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The Role of the Benzodiazepine–GABA System in the Memory Processes of the Day-Old Chick

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FARKAS, L. AND S. F. CROWE. *The role of the benzodiazepine–GABA system in the memory processes of the day-old chick.* PHARMACOL BIOCHEM BEHAV **65**(2) 223–231, 2000.—This series of experiments investigated the effect of the benzodiazepine diazepam on memory formation in day-old chicks trained on a single-trial, passive-avoidance task. The findings indicate that diazepam has a dose-specific and time-dependent effect on memory processes. A 0.125-mg/kg dose of diazepam administered immediately after training led to amnesia in these subjects only after 30 min following learning. Pretreatment with bicuculline and flumazenil were effective in ameliorating the memory deficits caused by diazepam, and consolidated memory function in saline-treated controls following strong and weak aversant training. These findings suggest that benzodiazepine effects on memory are mediated by their effects on arousal, possibly by the release of noradrenaline, which is critical to the establishment of long-term memory. © 2000 Elsevier Science Inc.

Benzodiazepines Diazepam GABA Bicuculline Flumazenil Memory Noradrenaline Chicks Passive-avoidance learning Weak training

BENZODIAZEPINES (BZDs) are extensively used in the clinical management of anxiety and panic, epilepsy, sleep disorders, and muscular tension (51), but are known to induce a profound deficit in memory and recall (38,51). Although the effects of BZDs on sedation and memory have not been clearly dissociated, the amnesic profile that they produce suggests an effect unrelated to sedation.

Previous research in humans has shown that BZDs impair the acquisition of new information. Benzodiazepines leave short-term memory relatively intact, and their effects on memory only become apparent when information has to be encoded in a more permanent store for accurate performance (51). Midazolam and diazepam, for instance, do not affect performance on tests of immediate recall, but significantly impair performance on tasks that involve the delayed recall of newly learned information (1,25,34). Material encountered early in the testing period is lost, and this loss is attributed to impairment in the long-term consolidation of memory.

If the memory deficits can be attributed to sedation, then the extent to which memory processes are effective will depend on the level of arousal of the central nervous system (24). Chemically similar BZDs have been found to produce comparable effects on sedation, but yield amnesia of different magnitudes (14). This suggests that differences in receptor affinity may mediate the qualitative and quantitative aspects of BZD actions. Furthermore, the BZD-receptor antagonists flumazenil (Ro15-1788) has been found to block BZD effects on sedation and psychomotor performance, but not on memory (1,13,26,30,31,34).

The central BZD receptor is part of a macromolecular protein complex that includes the subtype A of the gamma-aminobutyric acid (GABA) receptors ($GABA_A$ receptor), and an associated chloride ion (Cl^-) channel (20). The binding of BZDs to their receptor activates the $GABA_A$ receptor, and augments the ability of GABA to depress neuronal excitation (43). GABA opens the Cl^- channel, and hyperpolarizes the membrane potential. Consequently, neuronal firing is inhibited because a greater depolarization is needed to trigger an action potential (32).

Compounds that activate the GABAergic system have been found to either enhance or impair memory for a wide variety of learning tasks in animals. In rats, posttraining systemic or intraamygdala administration of the $GABA_A$-receptor antagonists bicuculline or picrotoxin enhance retention, while the agonist muscimol impairs retention on aversively motivated tasks (3–5, 56). Decreasing GABAergic transmission through administration of bicuculline or flumazenil is able to block the memory-impairing effects of midazolam in

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rats (18,19). Flumazenil also blocks the effects of diazepam on learning and memory tasks in mice and chicks (57). It is interesting that flumazenil blocks the effects of BZDs on memory in animal studies (18,19,57), but not in human studies (1,13, 26,34).

Memory can be defined as consolidated information that arises from relatively permanent changes in synaptic efficacy (45). Gibbs and Ng (21) have proposed a three-stage sequentially dependent model of memory formation, based on memory formation in young chicks using a one-trial, passive-avoidance task. This task utilizes the natural ability of young chicks to peck at objects in their immediate environment. Chicks are presented with a colored bead dipped in water and a different colored bead dipped in the chemical aversant, methyl anthranilate (MeA). Chicks are later presented with both beads, and memory for the task is calculated as a discrimination ratio between the number of pecks at the nonaversive bead and the overall number of pecks.

Short-term memory (STM) is thought to last between 5 and 10 min following learning, and its formation is attributed to neuronal hyperpolarization arising from an activity-induced increase in potassium conductance across the neuronal membrane (22). A second stage, intermediate-term memory (ITM), lasts from 20 to 50 min postlearning. The formation of ITM is attributed to neuronal hyperpolarization induced by sodium/potassium pump activity. The final stage, long-term memory (LTM), is defined as retention beyond 60 min postlearning, and is protein synthesis dependent (21).

