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Influence of Flumazenil on the Learning-Enhancing Effect of Ambocarb in Rats

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TITIEVSKY, A. V., O. RAILOMA, S. A. NIEMINEN AND M. M. AIRAKSINEN. *Influence of flumazenil on the learning-enhancing effect of ambocarb in rats.* PHARMACOL BIOCHEM BEHAV 47(3) 681-688, 1994.—The effects of flumazenil (Ro 15-1788) and a new β-carboline, ambocarb (AMB), on learning were investigated using the multichoice maze. The drugs, administered either alone or simultaneously, were injected once a day before training for eight days. AMB, administered alone, improved the performance and decreased the working errors, whilst flumazenil had no effect on performance during its sole administration but weakly prevented the learning-improving effect of AMB. More significantly, flumazenil antagonized the motor activity depressed by AMB. In the study ex vivo, flumazenil decreased and AMB increased the apparent affinity of [^{3}H]flunitrazepam to the central benzodiazepine receptors. Flumazenil reversed the action of AMB on the central benzodiazepine receptors, but failed to reduce significantly the modulative effects of AMB on [^{3}H]muscimol and [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) binding. These data indicate that flumazenil, due to its action on the central benzodiazepine receptors, more effectively reverses the inhibition of motor activity than the performance-improving effect of AMB.

Flumazenil β-Carbolines Benzodiazepine receptor Learning Spatial discrimination test

BENZODIAZEPINES have long been known to impair many forms of learning in many species, including man (41). Since benzodiazepines induce anterograde amnesia and sedation, some of the inverse agonists of the central benzodiazepine receptors (CBRs) with a β-carboline structure enhance memory and/or increase vigilance (28,34,43). Higher doses of β-carbolines have an opposite effect, impairing memory in these tasks which assess a function of acquisition (12).

The effects of β-carbolines on learning and memory are suppressed by administration of flumazenil (Ro 15-1788), which, with high affinity and great specificity, dose-dependently prevents all effects that CBR agonists and inverse agonists can produce via the CBR (16,20,34). Some investigators have reported that flumazenil also has an intrinsic positive effect on cognition (21,24). Thus, it could be proposed that the described effects on learning are mediated by the CBR and flumazenil merely attenuates the sensitivity to γ-aminobutyric acid$_A$ (GABA$_A$) agonists. However, not only the activation of CBRs, but also the modulation of peripheral benzodiazepine receptors (PBRs) leads to the enhancement of learning. Though PBRs are physically and pharmacologically distinct from CBRs (2,3,4,37), CBRs and PBRs appear to mediate some common behavioral actions on performance and anxiety (9,18). These effects are antagonized by the specific PBR antagonist PK 11195, but not by flumazenil (9). Nevertheless, several studies have suggested that effects of Ro 5-4864 may be the result of interactions with the GABA/benzodiazepine complex (4,13).

Therefore, since certain CBR and PBR ligands, through an interaction with the same macromolecular complex, can differently affect learning and memory, the present study investigated the effects of a novel β-carboline, ambocarb (AMB), in an appetitive discrimination task maze combined with studies on CBRs, PBRs, and muscimol binding sites of the GABA/benzodiazepine complex. Since one may presume that the effect of AMB on the anxiety level in animals reflects inherent properties as an agonist of CBR (23), the drug was administered both alone and in the presence of the specific antagonist of the CBR, flumazenil.

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METHODS

Animals and General Procedures

Fifty-four male Han : Wistar rats (National Laboratory Animal Center, Kuopio, Finland) aged 12–14 weeks at the beginning were used in four series of experiments. The rats were housed two per steel cage. Pelleted food (SDS, UK) and tap water were available ad lib. Animals were maintained on a 12-h light period (lights on 0700–1900). The temperature of the animal room was 21 ± 1°C and the relative humidity 50–60%. Four days before the training the animals were transferred to the testing laboratory and placed on a food-deprivation schedule so that they weighed approximately 85% of their free-feeding weight during the training period. The weight of rats decreased equivalently in all groups observed. For avoidance of inadvertent alterations within the GABA/benzodiazepine (BDZ) chlorine ionophor receptor complex, the animals were habituated to handling four days prior to the maze trials. At the same time, the rats were injected with sterile water (1 ml/kg of body weight) once a day.

Drugs

AMB, 4′-oxo-(2′,2′-dimethyl)-3,4-tetramethylene harman, was synthesized in the Department of Pharmacology of the Donetsk Medical Institute (Ukraine) and in the Department of Coal Chemistry of the Institute of Physical and Organic Chemistry (Donetsk, Ukraine). It was dissolved in sterile water. Flumazenil (Ro 15-1788), provided by Hoffmann-La Roche (Basel, Switzerland), was suspended in sterile water with a drop of Tween 80.

