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Pharmacology, Biochemistry and Behavior 77 (2004) 77–83
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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The influence of midazolam on active avoidance retrieval and acquisition rate in rats

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Received 6 June 2003; received in revised form 29 September 2003; accepted 30 September 2003

Abstract

The purpose of the present study was to examine the influence of midazolam on the retrieval and acquisition rate of two-way active avoidance in rats. In the schedule 2×100 trials, the effects of midazolam (0.5–5.0 mg/kg), benzodiazepine binding site antagonist flumazenil (2.5–10.0 mg/kg), specific antagonist of $GABA_A$ receptor, bicuculline (0.5–4.0 mg/kg), and the blocker of $GABA_A$ receptor containing Cl^- channels, picrotoxin (1.0–5.0 mg/kg), on the second day retrieval of avoidance performance were investigated, as well as the influence of the used blockers of $GABA_A$ receptor function on midazolam effects. Furthermore, the effect of midazolam (1.0 mg/kg) on acquisition rate in the 5×50 schedule, as well as the effects of third day treatment changing in that paradigm, was examined. Throughout the study, drugs were given intraperitoneally, 30 min before testing. Midazolam at the dose of 1.0 mg/kg facilitated avoidance retrieval, whereas flumazenil and bicuculline did not significantly change behavior. Picrotoxin (5.0 mg/kg) diminished performance. All three kinds of blockers used abolished facilitatory action of midazolam, confirming GABAergic mediation of the effect of benzodiazepine. Midazolam (1.0 mg/kg) increased acquisition rate during five consecutive days relative to saline, but without significant effect on the first day acquisition. In the case of third day changing of treatments, the intersection of regression rate lines was detected. Results from active avoidance paradigm experimentally support the findings from human studies that in certain circumstances, benzodiazepines, potentiating GABAergic neurotransmission, could produce retrieval-enhancing effects in memory tasks.

Keywords: Benzodiazepines; Midazolam; Active avoidance; Retrieval; Acquisition rate; Rat

1. Introduction

It is well established that memory is composed of three stages: acquisition, consolidation, and retrieval (Abel and Lattal, 2001). Substantial evidence has been accumulated showing that modulation of GABAergic neurotransmission affects memory processes (Castellano et al., 1996; Izquierdo and Medina, 1997; McGaugh, 2000). It can be quite difficult to isolate experimentally the different stages of memory because experimental techniques potentially affect two or more stages of memory, depending on the time course of the manipulations (Abel and Lattal, 2001). Nevertheless, $GABA_A$ receptors were pointed to as down-regulators of memory formation (Izquierdo et al., 1992; McGaugh and Izquierdo, 2000). Notably, benzodiazepines, the most prescribed anxiolytics, acting by potentiation of GABA effects on $GABA_A$ receptors, cause anterograde amnesia, as shown in humans (Lister, 1985; Rodrigo and Lusiardo, 1988; Curran, 1991; Kain et al., 2000) as well as in animals (Thiebot, 1985; Venault et al., 1986; Izquierdo and Medina, 1997).

In regard to the retrograde memory effects, despite some authors' reservations (Cole, 1986), the results of most animal studies ruled out an action of benzodiazepines on retrieval in different memory tasks (Venault et al., 1986; Nabeshima et al., 1990; Brioni and Arolfo, 1992; Chapouthier and Venault, 2002). Similarly, the frequent con-

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doi:10.1016/j.pbb.2003.09.018

clusion from human studies is that benzodiazepines do not significantly influence the retrieval of information from memory (Ghoneim and Mewaldt, 1975; Lister, 1985), although retrieval impairment was also reported (Block and Berchou, 1984). Nevertheless, memory facilitation in humans is sometimes seen as benzodiazepines' effect as well (Hinrichs et al., 1984; Curran, 1991; File et al., 1999; Fillmore et al., 2001). Hinrichs et al. (1984) hypothesized that this phenomenon is not a true facilitation of retrieval processes, but could be the result of reduced interference from items presented after drug administration, as a paradoxical consequence of drug-induced anterograde amnesia. However, more specific facilitating effects on retrieval processes have also been proposed (File et al., 1999).

