1.3
12.8	–	10.0	0	4/8++	50.1* ±2.1	64.5 ±3.5
12.8	32	2.5	0	8/8	30.5 ±1.5	94.8 ±5.4
12.8	32	5.0	0	8/8	39.2+ ±2.4	93.6 ±5.2
12.8	32	10.0	0	8/8	48.5+ ±4.5	92.0 ±5.5
12.8	0	0	5	8/8	61.5* ±1.5	50.0* ±1.6
12.8	0	0	10	8/8	60.3* ±1.8	50.0* ±1.8
12.8	32	0	5	8/8	50.3* ±2.4	52.3+ ±1.8
12.8	32	0	10	8/8	49.5+ ±1.2	51.1+ ±1.2

* Significantly different from the nitrazepam controls ($P<0.001$, Student's t test).
\+ Significantly different from the mice given 5-HTP and nitrazepam but no Cypro ($P<0.001$, Student's t test).
++ Significantly different from the nitrazepam controls ($P<0.01$, Chi-squared test).

Table 5 Influence of citalopram, metitepine and Ro15–1788 on nitrazepam sleep

Doses (mg kg^{-1})				*Onset of sleep* (min)	*Duration of sleep* (min)
Nitrazepam	*Citalopram*	*Metitepine*	*Ro15–1788*	*No. asleep/ No. used*	Mean ± s.e. mean	Mean ± s.e. mean
12.8	0	0	0	28/28	46.3 ± 1.5	62.2 ±2.0
0	2.5	0	0	0/8	– –	– –
0	5	0	0	0/8	– –	– –
0	10	0	0	0/8	– –	– –
12.8	2.5	0	0	8/8	44.4 ± 1.8	66.2 ±0.7
12.8	5	0	0	8/8	39.5 ± 2.3	78.2* ±6.2
12.8	10	0	0	8/8	38.8 ± 4.1	80.0* ±4.8
0	0	4	0	8/8	42.5 ± 3.4	24.4 ± 4.5
0	0	8	0	8/8	35.8 ± 4.2	30.6 ±2.2
12.8	0	4	0	8/8	8.5 ± 1.5	240*
12.8	0	8	0	8/8	6.2 ± 1.2	240*
12.8	0	0	10	0/8	– –	– –

* Significantly different from the nitrazepam controls $P<0.001$, Student's t test.

Table 6 Influence of 5-hydroxytryptophan (5-HTP) on pentobarbitone sleep

Doses (mg kg^{-1})			*Onset of sleep* (min)	*Duration of sleep* (min)
Pentobarbitone	*5-HTP*	*No. asleep/ No. used*	Mean ± s.e. mean	Mean ± s.e. mean
20	0	8/8	7.4 ± 2.8	25.4 ±3.6
0	4	0/8	– –	– –
0	8	0/8	– –	– –
0	16	0/8	– –	– –
20	4	8/8	9.2 ± 1.4	36.4 ±2.4
20	8	8/8	6.1 ± 1.6	30.6 ±3.8
20	16	7/8	5.2 ± 2.0	30.2 ±3.5
20	32	8/8	6.5 ± 2.1	43.0* ±4.5

* Represents $P<0.001$, Student's t test compared to mice given only pentobatbitone (20 mg kg^{-1}, i.p.).

(10 mg kg^{-1}, i.p.) effectively antagonized nitrazepam sleep (Table 5).

Influence of 5-HTP on petobarbitone sleep

5-HTP (32 mg kg^{-1} i.m.) significantly increased the duration of pentobarbitone sleep (Table 6).

Discussion

The present data indicate that the sedative-hypnotic effect of nitrazepam in mice is dose-dependent. Thus, at low doses (1.6–4.2 mg kg^{-1}, i.p.) the mice were sedated while higher doses (4.8–25.6 mg kg^{-1}, i.p.) hypnotized the animals. These results essentially agree with those of Valzelli & Garattini (1968) who studied the behavioural effects of diazepam in cats. Nitrazepam 1.6 mg kg^{-1} i.p. induced behavioural sleep in young chicks (Wambebe, 1983a, b). However, in this project, such a dose of nitrazepam lacked any hypnotic effect in mice. This difference may be due to species variation or to easier access of the drug into the young avian brain compared to entry of the drug into the mammalian brain. Such a proposal is supported by the lack of a fully functional blood-brain barrier in young chicks up to 28 days of age (Waelsch, 1955).