Test Apparatus

The six-unit T-maze was modified from an appetitive spatial discrimination maze task. It consists of six 50 × 40-cm units with 35-cm-high walls, separated from a start box and a goal box (25 × 25 × 20 cm) by a guillotine door (Fig. 1). The goal box was equipped with pelleted (45 mg) prize food (Campden Instruments Ltd, Loughborough, Leics, UK). The series of three units were connected with a passage as marked at Fig. 1. Each unit contained a T-wall, behind which a clear Plexiglas barrier was placed randomly on the left or right side. The correct path was always indicated with white labels on the back walls of the units, and incorrect choices were indicated with black labels (Fig. 1). The rats were trained to discriminate the correct (white) colour. The path was changed randomly after each training trial, and it was identical for all the animals trained on that day. The maze was dimly illuminated with indirect light and observed by a video camera hooked 2.5 m above the maze.

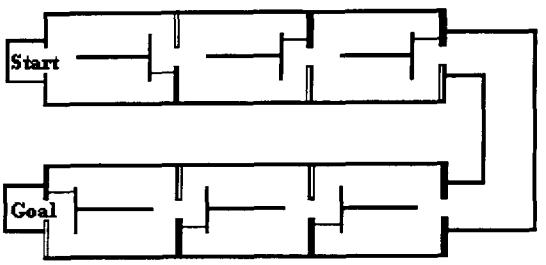

FIG. 1. Apparatus used for the testing of learning. The six-unit T-maze consists of six 50 × 40-cm units with 35-cm-high walls, separated from a start box and a goal box (25 × 25 × 20 cm each) by a guillotine door. The two series of three working units were connected with a U-shaped passage. Each unit (except start and goal boxes) contained a T-wall, behind which a clear Plexiglas barrier was placed on the left or right side. The correct alley was always indicated with white labels on the back walls of the units, and incorrect choices were indicated with black labels. The barriers were changed randomly after each training trial, and were identical for all the animals trained on that day. The rats were trained to discriminate the correct (white) colour and were allowed to find a path to the goal box for up to 10 min. The experimental procedure is described in Methods.

Procedure

Once a day during the two days before the training trials, all the rats in pairs were allowed to become familiar with the final doorway to the goal box equipped with prize food. During the training a rat was placed into the start box, the door was opened, and the rat was allowed to find a path to the goal box for up to 10 min. AMB was injected daily (3.0 mg/kg, IP) 30–45 min before the training trials. The dose and the time of administration of AMB were set from our initial studies, which showed that AMB at this dose has higher effect on retention performance (Airaksinen et al., submitted) and after 30 min of IP injection reaches the peak of concentration in the rat blood. Flumazenil was administered SC at a dose of 3 mg/kg 20 min before the training trials. The same dose and the time of administration of flumazenil were used in the previous studies which reported the performance-enhancing effect of flumazenil (21,24,32). The drugs were administered in the injection volume of 1 ml/kg. Control injections of sterile water were administered SC for AMB-treated and IP for flumazenil-treated groups. In the experiment with AMB and flumazenil, the drugs were injected at the same time and doses for comparison with administration of drugs alone. Animals in the control group were injected with sterile water (1 ml/kg) 30 min and 20 min before training IP and SC, respectively. The correct and error choices and the retracings and the time used until the animal reached the goal box were monitored. The rat was allowed to eat during 2 min in the goal box before it was removed into the home cage. The intertrial interval was 24 h, and the trials were repeated at eight days starting approximately at 1000.

Ligand Binding Assay.

Benzodiazepine binding assay. The animals were sacrificed by decapitation 1 h after the end of the last trial session, and the cerebral cortex or the whole brain were quickly dissected on ice and stored overnight at −20°C.

The synaptosomal membranes were prepared as previously described (36). In brief, tissue material was homogenized with a glass-Teflon homogenizer in 32 volumes (w/v) of ice-cold tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (50 mM, pH 7.4). The homogenates were centrifuged at 48 000 × *g* for 15 min and the resulting pellets washed twice by centrifugation (48 000 × *g* for 15 min) before binding experiments. Finally, the pellet was diluted to the approximate protein concentration of 0.15 mg per probe, measured by the method of Lowry et al. (26). Binding of [^{3}H]flunitrazepam (0.125–8 nM, sp act 79 Ci/mmol; Amersham, UK) and [^{3}H]Ro 5-4864 (0.5–16 nM, sp act 86.3 Ci/mmol; New England Nuclear, Boston) were carried out in a total incubation volume of 250 μl. Non-specific binding was determined in the presence of flunitrazepam (10 μM) or Ro 5-4864 (10 μM), respectively.

After 60 min incubation (in duplicate test tubes on crushed ice) all the reactions were stopped by rapid filtration over Whatman GF/B filters. The filters were washed three times with 4 ml of ice-cold Tris–HCl buffer. Filters were allowed to

solubilize in 5 ml of American Chemical Society scintillation liquid for 24 h before the radioactivity was measured.