Two-way active avoidance task, the form of avoidance behaviour perhaps most extensively studied (Gray and Lalljee, 1974; Izquierdo and Cavalheiro, 1976; Weinberg and Levine, 1977; Brush, 1991), is considered to measure procedural memory and relies on both classical fear conditioning and instrumental aversive conditioning (Squire, 1992). However, behavioral studies with benzodiazepines using this model are few. Moreover, there are certain contradictions with data obtained in active avoidance procedures, which may, at least partly, rely on substantial differences in experimental conditions (Izquierdo and Cavalheiro, 1976). Namely, these drugs facilitated early acquisition of active avoidance responses (Fernandez-Teruel et al., 1991a,b; Escorihuela et al., 1993), whereas diazepam, administered to rats before each daily training session for 5 days, decreased acquisition rate (Celik et al., 1999). On the other hand, diazepam improved performance (i.e., retrieval) of poorly learning male mice in the active avoidance paradigm (Oka et al., 1980). In one-way active avoidance paradigm in mice, diazepam at the dose of 1.0 mg/kg improved first-day acquisition of females but impaired acquisition of males (Podhorna et al., 2002). In aged mice, flumazenil (40 mg/kg) improved acquisition and retention performance of an active avoidance task (Lal and Forster, 1990).

The present study tested the hypothesis that the effects of benzodiazepines on memory acquisition and retrieval could be differentiated in active avoidance paradigm. Firstly, we examined the effects of midazolam, a widely used benzodiazepine, with especially salient application in preanesthetic medication and anesthesia (Wagner et al., 1997), on the second-day retrieval of active avoidance responses in rats. Furthermore, the influences of the benzodiazepine binding site antagonist flumazenil, the specific antagonist of $GABA_A$ receptor, bicuculline, and the blocker of $GABA_A$ receptor containing Cl^- channels, picrotoxin, on the effects of midazolam, were examined. Finally, the effect of midazolam on acquisition rate (Celik et al., 1999) in the 5×50 schedule, as well as the influence of third day treatment changing, was examined.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Wistar rats ($N = 300$; Military Farm, Belgrade, Serbia and Montenegro), weighing 180–220 g. All procedures in the study confirmed to EEC Directive 86/609 and were approved by the Ethical Committee on Animal Experimentation of the Medical Faculty in Belgrade. The rats were housed in transparent plastic cages, six animals per cage, and had free access to pelleted food and tap water before and after drug administration. The animals were placed in a room kept at a temperature of 22 ± 1 °C, relative humidity 40–70%, and 12/12-h light/dark period (lights on at 0630 h). All handling and testing took place during the light portion of the cycle. The animals were used only once throughout the study, with 10 rats in a treatment group.

2.2. Drugs

The substances used were midazolam, flumazenil (Hoffmann La Roche, Basel, Switzerland), bicuculline, and picrotoxin (Sigma Chemicals, St. Louis, MO, USA). All the substances were dissolved in saline solution (for bicuculline, pH adjusted to 2.5; for flumazenil, with the aid of Tween 80) and injected intraperitoneally in a volume of 1 ml/kg.

2.3. Two-way active avoidance paradigm

The active avoidance test was performed in automated two-way shuttle boxes and programming recording units (Campden Instruments, Sileby, UK). In the first part of the study, the active avoidance task was elaborated by 100-trial 2-day sessions (the first day values taken as the criterion for balanced assigning treatments on the next day). Animals were placed singly into the shuttle box and subjected to 100 avoidance trials/day. During the first 5 s of each trial, a sound signal was presented (broadband noise of 69 dB), allowing the animal to avoid shocks by moving to the other compartment. If the animal did not respond within this period, foot shock of 0.3 mA (7-s duration) was applied. Moving to the other compartment during the signal, before the shock, was considered a correct avoidance. If the rat changed compartments during the shock, that was discontinued (escape response). If no response occurred during the shock period, the shock was terminated after 7 s and this was considered a failure. The animal could move freely in the apparatus between trials (18-s intertrial intervals), intertrial crossings (ITCs) being automatically counted.

