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EFFECT OF THE BENZODIAZEPINE ANTAGONIST Ro 15-1788 ON FLUNITRAZEPAM-INDUCED SLEEP CHANGES

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1 The modifications of human sleep induced by benzodiazepines, and particularly by flunitrazepam, are complex. Stage 4 and paradoxical sleep are both decreased; however, these two effects have a different evolution during and after single or short-term drug administration.

2 The benzodiazepine antagonist Ro 15-1788 also tends to depress stage 4, but with immediate recovery in the post-drug night, and does not modify paradoxical sleep.

3 In combined administration, this drug totally reverses the hypnogenic effect of flunitrazepam, as well as its effect on paradoxical sleep but not the decrease of slow wave sleep.

4 Some of the benzodiazepine-induced alterations of sleep may be related to receptors different from central benzodiazepine receptors, or to mechanisms not directly connected to this type of receptors.

Introduction

The modifications induced by benzodiazepines of normal human sleep have been well described but their mechanism is still poorly understood (Monti & Altier, 1973; Bixler *et al.*, 1977; Nicholson & Stone, 1980). These compounds shift the sleep-waking balance (hypnogenic effect), delay the appearance and lower the density of paradoxical sleep (PS), decrease stage 4 mainly by a reduction of the amplitude of slow waves (Feinberg *et al.*, 1977), and enhance spindle activity. However, these modifications do not all have similar evolutions in relation to drug administration. In this respect, the contrast between PS and stage 4 is particularly striking. In the post-drug night following single, or withdrawal following short-term, administration of benzodiazepines, PS returns promptly to baseline, whereas stage 4 is slower to react to the drug, continues to decrease in the first post-drug night (Gaillard *et al.*, 1973; Gaillard & Aubert, 1975), and takes at least several days to recover (Gaillard, 1977). This effect is probably not related to the half-life of the compounds studied and has also been found recently with the short-acting benzodiazepine midazolam (Gath *et al.*, 1981). This rather suggests a heterogeneity in the mode of action of this class of drugs possibly through a heterogeneous population of receptors.

Recently, the imidazodiazepine Ro 15-1788 has been found to antagonize the central effects of benzodiazepines by competitive interaction at the receptor level (Hunkeler *et al.*, 1981). The known pharmacological properties of this compound indicate that it has no effect *per se* (Hunkeler *et al.*, 1981; Polc *et al.*, 1981, Mohler *et al.*, 1981). It binds selectively to central benzodiazepine receptors, in contrast to most of the currently marketed benzodiazepines, which bind equally to central and 'peripheral' receptors (Mohler *et al.*, 1981). However, the peripheral benzodiazepine receptors, particularly represented in the kidney (Braestrup & Squires, 1977; Regan *et al.*, 1981) are not related to any known pharmacological effect and have therefore been termed acceptors (Richards *et al.*, 1982). The present experiment was designed to determine if Ro 15-1788 equally antagonizes the modifications of stage 4 and those of PS induced by flunitrazepam.

Methods

Twelve normal subjects, eight males and four females, were paid volunteers for this experiment. Their mean age was 24 ± 5 years. Interview and clinical examinations ensured that they were in good health, usually slept well, did not take any medication, and were not heavy alcohol consumers. They were instructed to refrain from naps, drugs, excessive alcohol and coffee consumption, and to maintain their usual way of living throughout the experiment. Care was taken to record the female subjects in the first half of their menstrual cycle.

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These subjects were recorded, using standard sleep monitoring techniques for 10 nights, distributed in three sessions separated by at least 15 days. The first session consisted of two placebo pre-drug, one drug and one placebo post-drug night. The last two sessions consisted each of one placebo pre-drug, one drug, and one placebo post-drug night. In addition, each session was preceded by one habituation night not recorded. Within a session, all nights were consecutive.

Three different drug conditions were studied in a double-blind, randomized balanced design: flunitrazepam (2 mg) + placebo, Ro 15-1788 (100 mg) + placebo and a combination of flunitrazepam + Ro 15-1788 simultaneously administered. Placebos were pills of identical aspect. The treatments were given orally 20 min before lights out.