[^{3}H]Muscimol binding assay. Brains were rapidly removed and placed in beakers containing ice-cold 50 mM Tris-HCl buffer (pH 7.4). Brain regions were dissected and weighed. Membranes for assays were prepared as described previously (40). Tissues were homogenized in 32 volumes 50-mM Tris-HCl buffer with a glass-Teflon homogenizer. The homogenates were then centrifuged at 40 000 × *g* for 20 min (4°C). The pellet was washed four times by suspension in Tris-HCl buffer and centrifugation, and then frozen at −20°C.

After thawing, the pellets were resuspended in 50 mM Tris-citrate buffer (pH 7.4) and centrifuged as before. A final wash and resuspension was performed in 20 volumes 50-mM Tris-citrate buffer (pH 7.4). Binding of [^{3}H]muscimol (using concentration 1-64 nM, sp act 25 Ci/mmol; Amersham) was determined in a final volume 500 μl (final protein concentration approximately 0.2 mg). After 30 min of incubation on ice the reactions were stopped by rapid filtration through the GF/B Whatman filters. The filters were washed three times with 4 ml of ice-cold Tris-citrate buffer. Nonspecific binding was determined using 10 μM GABA.

[^{35}S]TBPS binding assay. Cerebral cortical membranes for these experiments were prepared by four-times washing as described in the [^{3}H]muscimol binding section above, frozen at −20°C, and stored at least 24 h before binding assay.

After thawing the pellets were resuspended in 50 mM Tris-citrate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7.5) and centrifuged at 40 000 × *g* for 20 min (4°C). The washing procedure was repeated once. A final resuspension was performed in 20 volumes of the same buffer. [^{35}S]TBPS binding was determined using a modification of the method of (39) as described by Havoundjian et al. (17) with reducing the volume of incubation. Incubations consisted of 50 μl [^{35}S]-TBPS (sp act 118-85 Ci/mmol, New England Nuclear), 150 μl tissue homogenate (approximately 0.50 mg protein), and drugs or buffer to a final volume of 0.25 ml. Incubation at 25°C was initiated by addition of radioligand and terminated after 90 min by filtration through Whatman GF/B glass fiber filters with three 5-ml washes with Tris-citrate buffer (pH 7.5) maintained at room temperature. Nonspecific binding was determined using 20 μM picrotoxin (Sigma Chemical Co., St. Louis) and was usually <20% of total binding. After filtration all filters were placed into the 5 ml of American Chemical Society scintillation liquid for 24 h before radioactivity was measured.

Protein concentration was measured in all experiments by the method of Lowry et al. (26).

Data Analysis

The statistical significance of differences was assessed with analysis of variance (ANOVA) (repeated measures for behavioral data [two-factor: one factor, trial; second factor, dose] and one-factor for binding studies). Mann-Whitney *U* test was used for post hoc comparisons. The criterion of statistical significance was $p < 0.05$. Statistical analysis was performed with StatView SE+ Software (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Behavioral Data

In the behavioral experiments animals received either sterile water, AMB alone, flumazenil alone, or AMB + flumazenil. Although there were no differences in the running time of rats (Fig. 2) from start to goal box between control group and drugs-alone groups, $F(3, 320) = 2.53$, $p > 0.05$, NS (repeated ANOVA), animals receiving simultaneous administration of AMB and Ro 15-1788 used less time for decision of the linear maze task than animals of the control group, $F(1, 184) = 5.4$, $p < 0.05$ (repeated ANOVA), or AMB-alone animals, $F(1, 144) = 6.54$, $p = 0.021$ (repeated ANOVA).

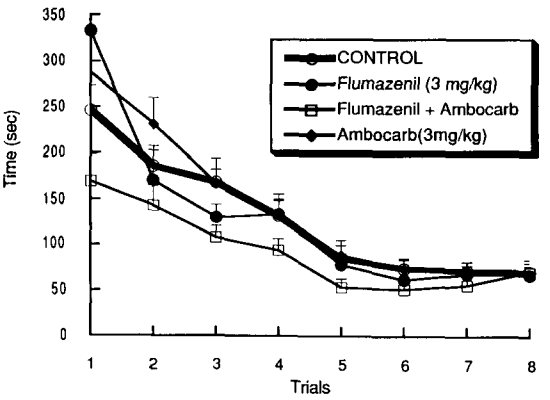

FIG. 2. The time (in seconds) spent in the multichoice maze until the animal reached the goal box. Flumazenil (3 mg/kg, SC) and AMB (3 mg/kg, IP) were injected daily 20 min and 30 min, respectively, before the trial. The drugs were injected either alone or jointly; the control group received sterile water (1 ml/kg) at the exact time as the drugs. Scores are mean ± SE; n = 13-14 per group.

AMB reduced the number of errors (i.e., the number of visits to the wrong-choice alleys) (Fig. 3) during the administration alone as compared to the control, $F(1, 312) = 18.26$, $p < 0.001$, whereas flumazenil alone showed no effect, $F(1, 296) = 0.71$, NS (repeated ANOVA), but weakly prevented the improving effect of AMB on performance in the last days of training, $F(1, 280) = 2.06$, NS (in comparison with the control).