For assessment of the influence on retrieval (schedule 2×100 trials), midazolam (0.5, 1.0, 2.5, and 5.0 mg/kg), flumazenil (2.5, 5.0, and 10.0 mg/kg), bicuculline (0.5, 1.0, 2.0, and 4.0 mg/kg), and picrotoxin (1.0, 2.0, 4.0, and 5.0 mg/kg), as well as saline in control group, were given 30 min before retention session. Afterwards, in the same

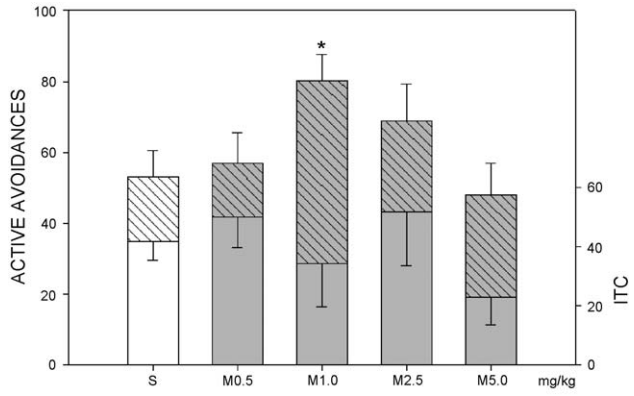

Fig. 1. The effects of midazolam (M: 0.5, 1.0, 2.5, and 5.0 mg/kg), on active avoidance retrieval (hatched bars) and ITCs (open bars) in rats. * $P < .05$ compared to saline group (S).

2 × 100 schedule, the capability of flumazenil, bicuculline, and picrotoxin to antagonize effects of midazolam was checked.

In the second part of the study, the influence of retrieval-enhancing dose of midazolam (1.0 mg/kg) on the acquisition rate was checked in a procedure lasting five consecutive days, with 50 trials per day (the same trial conditions as in the first part). Two groups of rats were injected with the drug and saline, respectively, 30 min before each of five consecutive sessions. Possible state dependency of the obtained behavioral effects of midazolam was assessed in the separate series of the 5 × 50 trials. On the first 2 days, half of the rats were injected with midazolam (1.0 mg/kg), whereas the second half received saline. On the third day, treatments were replaced. On the remaining 2 days (the fourth and the fifth), the rats received treatments as on the first and the second day.

2.4. Statistical analysis

All numerical data presented in the figures were given as the mean ± S.E.M. An alpha level of .05 was used for all statistical tests. In the first part of the study, in the model 2 × 100 trials, statistical significance of avoidance differences was determined using one-way ANOVA. If the ANOVA was significant, each treatment condition was compared with the vehicle control by a Dunnett's test, whereas for post hoc estimation of antagonism, where appropriate, Tukey's test was used. Because individual data concerning ITCs did not follow a normal distribution, these data were analyzed by a nonparametric test, the Kruskal–Wallis ANOVA on ranks. For assessment of the influence of midazolam on acquisition of avoidance behavior (5 × 50 paradigm), the approach given by Celik et al. (1999) was applied, namely, mean acquisitions (in a session) for five consecutive days were expressed as regression lines. The significance of difference between regression coefficients for midazolam and saline groups was assessed using Student's *t* test. For the assessment of difference between two groups' avoidance values on an observed day, where appropriate, Student's *t* test for independent samples was used. Statistical analyses were performed with commercial statistical software for PC, Stat for Windows R. 5.0.

3. Results

3.1. Two-way active avoidance task in the 2×100 schedule

First day average of avoidance responses throughout the groups selected from untreated animals (training sessions) was 25.8–26.9, whereas ITC was 27.7–44.9 (data not shown).