The recordings were made on magnetic tape and scored off line with an automatic scoring system previously described (Gaillard & Tissot, 1973). The scoring program followed as closely as possible the criteria of Rechtschaffen & Kales (1968), and generated in the data base one file for each recording, containing the numerical values of a number of classical sleep parameters, as well as the hypnogram. A special software package for sleep data handling was used for intersubject averaging and statistical estimation. All treatment and post-drug conditions were compared to the appropriate pre-drug placebo night using two-tailed Student's *t*-test. In addition, the general trends of sleep stages were calculated according to a method previously described (Gaillard, 1979). These trends are functions describing the evolution of each sleep stage with respect to sleep time, and waking with respect to recording time. The fitting parameters, level (degree 0), slope (degree 1) and curvature (degrees 2 and 3) describe the shape of this function and can be statistically estimated using two-tailed Student's *t*-test. The level of significance was $P < 0.05$.

Results

The comparison of all pre-drug placebo nights showed no significant difference for any sleep parameter. More specifically, the similarity of the first two nights under placebo in the first session indicated that habituation to laboratory environment was good and that there was no sequential effect with systematic variations from one night to the next related to the order of the nights during the session.

Flunitrazepam induced the full spectrum of modifications already known with this and other benzodiazepines (Table 1). Especially noticeable was the discrepancy between short-lasting and long-lasting effects. The drug significantly shifted the sleep-waking balance by enhancing total sleep and efficiency index, and by reducing total waking time, sleep latency, and number of awakenings. Waking latency, the number of minutes between sleep onset and the first awakening of more than 3 min, was also prolonged, but not significantly so due to high individual variability. The general trend of waking was significantly decreased by flunitrazepam (level: $P < 0.01$; slope and curvature: $P < 0.05$). In the post-drug nights the parameters describing the sleep-waking balance were back to control values, except the number of awakenings, still significantly decreased.

In addition to sleep promoting effects, flunitrazepam also modified the architecture of sleep. The drug delayed the first appearance of PS; as a consequence of this lengthening of PS latency, the number of sleep cycles was smaller, and the average sleep cycle duration longer than in control nights. In fact, this figure resulted from the first sleep cycle, extending from sleep onset to the end of the first episode of PS; this first cycle was markedly longer under flunitrazepam ($P < 0.005$), while the other cycles had durations similar to controls. The characteristics of PS returned to control values in the post-drug night.

Stage 2 was enhanced both under and after flunitrazepam. In contrast, a significant reduction of stages 3 and 4 appeared only in the post-drug night. The non-significant decrease of stage 4 under flunitrazepam was probably the beginning of the effect becoming significant in the next night (level, slope and curvature: $P < 0.005$) (Figure 1). Interestingly, the general trend of stage 3 was enhanced under flunitrazepam (level and curvature: $P < 0.01$), suggesting redistribution of slow wave sleep between stages 3 and 4 as one of the immediate effects of this compound (Figure 1).

Very few measures of sleep were affected by Ro 15-1788 + placebo (Table 2). The amount of stage 4 was slightly less than in the controls, but the difference was not significant. However, the level of the general trend was significantly lower (Table 4, Figure 2). Thus, an effect of Ro 15-1788 on stage 4 is not clearly demonstrated, but cannot be totally discarded. In contrast to what happened under flunitrazepam, no enhancement of stage 3 was seen, which suggests that Ro 15-1788 did not redistribute slow wave sleep. The significant shortening of the average sleep cycle duration under Ro 15-1788 may be related to the decrease of stage 4, if such a decrease really exists.

In the placebo night following Ro 15-1788, the production of stage 4 returned to baseline (Figure 2). PS showed a secondary increase, measured by total duration as well as by general trend (level: $P < 0.05$).