Similar effects were observed when we registered not only errors of performance or learning but also the number of all mistakes, which includes the number of revisited alleys and boxes (Fig. 4). These errors, in our opinion, might particularly

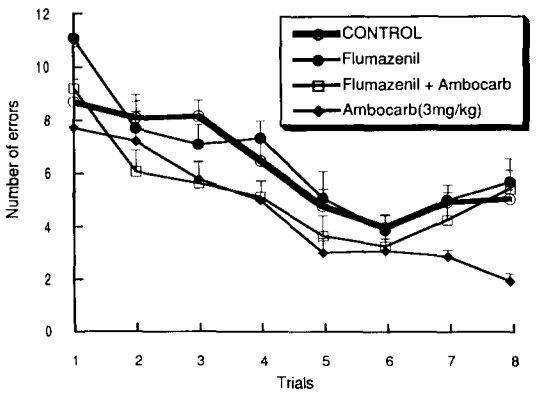

FIG. 3. The number of "working errors," which are the number of selections of the incorrect alleys after the daily treatment by drugs and solvent during the eight day trials. The rats were trained to discriminate the alleys marked by the light colour at the corner of the correct alleys. Scores are mean ± SE; n = 13-14 per group.

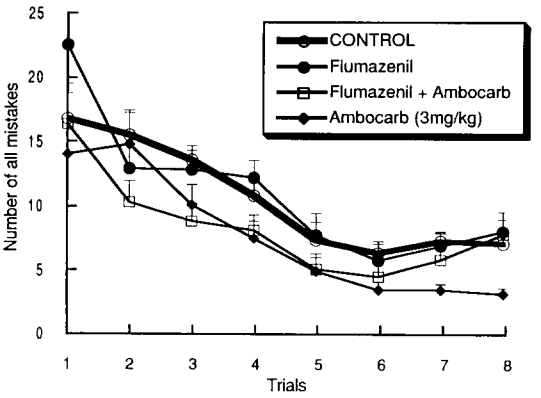

FIG. 4. The number of all errors, which includes also the number of retracings to the previous box. For more details, see text. Scores are mean ± SE; n = 13–14 per group.

reflect the level of fear in the animals. AMB improved the performance from the third day to the last day of trials as compared to the control, $F(1, 234) = 12.74$, $p = 0.001$ (repeated ANOVA). Flumazenil did not have any effect on performance and learning during its sole administration, $F(1, 296) = 0.52$, NS (flumazenil vs. controls, repeated ANOVA), nor did it prevent the improving effect of AMB up to the last two days of trials, $F(1, 216) = 0.13$, NS (flumazenil + AMB vs. AMB alone, repeated ANOVA); $F(1, 216) = 3.89$, $p = 0.05$ (flumazenil + AMB vs. control, repeated ANOVA).

AMB-treated rats significantly enhanced their retention performance in comparison to the control (Mann–Whitney U test: $U = 90.5$, $p = 0.01$ and $U = 40.5$, $p < 0.001$ at three and eight days of trials, respectively) as also estimated by the increase in the percent of correct choices up to 70% at the last day of trials (Fig. 5). The control and flumazenil-alone groups did not increase to the same extent their levels of performance. There was no difference between the control and Ro 15-1788 groups, $F(1, 296) = 0.67$, NS (repeated ANOVA). Flumazenil did not prevent the performance-enhancing effect of AMB during the entire period of the trials, $F(1, 280) = 5.24$, $p = 0.03$ (flumazenil + AMB vs. control, repeated ANOVA).

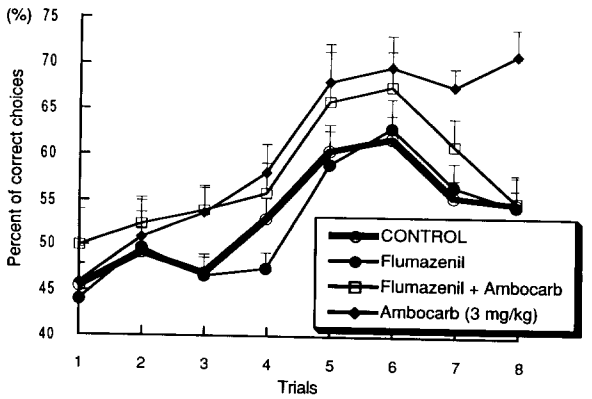

FIG. 5. The percent of correct choices, which was evaluated as a percent of choice of the correct alleys from all possible choices (correct, wrong, and retracings). Scores are mean ± SE; n = 13–14 per group.

Only on the last day of trials did we observe an antagonizing action of flumazenil on the effect of AMB (Mann–Whitney U test: $U = 30.5$, $p < 0.002$, AMB alone vs. AMB + flumazenil).