Treatment with midazolam significantly affected retrieval of avoidance responses on the second day of shuttle box testing [$F(4,45) = 2.88$, $P < .05$], without causing major variations in locomotor activity assessed through ITC [$H(4) = 6.21$, $P = .184$] (Fig 1). Dunnett's test indicated that the avoidance-facilitatory dose of midazolam was 1.0 mg/kg. On the other hand, flumazenil and bicuculline (Fig. 2), in the range of doses tested, did not modify registered parameters of behavioral activity (for flumazenil [$F(3,36) = 2.06$, $P = .123$], [$H(3) = 3.38$, $P = .337$] and for bicuculline: [$F(4,45) = 2.14$, $P = .096$], [$H(4) = 2.93$, $P = .570$]). However, the highest tested dose of picrotoxin (5.0 mg/kg) significantly reduced number of correct avoidances [$F(4,45) = 2.96$, $P < .05$], but did not affect locomotor activity [$H(4) = 7.20$, $P = .126$] (Fig. 2).

Coadministration of flumazenil, bicuculline, or picrotoxin (Fig 3) significantly affected avoidance activity obtained with 1.0 mg/kg midazolam ([$F(3,36) = 3.51$, $P < .05$]; [$F(3,36) = 3.63$, $P < .05$]; [$F(3,36) = 3.04$, $P < .05$], respectively), but not ITC values ([$H(3) = 4.66$, $P = .198$]; [$H(3) = 2.04$, $P = .563$]; [$H(3) = 6.04$, $P = .110$, respectively]). Tukey's test revealed that flumazenil at 2.5, 5.0, and 10.0 mg/kg, bicuculline at 1.0 mg/kg and 2.0 mg/kg, and picrotoxin at 4.0 mg/kg completely antagonized retrieval enhancement induced by midazolam.

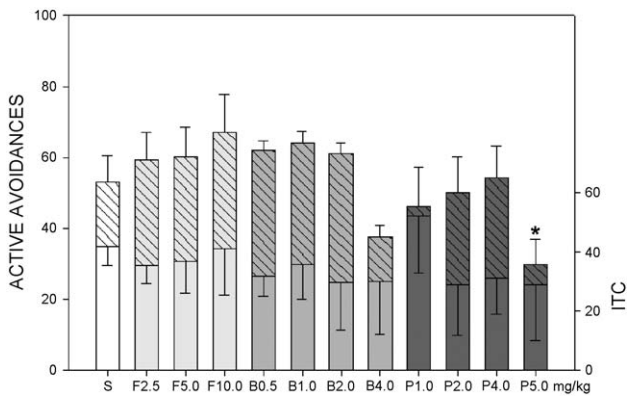

Fig. 2. The effects of flumazenil (F: 2.5, 5.0, and 10.0 mg/kg), bicuculline (B: 0.5, 1.0, 2.0, and 4.0 mg/kg), and picrotoxin (P: 1.0, 2.0, 4.0, and 5.0 mg/kg) on active avoidance retrieval (hatched bars) and ITCs (open bars) in rats. * $P < .05$ compared to saline group (S).

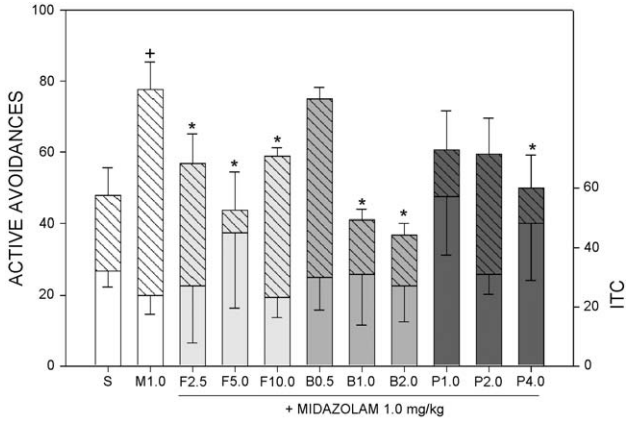

Fig. 3. The influence of flumazenil (F), bicuculline (B), and picrotoxin (P) on the effects of midazolam 1.0 mg/kg on active avoidance retrieval (hatched bars) and ITCs (open bars) in rats. $^{*}P<.05$ compared to midazolam 1.0 mg/kg; $^{+}P<.05$ compared to saline group (S).