Most of the effects of flunitrazepam were abolished by the simultaneous administration of Ro 15-1788 (Table 3). In particular total sleep, waking, sleep latency, waking latency and efficiency index were very close to control values, indicating full antagon-

Table 1 Modifications of sleep parameters induced by flunitrazepam in 12 normal young adults (Mean ± s.d.)

Sleep parameters	*Placebo pre-drug*	*Flunitrazepam 2 mg*	*Placebo post-drug*
Total sleep (min)	505 ± 17	523 ± 32*	499 ± 34
Waking (min)	36 ± 17	12 ± 11***	26 ± 21
Stage 1 (min)	21 ± 17	11 ± 9*	25 ± 21
Stage 2 (min)	266 ± 50	320 ± 46*	294 ± 33*
Stage 3 (min)	33 ± 15	45 ± 16	24 ± 10*
Stage 4 (min)	77 ± 38	52 ± 40	40 ± 24***
Paradoxical sleep (min)	107 ± 21	95 ± 32	116 ± 24
Sleep latency (min)	25 ± 17	7 ± 9***	20 ± 20
Paradoxical sleep latency (min)	97 ± 40	182 ± 79***	101 ± 48
Waking latency (min)	403 ± 194	502 ± 76	423 ± 118
Mean duration of sleep cycles (min)	106 ± 22	134 ± 30***	108 ± 33
Number of sleep cycles	4.5 ± 0.9	3.9 ± 1.1*	4.5 ± 1.0
Efficiency index	0.935 ± 0.031	0.979 ± 0.019***	0.950 ± 0.038
Number of stage shifts	49 + 7	37 + 6***	43 + 11
Number of awakenings	3.7 ± 2.2	2.1 ± 1.2*	2.6 ± 1.9*
Number of ample body movements in sleep	31 ± 25	23 ± 15	33 ± 17
Number of small body movements in sleep	128 ± 56	66 ± 28***	96 ± 31*

*$P < 0.05$, *** $P < 0.005$ (two tailed paired Student's t-test).

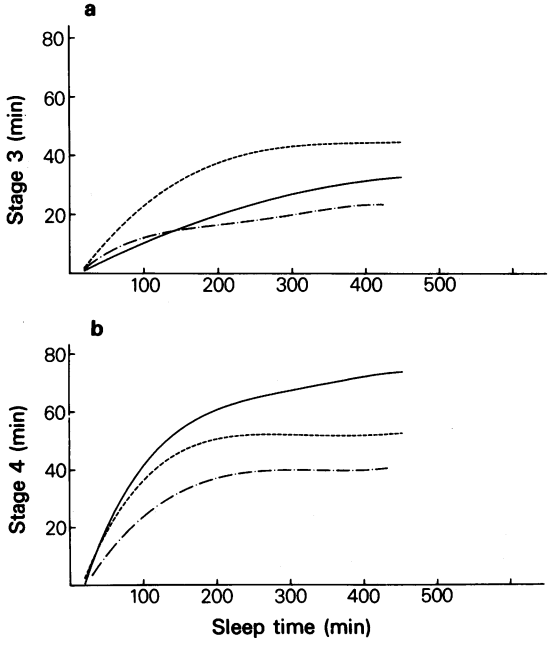

Figure 1 Evolution of stage 3 (a) and stage 4 (b) as a function of sleep time in 12 normal subjects, under the effect of 2 mg of flunitrazepam.
———placebo pre-drug, – – – – flunitrazepam and – · – · – · placebo post-drug.

ism against the hypnogenic properties of flunitrazepam. Similarly, Ro 15-1788 abolished the effects of flunitrazepam on latency and production of PS (Figure 3).

However, the number of stage shifts, the number of awakenings, the number of ample and small body movements in sleep were significantly decreased under the combined treatment. Stage 2 was enhanced. The duration of stage 3 was not modified whereas stage 4 was significantly depressed (Table 3, Figure 3). The deficit of stage 4 was close to the sum of the deficit under Ro 15-1788 + placebo and under flunitrazepam + placebo.