As shown in Table 2, 5-HTP (8–64 mg kg^{-1}, i.m.) did not induce sleep but sedated mice. This observation agrees with the findings of Sanghvi & Gershon (1970) who used cats in their study. When nitrazepam was administered to 5-HTP-pretreated mice, there was a marked potentiation of nitrazepam sleep. Since NSD 1034 (central 5-HT decarboxylase inhibitor) antagonized the effect of 5-HTP on nitrazepam sleep, the observed potentiation of nitrazepam sleep by 5-HTP might be mediated centrally. According to Bogdanski *et al.* (1958), 5-HTP is taken up by 5-HTergic nerves where it is decarboxylated to 5-HT. These results therefore suggest that the hypnogenic action of nitrazepam in mice might be mediated partly via a central 5-HT mechanism. According to Trulson *et al.*, (1982), the usual anxiolytic doses of diazepam in cats (i.e. 0.5–1 mg kg^{-1}) had no effect on raphe unit activity. However, higher doses of diazepam (2.5–10 mg kg^{-1}) which depressed raphe unit activity exhibited a biphasic behavioural effect in cats, an initial excitatory effect followed by a sedative-hypnotic effect (Trulson *et al.*, 1982). It has also been reported by Chadwick *et al.*, (1978) and Pratt *et al.*, (1979) that clonazepam increased cerebral 5-HT and tryptophan levels in mice by some unknown mechanisms. It is therefore apparent that the hypotheses which relate the degree of behavioural arousal to the activity of the raphe cells as well as explaining the anxiolytic effect of benzodiazepines on the basis of their suppression of 5-HT neurones may require critical re-examination.

Hyttel (1977a, b) found that citalopram was a potent and selective inhibitor of 5-HT uptake. The potentiation of nitrazepam sleep in this study by citalopram might be due to an increase in the brain levels of free 5-HT available at the postsynaptic receptor sites. According to Stenberg *et al.*, (1980) citalopram normalized sleep of PCPA-pretreated animals.

PCPA blocks the synthesis of tryptophan hydroxylase thereby inhibiting 5-HT synthesis (Koella, 1969; Koella, 1981). In the present study, PCPA (100–400 mg kg^{-1}) effectively antagonized nitrazepam sleep dose-dependently. Apparently, the failure of nitrazepam to induce sleep in PCPA-treated mice might be partly related to the inhibition of 5-HT synthesis. The depression of sleep by PCPA has been reported in cats (Jouvet, 1969), rats (Mouret *et al*, 1968) and monkeys (Weitzman *et al.*, 1968). Thus, the present data agree with the reported antagonistic effect of PCPA on sleep. It is significant to note that 5-HTP reversed PCPA-induced antagonism of nitrazepam sleep which further implicates 5-HT in nitrazepam sleep. Similar reversal of PCPA-induced insomnia in cats by 5-HTP has been reported by Koella *et al.* (1968). Thus, it is possible that similar mechanisms may underly these observations. According to Dray *et al.* (1978) and Tanner (1978), inhibition of 5-HT synthesis leads to hyperactivity of dopaminergic mechanisms. Dopamine receptor agonists have been reported to antagonize pentobarbitone-induced sleep in chicks (Osuide & Wambebe, 1980) and rats (Wambebe & Osuide, 1983). It is therefore possible that dopaminergic hyperactivity may be partly responsible for PCPA-induced antagonism of nitrazepam sleep.

The sedative effect of cyproheptadine may be related to its non-specific enhancing effect upon 5-HT neurotransmission (Jacoby *et al.*, 1978). However, the weak antagonistic effect of cyproheptadine against nitrazepam sleep may be due to its ability to block central 5-HT receptors (Jacoby *et al.*, 1978). On the other hand, LSD-25 behaviourally excited the mice and partially antagonized nitrazepam sleep. In addition, 5-HTP-induced potentiation of nitrazepam sleep was antagonized by LSD-25. These results indicate an involvement of 5-HT in the mechanism of nitrazepam sleep.

Metitepine is a potent antagonist of central 5-HT autoreceptors (Monachon *et al.*, 1972). In this study, metitepine induced behavioural sleep and significantly potentiated nitrazepam sleep. Such results suggest that central postsynaptic 5-HT receptors may be implicated in the sedative-hypnotic effect of nitrazepam in mice, possibly via enhancement of

GABAergic mechanisms. It is equally possible that enhanced GABA receptor activation facilitates transmission of central 5-HT synapses to induce sleep. Since Ro15-1788 (a benzodiazepine receptor antagonist) blocked nitrazepam sleep, the involvement of benzodiazepine receptors is indicated.

Since barbiturates and benzodiazepines may increase chloride flux through chloride channels at $GABA_A$ receptor sites (see Bowery, 1984), the potentiation of pentobarbitone sleep by 5-HTP is not surprising.

The data accumulated in this study essentially agree with those obtained by Wambebe (1983b) using chicks. It may therefore be possible that similar mechanisms involving endogenous 5-HT may underlie nitrazepam sleep in both chicks and rats. Thus, it appears that activation of central $5\text{-}HT_2$ receptors may induce sedative-hypnotic effect in mice. In addition, central 5-HT neurotransmission may be involved directly or indirectly in the sedative-hypnotic effect of nitrazepam in mice.

The generous gifts of nitrazepam (Mogadon), metitepine and Ro15-1788 from Roche Products as well as cyproheptadine HCl from Merck Sharpe & Dohme are gratefully acknowledged. I am also grateful to Ahmadu Bello University Board of Reaearch for funding this project, Prof. G. Osuide for his encouragement and Mr M. A. Rahman for his valuable assistance.

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(*Received May 31, 1984.*
Revised August 14, 1984.)