However, we registered a slight decrease in motor activity in the AMB-treated group (Fig. 6). This effect was significant from the latter part of the trial days, $F(1, 156) = 1.78$, NS (for first four days, repeated ANOVA); $F(1, 156) = 4.2$, $p < 0.05$ (for last four trials days as compared to the control, repeated ANOVA). On these last four days we observed a very significant effect of flumazenil to antagonize the activity depressed by AMB, $F(1, 156) = 8.07$, $p < 0.01$ (repeated ANOVA). However, rats treated with flumazenil alone showed no enhancement of their activity when compared to the control, $F(1, 148) = 1.47$, NS. To examine whether these effects reflect the specific actions of AMB and flumazenil on the GABA/benzodiazepine complex we investigated the action of these drugs ex vivo on the $GABA_A$ receptors.

Ligand Binding Data

In the cerebral cortices of vehicle-treated rats used in the maze experiments we observed an increase in the number of the CBRs, $F(1, 11) = 17.32$, $p = 0.002$ (one-way ANOVA) and $U = 0$, $p = 0.004$ (Mann–Whitney U test), but no increase in PBRs, $F(1, 11) = 0.37$, NS, as compared to the number of receptors in the cerebral cortex of handling-habituated rats, which did not participate in the experiment (Fig. 7, bottom panel). Learning did not change the apparent affinity either of CBRs or of PBRs in the rat cerebral cortex (Fig. 7, top panel). AMB alone did not change the number of CBRs, $F(1, 8) = 2.39$, NS (one-way ANOVA) and $p > 0.05$ (Mann–Whitney U test), whereas flumazenil alone reduced the number of CBR in the cerebral cortex, $F(1, 8) = 30.54$, $p < 0.001$ (one-way ANOVA). However, AMB significantly decreased (Fig. 7, bottom panel) the number of CBRs in the cerebral cortex of the handling-habituated rats not used in the maze procedure, $F(1, 9) = 8.18$, $p < 0.05$ (one-way ANOVA).

The drugs had no effect on the number of PBRs. Nevertheless, opposite effects of AMB and flumazenil were observed

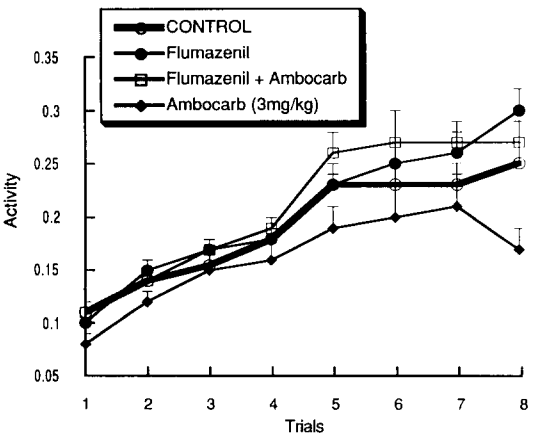

FIG. 6. The motor activity of rats in the control and in the drug-treated groups. The activity was expressed as number of crossings of the lines of choice (the conventional lines which delimit the boxes) per second during the acquisition of the maze task. Scores are mean ± SE; n = 13–14 per group.

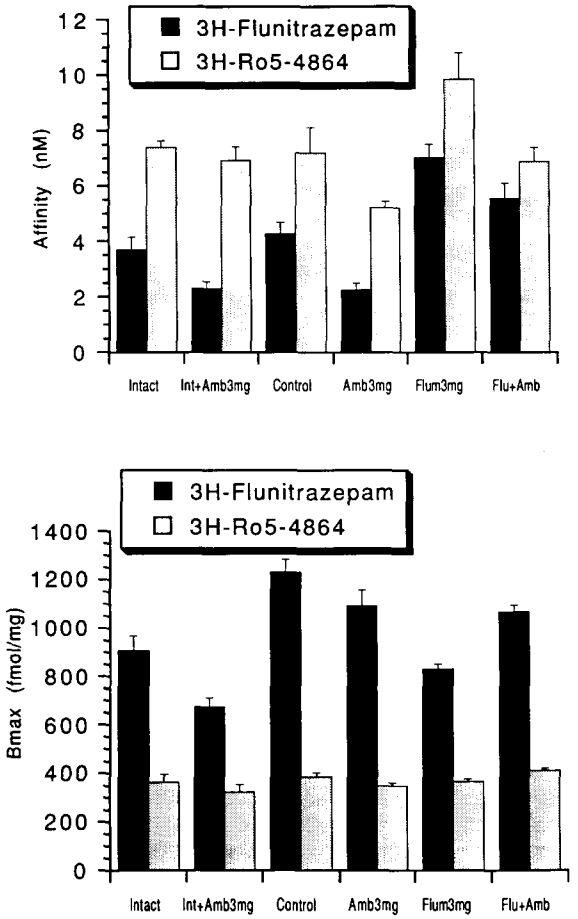

FIG. 7. The characteristics of the [^{3}H]flunitrazepam and [^{3}H]Ro 5-4864 binding to rat cerebral cortical membranes ex vivo after the end of the training trials. The brains were removed after 1 h from the last training in the maze. (Top) The apparent affinity (K_d; nM) of the ligands. (Bottom) The number of binding sites (B_{max}; fmol/mg protein) estimated from the Scatchard analysis. First and second groups of columns from the left represent the binding of ligands to the cortical membranes of handling-habituated and AMB-treated rats (3 mg/kg, IP), which were not used in any experiments. Scores are mean ± SE; n = 3-5 per each group.