The direct comparison of the nights under flunitrazepam + placebo and under flunitrazepam + Ro 15-1788 showed significant differences for waking ($P < 0.01$), stage 1 ($P < 0.05$), sleep latency ($P < 0.05$), and efficiency index ($P < 0.05$), confirming the suppression of the hypnogenic effect of flunitrazepam, as well as for the latency of PS ($P < 0.05$) (Tables 1 and 3). In contrast, the latency of stage 3 was longer under the combined treatment than under flunitrazepam alone ($P < 0.005$).

At this point, the most parsimonious conclusion is that stage 4 is at least as low under combined treatment as under flunitrazepam + placebo. However, as indicated above, we cannot totally exclude an effect of Ro 15-1788 on stage 4. Two possibilities can be considered. According to the first one, all the effects of flunitrazepam, including the decrease of slow wave

Table 2 Parameters of sleep under and following Ro 15-1788 in 12 normal young adults (Mean ± s.d.)

Sleep parameters	*Placebo pre-drug*	*Ro 15-1788 100 mg*	*Placebo post-drug*
Total sleep (min)	429 ± 41	485 ± 64	508 ± 56
Waking (min)	40 ± 26	57 ± 46	32 ± 16
Stage 1 (min)	16 ± 11	14 ± 11	12 ± 7
Stage 2 (min)	267 ± 43	269 ± 50	256 ± 47
Stage 3 (min)	32 ± 13	33 ± 14	38 ± 13
Stage 4 (min)	71 ± 24	61 ± 31	75 ± 17
Paradoxical sleep (min)	106 ± 28	109 ± 25	127 ± 32*
Sleep latency (min)	30 ± 25	48 ± 40	24 ± 15
Paradoxical sleep latency (min)	96 ± 39	85 ± 43	90 ± 40
Waking latency (min)	440 ± 151	423 ± 148	508 ± 56
Mean duration of sleep cycles (min)	108 ± 20	94 ± 16*	106 ± 20
Number of sleep cycles	4.3 ± 1.0	4.8 ± 0.9	4.7 ± 1.0
Efficiency index	0.925 ± 0.048	0.893 ± 0.092	0.939 ± 0.034
Number of stage shifts	46 ± 10	48 ± 10	45 ± 7
Number of awakenings	3.7 ± 2.3	3.3 ± 3.8	3.3 ± 2.0
Number of ample body movements in sleep	30 ± 17	30 ± 14	32 ± 17
Number of small body movements in sleep	98 ± 26	96 ± 35	103 ± 69

* $P < 0.05$ (two tailed paired Student's t-test).

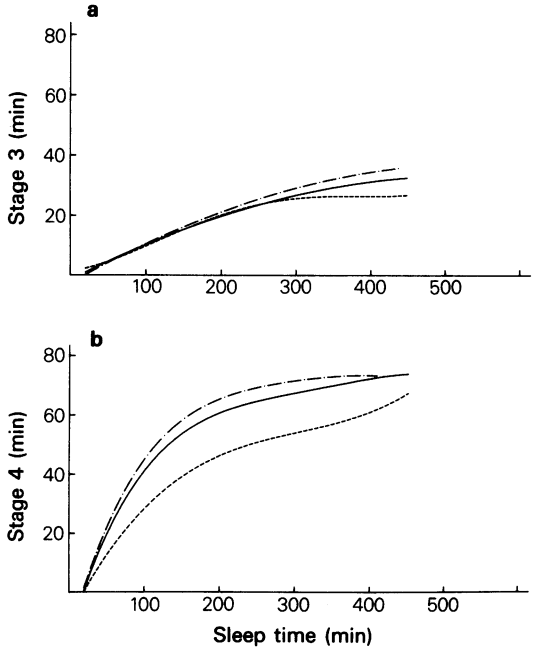

Figure 2 Evolution of stage 3 (a) and stage 4 (b) as a function of sleep time in 12 normal subjects, under the effect of 100 mg of Ro 15-1788.
——— placebo pre-drug, - - - - - Ro 15-1788 and — · — · — placebo post-drug.