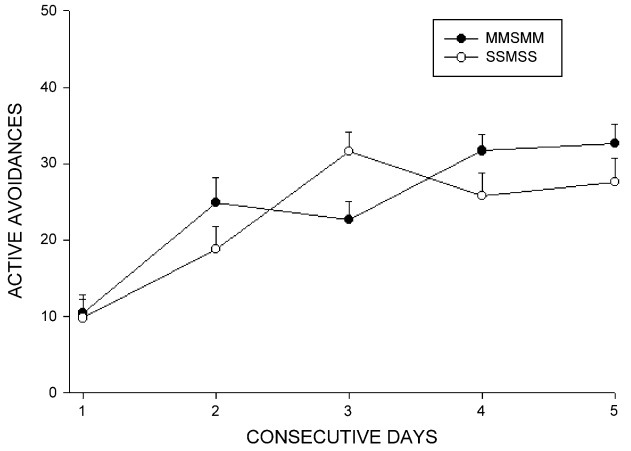

Fig. 5. The influence of third day treatment change on the active avoidance performance in rats treated with midazolam (M) 1.0 mg/kg or saline (S) before the other four sessions in the 5 × 50 paradigm.

3.2. Two-way active avoidance task in the 5×50 schedule

At the dose facilitating retrieval of avoidance memory, midazolam significantly ($P<.05$, comparison of regression coefficients by Student's t test) and progressively increased acquisition rate during 5 days training, compared to the saline group (Fig. 4). Student's t test for independent samples revealed that a significant difference between the respective two groups' avoidance values is not achieved before the third day. In the experiment with third day change of treatment (Fig. 5), the group previously injected with midazolam performed poorer after third day saline injection, whereas midazolam-treated rats continued enhancement of performance acquired under saline. On the fourth and fifth day, resumed administration of the first 2-day treatments resulted in recovering of performance-enhancing trend in the midazolam group, whereas in the second group, rats treated with midazolam only once were not able to attain third day level of avoidance again.

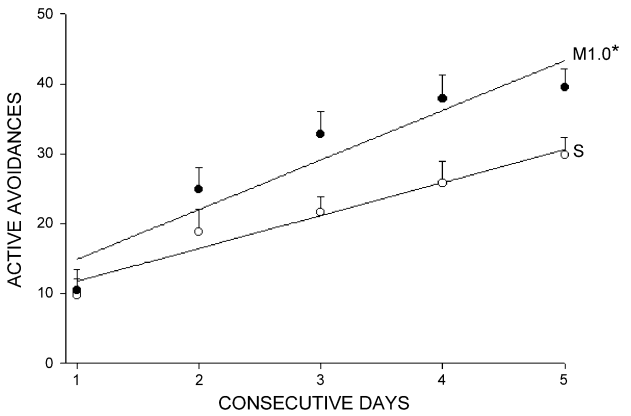

Fig. 4. The effects of midazolam (M) 1.0 mg/kg on active avoidance acquisition in rats during five consecutive days (saline = ○; midazolam = ●). $^{*}P<.05$ compared to saline (S) group. Student's t test for independent samples revealed that a significant difference between respective two groups' avoidance values exists on Days 3, 4, and 5.

4. Discussion

The results of the present study show that midazolam at the dose of 1.0 mg/kg, in a manner resembling an inverted U shape, facilitates retrieval of memory task imposed to rats in two-way 2 × 100 trials active avoidance paradigm. Of the $GABA_A$ antagonists used, only picrotoxin (5.0 mg/kg) engendered a significant effect, decreasing the second day retrieval. However, that dose of picrotoxin could have elicited convulsive activity in rats as well (Paul et al., 2001). Also, bicuculline at the dose of 4.0 mg/kg could have induced convulsions (Drugan et al., 1985), but the avoidance-diminishing effect of that dose did not reach significance in our experiment. Although clear signs of convulsive activity were not observed in our study, the potentially incapacitating doses of picrotoxin (5.0 mg/kg) and bicuculline (4.0 mg/kg) were not used further.