in their actions on the apparent affinities of CBRs and PBRs (Fig. 7, top panel). AMB alone increased and flumazenil alone decreased the affinity of the CBRs, $F(1, 8) = 10.93$, $p = 0.013$ (AMB alone, one-way ANOVA) and $F(1, 8) = 5.99$, $p < 0.05$ (flumazenil alone, one-way ANOVA), but did not decrease the affinity of the PBRs, $F(1, 8) = 2.15$, NS (AMB alone, one-way ANOVA) and $F(1, 8) = 2.54$, NS (flumazenil alone, one-way ANOVA) in comparison with the vehicle-treated control. Flumazenil inhibited the affinity-increasing effect of AMB on CBRs, $F(1, 8) = 3.31$, NS (flumazenil + AMB vs. control, one-way ANOVA).

Neither control, AMB, nor flumazenil treatment had any direct influence on the number of [^{3}H]muscimol sites (Fig. 8) or [^{35}S]TBPS binding sites (Fig. 9) in the cerebral cortex, $F(3, 17) = 1.43$, NS and $F(3, 14) = 2.39$, NS for muscimol and TBPS binding sites, respectively (one-way ANOVA). However, in the cerebral cortices of vehicle-treated controls (Mann-Whitney U test: $U = 0$, $p = 0.004$, as compared to intact group) and AMB-treated rats (Mann-Whitney U test, $U = 0$, $p = 0.02$, as compared to vehicle-treated control) an increase in the apparent affinity of GABA binding sites was observed. Flumazenil alone did not change the apparent affinity of GABA binding sites ($p > 0.05$) in the cerebral cortex (Fig. 8) and did not reverse the affinity-increasing effect of AMB (Mann-Whitney U test: $p > 0.05$).

Learning increased the apparent affinity of [^{35}S]TBPS binding sites in the rat cerebral cortex (Fig. 9, top panel) as estimated for the control, AMB-treated, and flumazenil-treated groups, $F(3, 14) = 4.96$, $p < 0.05$ in comparison with the intact group. However, treatment by AMB increased the affinity of [^{35}S]TBPS binding sites (Fig. 9), $F(1, 7) = 8.62$, $p < 0.05$ (one-way ANOVA) and $U = 1$, $p < 0.05$ (Mann-Whitney U test), as compared to the control group (rats participated in the learning procedure). Flumazenil on its own had no effect on the affinity of [^{35}S]TBPS binding sites and weakly inhibited the action of AMB, $F(1, 7) = 4.12$, $p = 0.09$; $U = 3$, NS (Mann-Whitney U test) as compared to the control; $F(1, 7) = 0.45$, $p > 0.05$, NS (AMB-treated vs. AMB + flumazenil).

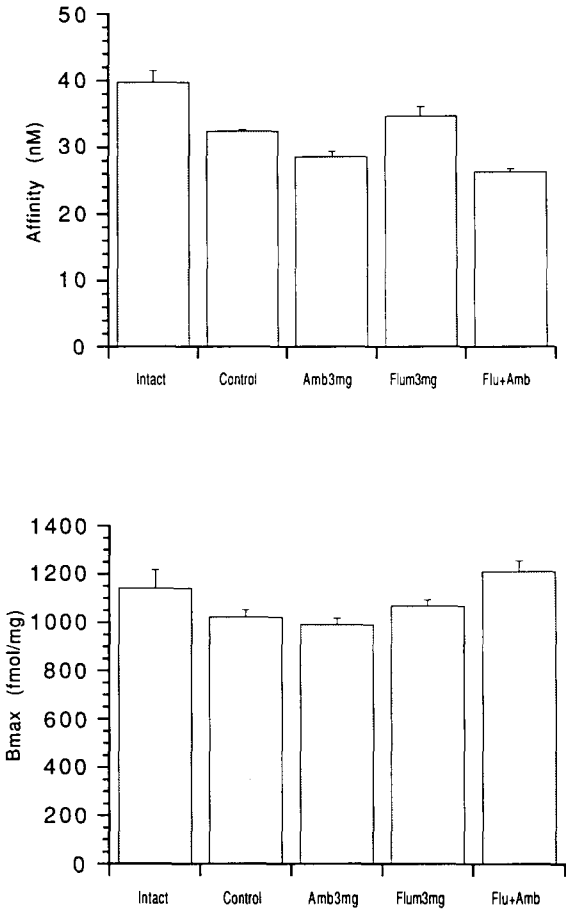

FIG. 8. The characteristics of the [^{3}H]muscimol binding to cerebral cortical membranes of rats 1 h after the end of trials and in the handling-habituated rats (the first columns from the left in each panel). The membranes were six-time washed to remove the endogenous GABA and the drugs as described in Methods. (Top) The apparent affinity (K_d). (Bottom) The number of binding sites (B_{max}) of [^{3}H]muscimol. Scores are mean ± SE; n = 3-5 per group.