sleep, are antagonized by Ro 15-1788, and the diminution observed under combined treatment is only due to the intrinsic effect of Ro 15-1788. The second possibility is that Ro 15-1788 may not antagonize the flunitrazepam-induced decrease of slow wave sleep, in addition to its own depressing effect. If this is true, slow wave sleep should be lower in the night under combined treatment than under flunitrazepam + placebo. To test this hypothesis, and to take into account the redistribution between stages 3 and 4 induced by flunitrazepam, the sum of the two stages was compared and the difference estimated using one tailed Student's t-test for paired samples. Under combined treatment, this sum was 75 ± 28 min, significantly lower than under flunitrazepam, 97 ± 41 min ($P < 0.05$). For comparison, the baseline value was 110 ± 25 min. Table 4 gives the numerical values of the fitting parameters of stage 4, as well as the total number of minutes spent in this stage.

In the placebo night following the combined treatment, the values of the different sleep parameters were not different from those of the night following flunitrazepam + placebo, indicating that the effect of Ro 15-1788 was no longer visible in this condition.

Discussion

The values of the different sleep parameters in the control nights were very close to those previously

Table 3 Parameters of sleep under and following combined administration of flunitrazepam and Ro 15-1788 in 12 normal young adults (Mean ± s.d.)

Sleep parameters	*Placebo pre-drug*	*Flunitrazepam 2 mg Ro 15-1788 100 mg*	*Placebo post-drug*
Total sleep (min)	488 ± 44	498 ± 36	502 ± 37
Waking (min)	34 ± 20	43 ± 36	26 ± 17
Stage 1 (min)	17 ± 10	19 ± 13	19 ± 13
Stage 2 (min)	253 ± 48	302 ± 44**	294 ± 40*
Stage 3 (min)	39 ± 14	32 ± 18	31 ± 13
Stage 4 (min)	78 ± 22	44 ± 27***	39 ± 27***
Paradoxical sleep (min)	102 ± 13	102 ± 38	119 ± 27
Sleep latency (min)	28 ± 20	42 ± 40	19 ± 14
Paradoxical sleep latency (min)	112 ± 39	126 ± 52	117 ± 63
Waking latency (min)	487 ± 45	450 ± 148	470 ± 74
Mean duration of sleep cycles (min)	111 ± 18	113 ± 31	110 ± 30
Number of sleep cycles	4.3 ± 0.7	4.5 ± 1.2	4.5 ± 1.1
Efficiency index	0.935 ± 0.040	0.922 ± 0.065	0.951 ± 0.032
Number of stage shifts	48 ± 11	38 ± 7*	37 ± 7*
Number ofawakenings	3.1 ± 1.8	1.7 ± 1.1*	2.3 ± 1.7
Number of ample body movements in sleep	38 ± 13	23 ± 14***	21 ± 16***
Number of small body movements in sleep	115 ± 35	79 ± 21***	92 ± 39**

*$P < 0.05$, ***$P < 0.005$ (two tailed paired Student's *t*-test).

observed in our laboratory in groups of subjects of similar age. The comparison of the placebo pre-drug nights, as well as accumulated experience of this laboratory also indicated very good stability of recording conditions within as well as across sessions. Particularly, there was no chronological or habituation effect which would have resulted in systematic modifications of sleep in a given night due to its particular position within a session. The selected experimental design further decreased the likelihood of a drug effect being confounded with a sequential effect. In each treatment condition, two thirds of the drug nights followed one placebo night and were thus the second nights of the session; one third of the drug nights followed two placebo nights and were thus the third nights of the session. This alteration of the sequential position of the drug nights was aimed at destroying any effect due only to the order of the recorded nights. Moreover, all treatment conditions were randomized and balanced. The choice of the experimental design had also to take into account the necessity to observe both drug and post-drug modifications.