The effect of midazolam was fully antagonized by the antagonist of benzodiazepine binding site, flumazenil, as well as by the blocker of $GABA_A$ receptor containing Cl^- channels, picrotoxin, and specific antagonist of $GABA_A$ receptor, bicuculline, all three kinds of blockers being used in per se ineffective doses. Hence, the effect of midazolam appears to be exerted through potentiation of GABAergic neurotransmission. These results correspond to the antagonizing effects of the same blockers of $GABA_A$ receptor function on acquisition impairing effects of benzodiazepines in extensively studied passive avoidance paradigm (Tohyama et al., 1991; Dickinson-Anson et al., 1993; Nakagawa et al., 1993; Venault et al., 1986). Interestingly, it has previously been shown that diazepam antagonizes performance deficit exerted by picrotoxin (1.5 mg/kg) in a different active avoidance

paradigm, in rats learned to the criterion of 50% of correct responses (Davis, 1982).

The improvement of the performance in the retention session exerted by midazolam (1.0 mg/kg) could not be clearly associated with the changes in motor activity, since there were no significant variations in ITC measured during the session. However, it should be noted that crossings during the habituation period (not used in our design) better correspond to spontaneous locomotor activity, whereas ITC could be seen as a form of "pseudoavoidance" or conditioned activity (cf. Satorra-Marin et al., 2001; Torras-Garcia et al., 2003).

Another possible explanation of the effect relates to the anxiolytic activity of the drug. Benzodiazepines reduce conditioned fear, which interferes with the acquisition of the active coping behaviour, and hence enhances acquisition (Fernandez-Teruel et al., 1991a,b; Escorihuela et al., 1993). However, it should be noted that the score of early trials in the acquisition session, but not the retention score, was validated as an index of anxiety in two-way active avoidance procedure (Fernandez-Teruel et al., 1991b). Moreover, pharmacologically untreated Wistar rats selected as "non-anxious" according to their behavior in an elevated plus maze exhibited worse retention scores in two-way active avoidance task compared to "normal" rats (Ribeiro et al., 1999). However, in interpreting these findings, one could not exclude the possibility that various behavioral procedures, encompassing different aspects of anxiety, are differently modulated by benzodiazepines (cf. Dal-Col et al., 2003). Nevertheless, the predominance of the anxiolytic action of midazolam in the mechanism of retrieval-facilitating effect could not be concluded from the data obtained in our study.

On the other hand, retrograde memory facilitation is benzodiazepines' effect sometimes seen in human investigations (Hinrichs et al., 1984; Curran, 1991; File et al., 1999; Fillmore et al., 2001). Hinrichs et al. (1984) hypothesized that this phenomenon is not a true facilitation of retrieval processes, but could be the result of reduced interference from items presented after drug administration, as a paradoxical consequence of drug-induced anterograde amnesia. However, more specific facilitating effects on retrieval processes have also been proposed (File et al., 1999). Hence, the importance of the amnesic effect of benzodiazepines on performance in the retrieval session of our experiment should be discussed. In order to solve the task, the rat has to leave one compartment where it is about to receive a shock and go into the second half where it could have just been submitted to the shock. In other words, a behavioral conflict tendency not to reenter the previous shock compartment (two-way paradigm) develops during the test. As Guillazo-Blanch et al. (2002) argued in their paper regarding effects of lesions of fimbria-fornix on two way active avoidance, the untreated rats, as expected, remembered that they were shocked before in the other compartment. Hence, they hesitate on every trial, and they must learn to suppress this memory and simply run after the conditioning stimulus. By contrast, analogously to the lesion of the main subcortical input to the hippocampus (Guillazo-Blanch et al., 2002), midazolam-treated animals, due to the amnesic effect of the drug, could have reduced recent memory and therefore lack the described conflict element. This hypothetical effect, supposedly in addition to the direct anxiolytic activity, could lead to the facilitation of retrieval in the active avoidance paradigm.