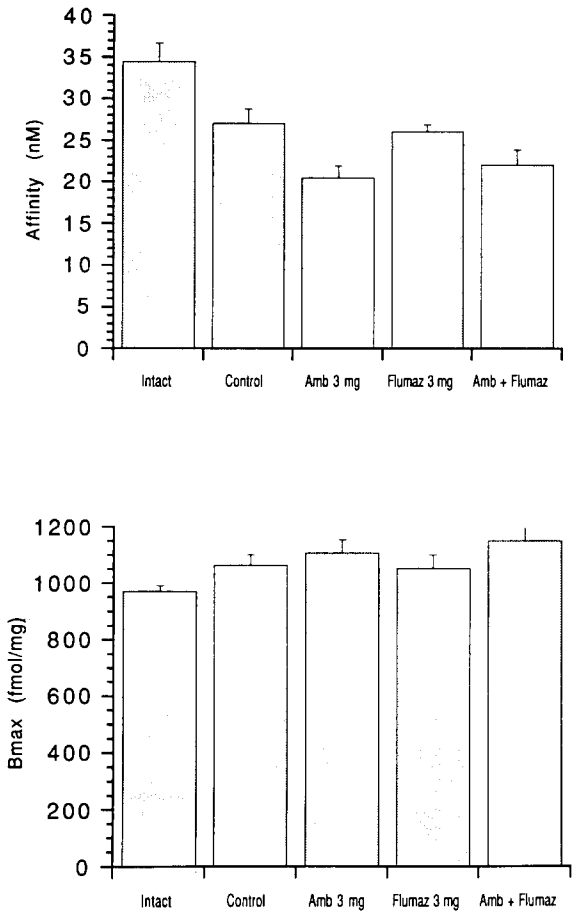

FIG. 9. The characteristics of the [^{35}S]TBPS binding to rat cerebral cortical membranes 1 h after the end of trials and in the handling-habituated rats (the first and the second groups of columns from the left in each panel). The membranes were extensively washed as described in Methods. (Top) The apparent affinity(K_d). (Bottom) The number of binding sites (B_{max}). Scores are mean ± SE; n = 4–5 per group.

DISCUSSION

Although flumazenil is described as a ligand of CBRs which prevents and reverses, dose-dependently, all the effects of BDZ agonists and inverse agonists on the CBRs (15), in our study we have shown only a weak reversing effect of flumazenil at a low dose on the performance-enhancing effect of AMB. The weak effect of flumazenil on learning might not be due simply to the low dose. The low, nonanxiogenic dose of flumazenil used in our experiments was selected from the investigations (24) which have shown an enhancement of retention in an active avoidance task in mice with pretraining flumazenil at similar, presumably purely antagonistic, doses. As has also been reported, this same low dose of flumazenil (5 mg/kg or less) given prior to training, has enhanced retention of habituation to a buzzer (21) and active (24) and inhibitory avoidance learning (21,32) in rats.

The question remains as to how AMB and flumazenil exert their effects on learning. β-Carbolines are known to bind with high affinity to the CBRs (1,5,14,35), and some have been proposed as endogenous ligands for these receptors (5,7,31). We have found (Airaksinen, unpublished results) that AMB posesses the properties of a positive modulator or partial agonist of CBR. But since the effects of AMB on learning were weakly suppressed by administration of flumazenil, a specific antagonist of CBRs, it may be assumed that these effects are only partly mediated by CBRs in this learning task. One possibility to explain this is that these drugs, and the possible endogenous modulatory mechanism that their actions suggest, affect only the acquisition of behaviors that involve or require a good perception of stress or anxiety.

AMB in the small doses possibly increased the level of arousal during the training session and seemed to decrease the anxiety induced by reinforcements as observed for reduction of the number of retracings (Fig. 4). This explanation is in agreement with the observation that, in rodents, arousal-enhancing drugs improve learning (27) and that several β-carbolines increase arousal (22,30). Similar effects were found in humans, where the β-carboline ZK 93426 improved performance in two cognitive tasks (10).

Another possibility would be to link the effects of AMB on learning to its anxiolytic effects. Thus, depending on the fearfulness of the condition, AMB could induce performance-enhancing effects. However, the performance-enhancing effects of an anxiogenic β-carboline, methyl β-carboline-3-carboxylate (BCCM), in mice were not seen in the dose range of the anxiogenic or convulsive effects of this drug (33).