The effects of flunitrazepam observed here fully agree with previous observations (Gaillard *et al.*, 1973; Monti & Altier, 1973; Bixler *et al.*, 1977). They fall under three headings: hypnogenic effect, suppression of PS early in the night and decrease of slow wave sleep. A number of other modifications of sleep stages,

Table 4 Numerical values of total amount and general trend of stage 4 in the different treatment conditions. Under placebo are given for simplication the average values of all four pre-drug nights, but the statistical significances are calculated for each condition with respect to the corresponding placebo pre-drug. Level, slope and curvature of the second degree are the fitting parameters (dimensionless) of the general trend of stage 4 for degrees 0, 1 and 2 respectively.

	Placebo	*Flunitrazepam*	*Ro 15-1788*	*Flunitrazepam + Ro 15-1788*
Minutes	74 ± 20	52 ± 40	61 ± 31	44 ± 27***
Level	60.5 ± 16.0	61.3 ± 24.1	49.5 ± 19.2*	40.6 ± 19.4***
Slope	1.17 ± 0.50	0.79 ± 0.51	1.12 ± 0.55	0.66 ± 0.49***
Curvature of the second degree	−0.0479 ± 0.0230	−0.0484 ± 0.0266	−0.0390 ± 0.0276	−0.0376 ± 0.234

*$P < 0.05$, ***$P < 0.005$ (two tailed paired Student's *t*-test).

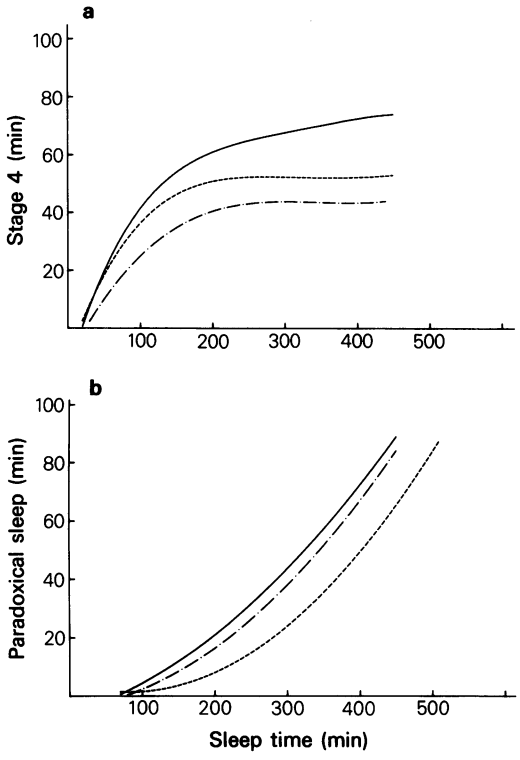

Figure 3 Evolution of stage 4 (a) and paradoxical sleep (b) as a function of sleep time in 12 normal subjects, under the effect of flunitrazepam + placebo and flunitrazepam + Ro 15-1788.
——— placebo pre-drug, - - - - - flunitrazepam + placebo and — · — · — flunitrazepam + Ro 15-1788.
The effect of flunitrazepam on paradoxical sleep is antagonized by Ro 15-1788, whereas its effect on stage 4 is not antagonized.

such as increase of sleep spindles, decrease of slow waves, reduction of rapid eye movements in PS, were also observed and will be described elsewhere. The effects of flunitrazepam on total sleep and on PS were short-lasting and probably directly related to the presence of the drug in the body, since they were no longer observed in the post-drug night. In contrast, the decrease of slow wave sleep had a slower onset and was more accentuated in the post drug night than in the drug night. This alteration has been found in varying degrees with all benzodiazepines adequately studied from this point of view. It has also been described after the short-acting benzodiazepine midazolam (Gath *et al.*, 1981). This suggests that the decrease of slow wave sleep may have less direct relationship with the amount of the drug still present in the body. There is little doubt that this decrease is a result of flunitrazepam action, but it is possible that a complex chain of events is triggered and continues to manifest itself even when the level of the active compound becomes very low. The differences between the short-lasting hypnogenic and PS depressing effects of flunitrazepam and the long-lasting decrease of slow wave sleep is not due to a tolerance mechanism, since these changes persist under chronic treatment (Gaillard & Tissot, 1975).