The relative specificity of the facilitatory effect of midazolam at the dose of 1.0 mg/kg on avoidance retrieval was attempted to be checked experimentally using the 5×50 trials procedure, with and without third day treatment changing. Although benzodiazepines are known as drugs exerting state-dependent learning (Nakagawa et al., 1993; Nakagawa and Iwasaki, 1995), the clear intersection of performance rate lines after two treatment changes in the 5×50 paradigm points to the specificity of midazolam activity at the selected dose in active avoidance task. In contrast to the effects of diazepam in a similar model (Celik et al., 1999), midazolam engendered enhanced acquisition rate in relation to the saline group, determined by the significance of difference between regression coefficients describing corresponding regression lines. This discrepancy could be, at least partly, related to the salient distinction between these two benzodiazepines' pharmacokinetics parameters (Jack et al., 1983). Obviously, in contrast to the present midazolam study, the accumulation of the parent substance and metabolites could not be avoided in the case of diazepam administration during several consecutive days (Celik et al., 1999), thus impeding interpretation of results. Hence, we presume that the task performance at a given day could result from the effects on acquisition at earlier sessions and the action on retrieval at the observed session. It should be noted that the lack of acquisition facilitation at the first session, otherwise seen with benzodiazepines (Fernandez-Teruel et al., 1991a,b; Escorihuela et al., 1993), could be explained by the higher number of trials in our design (50 trials), comprising not only early acquisition, validated as an animal model of anxiety (up to 40 trials) (Fernandez-Teruel et al., 1991b). Consistently, in the recent study with Wistar rats divided into two subgroups with either "low" or "high" anxiety based on their elevated plus-maze behavior (Ho et al., 2002), the avoidance number in low-anxiety rats, compared with high-anxiety ones, was higher during the first 10 trials, but not during the other 10 trials of the 20-trial acquisition session. Consequently, we could argue that the 5×50 design was elaborated with the dose of midazolam not significantly affecting acquisition per se, so that the obtained enhancement of acquisition rate would better correspond to the previously found facilitatory effect of midazolam, at the 1.0 mg/kg dose, on retrieval.

Finally, we discuss the seemingly inverted U-shape effect of midazolam on retrieval in the 2×100 paradigm. The lack of clear dose–response or even U-shaped effects of the

drugs, which exert their effects through some components of $GABA_A$–benzodiazepine receptor complex, were shown in several experimental models (File et al., 1982; Lamb and Griffiths, 1987; Farkas and Crowe, 2000; Munzar et al., 2001; Obradović et al., 2002, 2003). The findings imply that a distinct level of exogenous benzodiazepine modulation on effects exerted by inherent content of ligands for benzodiazepine binding sites is needed for realizing the significant influence on measured parameters. Furthermore, in the previously mentioned investigation in humans, File et al. (1999) obtained improved retrieval after the middle dose (1 mg) of three doses of lorazepam used, the effect also resembling an inverted U shape. The authors suggested that the lack of the improvement of retrieval at higher doses of benzodiazepines could be explained by sedative and/or amnesic effects of these doses, which may have masking effects (File et al., 1999).

Although direct extrapolations to cognitive changes in humans are not possible, the fact that midazolam at the dose (1 mg/kg) inducing acquisition impairment in passive avoidance paradigm in rats (Quevedo et al., 2002) could also, under certain circumstances, improve retrieval would be worthy of clinical notice. It could be hypothesized that such an effect of midazolam, administered as a preanesthetic medication, where amnesia is welcomed, would tend to improve recall of potentially unpleasant procedures preceding medication. Taking into account the difference in dose dependence of these two effects, it seems reasonable to suggest that in the settings of preanesthetic medication, the first effective amnesic dose of midazolam should not be regarded as an optimal one. However, as levels of anxiety and arousal, modified by benzodiazepines, also influence cognitive functioning (Curran, 1991), the actual memory effects of the selected dose could decisively depend on the individual's initial (predrug) level of anxiety.

In conclusion, our results from active avoidance paradigm experimentally support the findings from human studies that in certain circumstances, benzodiazepines, potentiating GABAergic neurotransmission, could produce retrieval-enhancing effects in memory tasks.

Acknowledgements

This work was supported by Scientific Fund of Farman Engineering.

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