Flumazenil did not reduce the performance-enhancing effect of AMB for most of the training. However, inhibition of AMB-induced improvement of performance in the latter phase of the trials indicated that flumazenil acts better when the information acquired during the trials can be transformed into long-term memory. Nevertheless, flumazenil effectively increased the animal motor activity inhibited by AMB during the whole training period. In rats, Ro 15-1788 has clearly prevented the anxiolytic properties of diazepam without abolishing the drug's amnestic effects (42), whilst in man flumazenil may antagonize the subjective and objective measures of sedation in diazepam-treated subjects without any effect on amnesia (19). Thus the learning effects of AMB may be mediated by a different (flumazenil-insensitive) subset of the modulation of acquisition and consolidation.

In our experiments flumazenil was ineffective on its own on the modulation of learning and acquisition. These data do not agree with the observations, which showed (24) that flumazenil could enhance learning and memory over a large range of doses (2.5 to 40 mg/kg). However, the fact that flumazenil was ineffective on its own at this low dose and only weakly inhibited the effect of AMB in this task which was very sensitive to AMB, but effectively affected the motor activity, suggests that a mechanism involving BDZ agonists acting at CBRs normally differentially modulates these behaviors.

Binding studies with flumazenil and AMB in the cerebral cortices of rats used in the experiment suggested that flumazenil is a very potent ligand for the CBRs [as is well known from the literature; for a review, see (11)] and appears to interact with the same number of sites of the CBRs as AMB. Although our binding studies indicate that flumazenil binds to the same receptor population of CBRs as AMB and reverses the AMB-mediated increasing of the apparent affinity of the CBRs, further studies indicated that the mode of interaction of flumazenil with the CBRs is different from that of AMB.

AMB increased the affinity of [^{3}H]muscimol binding sites, but flumazenil failed to inhibit this effect. Originally described as being essentially a pure antagonist ligand of the CBR (6,15),

flumazenil has been shown, however, to have intrinsic properties on the BDZ receptor, behaving either as a weak agonist (16) or as a weak inverse agonist (8,11). The direction of intrinsic efficacy (agonist to inverse agonist) varies according to the test situation and, frequently, to the dose of flumazenil administered. We propose that at this low dose and in this learning task flumazenil acts as an antagonist of CBRs and its weak action on the allosteric effects of AMB shares a different mechanism in modulating the benzodiazepine sites of the $GABA_A$ receptor complex.

Allosteric action of $GABA_A$ receptor ligands on [^{35}S]TBPS binding at or near the $GABA_A$ receptor-coupled chloride channel has been of considerable interest because of its potential to predict the pharmacological efficiency of the ligands. AMB in well-washed cortical membranes significantly increased the affinity of [^{35}S]TBPS binding sites. Recent studies have indicated that the effects of benzodiazepines and positive modulators of the $GABA_A$ receptor complex are highly dependent on GABA levels. Benzodiazepines either cause enhancement (25,29,39) or have no effect (38) on [^{35}S]TBPS binding in well-washed membrane preparations of the rat brain. In our same experimental conditions (frozen, extensively washed membranes) flumazenil had no effect on either the changes of [^{35}S]TBPS binding sites or its apparent affinity. However, flumazenil weakly inhibited the effect of AMB. This may suggest that only part of the action of AMB on [^{35}S]TBPS binding was mediated by the occupation of the central benzodiazepine receptor.

The intrinsic activity of Ro 15-1788 was not apparent in our test situations, which were sensitive to agonist-like or partial agonist-like effects. Apart from certain tests in which the intrinsic actions of flumazenil have been consistently evident (the social interaction test of anxiety, the holeboard in rats), in our T-maze situation this intrinsic action was weak when compared with that of AMB.

The AMB-induced change results in an activation of GABA-mediated neurotransmission, as may be shown by the increase of apparent affinity of GABA and [^{35}S]TBPS binding sites in the cerebral cortex of rats treated with AMB. The CBR antagonist flumazenil is thought to push the receptor towards a predominantly neutral state, in which a functionally relevant conformational change has not been produced (11). Flumazenil would thereby block the ability of AMB to induce changes in the benzodiazepine binding sites, but we can suppose that it did not block other possible binding sites for the β-carboline AMB, coupled or nonrelated with the BDZ receptors.

There are critical differences in the pharmacological mechanisms underlying the behavioral actions of AMB and flumazenil on learning and also in their influences on the components of the GABA/benzodiazepine chlorine ionophor receptor complex. The results of our experiment demonstrate that flumazenil inhibited the consolidation but failed to affect the acquisition of a spatial memory in a multichoice paradigm enhanced by daily pretreatment with AMB, indicating that there is a different action of the BDZ ligands on working memory and the process of consolidation. It is possible that AMB mainly affected working memory as the result of the high levels of performance achieved earlier in training. Flumazenil was much more effective at reversing the effect of AMB on the animal motor activity than its effects on learning.

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