Ro 15-1788 totally reversed the hypnogenic effect of flunitrazepam, as well as the modifications of PS. This is in perfect agreement with the known properties of this compound as an antagonist of benzodiazepines (Hunkeler *et al.*, 1981; Polc *et al.*, 1981). However, Ro 15-1788 did not antagonize the effect of flunitrazepam on slow wave sleep. An intrinsic effect of this compound on stage 4 is not clearly demonstrated but this possibility must be left open. Such an effect is difficult to establish due to the large natural variability of stage 4. These results raise important issues regarding sleep regulation as well as benzodiazepine action.

In a very large number of experiments, Ro 15-1788 has been found to be totally devoid of intrinsic activity, with the exception of a dose-dependant depression of multiunit activity of the nucleus raphe dorsalis (Polc *et al.*, 1981; Laurent *et al.*, 1983). Benzodiazepines also markedly inhibit the firing of raphe units (Polc *et al.*, 1981; Laurent *et al.*, 1983) and decrease slow wave sleep. Since both benzodiazepines and benzodiazepine antagonists belong to the same chemical family, it is not necessarily unexpected that they have some similar effect. However, this effect may still be not identical since benzodiazepines seem to be much more potent than the antagonist in depressing multiunit activity in the raphe. Moreover, the effect of both compounds on slow wave sleep is not the same. Ro 15-1788 does not modify stage 3 and may reduce somewhat stage 4. Stage 4 returns to control values in the post-drug night. In contrast, flunitrazepam redistributes slow wave sleep between stages 3 and 4 to start with, and then profoundly and lastingly decreases both stages. In all likelihood this corresponds to two different mechanisms. There is at present no evidence of any partial benzodiazepine-like activity of Ro 15-1788, except with very high doses, much higher than used here (Haefely, personal communication).

Although the duration of action of Ro 15-1788 is not known with precision, the present and other data not shown here, such as spindle count, suggest that it is present at least in the first two thirds of the night, when most of stages 3 and 4 are produced. Moreover, the decrease of slow wave sleep under combined treatment is roughly equal to the sum of the effects induced by either drug + placebo. The evolution of stage 4 further indicates that differences in pharmacokinetics cannot account for the lack of antagonism against the flunitrazepam-induced depression of this stage. An interesting possibility is that it could be related to the different binding specificities of the two

substances. Ro 15-1788 displaces flunitrazepam only from the central benzodiazepine receptors, but not from the so-called peripheral acceptors, which have been found in the cortex and in the choroid plexus (Schoemaker *et al.*, 1981; Richards *et al.*, 1982). Recent results suggest that the choroid plexus may be involved in the generating mechanisms of slow wave sleep, possibly through the 5-hydroxytryptaminergic terminals they contain (Bobillier *et al.*, 1980); Petitjean *et al.*, 1981; Sallanon, 1981). According to this view, the effect of flunitrazepam on PS could be related to the central type of benzodiazepine receptors, whereas at least part of its effect on slow wave sleep could result from binding to the peripheral type of benzodiazepine receptors in the central nervous system, or from other mechanisms not directly connected to central receptors. It is true that we cannot exclude a participation of the central type of benzodiazepine receptors in the decrease of slow wave sleep induced by flunitrazepam, since this drug decreases the firing of raphe units, as does Ro 15-1788. Nevertheless this is not the main visible effect of flunitrazepam, the alteration of stage 4 induced by the two substances appearing to be very different.

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Note added in proof

Preliminary results from experiments in progress in our laboratory support this interpretation. Zopiclone is a non-benzodiazepine compound which binds to central type, but not to 'peripheral' type of benzodiazepine receptors (Blanchard, Boireau & Julou, communication at the 13th CINP Meeting, Jerusalem, June 20–25, 1982). In normal subject (n = 5), this drug does not decrease stage 4, but rather enhances it. This stage returns to control level in the post-drug night.

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