In the avian brain, direct injection of drugs that act at the $GABA_A$ receptor into the intermediate hyperstriatum ventrale (IMHV), a region containing a high density of GABA receptors and a precursor for its synthesis (48,52,53), can modulate memory for the passive-avoidance task. Pretraining injections of muscimol into the IMHV impairs passive-avoidance learning, and appears to do so early in the process of memory formation (6). When the intensity of the stimulus is reduced to yield weakly reinforced training, pretraining injections of bicuculline into the IMHV are able to enhance memory for the task. Direct stimulation or inhibition of the GABAergic system at or around the time of learning can thus respectively inhibit or enhance memory for the passive-avoidance task. There is evidence to suggest that the distribution of GABA receptors in the chick forebrain increases during ontogeny and reaches a peak at birth (52). This pattern of development renders the chick brain relatively mature at birth, and consequently, these receptors appear to play a fundamental role in early learning in chicks.

Injections of muscimol or bicuculline appear to be ineffective when administered just prior to, or during the induction of the protein synthesis processes necessary for long-term consolidation (6). This indicates that stimulation of the GABAergic system inhibits the formation of memory in the acquisition stage prior to LTM consolidation, but does not appear to inhibit LTM, per se. This finding parallels the behavioral and human neuropsychological findings that BZDs impair memory in the early stage between processing and storage, at acquisition in particular. Both types of studies indicate that STM and events that have been encoded remain unaffected by BZDs.

An understanding of how BZDs interfere with the ability to learn new information, therefore, requires an understanding of where in the learning process the amnesia occurs. The aim of the present series of experiments was to determine how the indirect modulation of GABA through BZD-receptor activation impinges on memory formation. Evidence from passive avoidance training using young chicks suggests that modulation of the $GABA_A$ receptor can impinge on early stages of the memory process. Furthermore, several areas of the chick brain associated with passive avoidance learning show a high density of GABA receptors, or are involved in its synthesis (48,52,53).

It was, therefore, of interest in the present study to use the passive-avoidance learning task to observe the effects of the BZD diazepam on memory processes delineated by Gibbs and Ng (21). Specific antagonists for the BZD and GABA receptors were used to determine whether decreasing GABAergic neurotransmission on concentrated MeA training would facilitate retention for the task. The relationship between diazepam and reinforcement was then investigated by decreasing the intensity of the training stimulus. Weak reinforcement training was employed to assess the effect of inadequate hormonal reinforcement on memory with and without BZD treatment, and to allow comparisons of the relative effectiveness of BZD- and $GABA_A$-receptor antagonists on retention.

METHOD

Animals

The subjects were male day-old Black Australorp/White Leghorn chickens obtained from a local poultry farm on the morning of each experiment. Chicks were randomly placed in pairs into open-topped wooden boxes ($20 \times 25 \times 20$ cm). The wooden boxes were arranged in rows, each row consisting of five wooden boxes. Housing the chicks in pairs reduces the behavioral indices of stress such as distress calling and attempts to escape. One chick in each pair was marked with a small black stripe on its head for identification during data recording. A constant temperature of 25–29°C was maintained using a white 25-Watt incandescent light globe suspended above each box. A group of 20 chicks constituted one experimental group, and represented one data point. Food was available ad lib for the duration of the experiment.

Drugs

Subcutaneous (SC) drug injections were administered by freehand into a ventral skinfold just below the rib cage using a Becton Dickinson Tuberculin 1-ml syringe with a 27.5-gauge needle. Diazepam was prepared in sterile 154 mM saline (NaCl) to concentrations of 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/kg. Subcutaneous injections were injected in a volume of 100 μl. Intracranial (IC) injections of saline, bicuculline (0.1 mM), or flumazenil (0.1 mg/kg) were administered bilaterally to the center of each forebrain in 10 μl vol. Bicuculline was prepared in 154 mM saline, and flumazenil was suspended in saline with two drops of Tween 80. The injections were performed by free-hand injection using a Hamilton repeating dispenser syringe to a depth of approximately 3 mm. In experiments where a weaker dilution of MeA was required, it was diluted to 20% in ethanol. Flumazenil was generously provided by Mr. Steven Lam (Roche Pharmaceuticals).

Procedure

All of the experiments employed the same general procedure. Chicks were placed in pairs in the prewarmed wooden boxes upon arrival at the laboratory. One chick in each pair was marked with a black stripe on its head using a black felt pen for identification purposes. Chicks were allowed to settle for 30 min, and were subsequently pretrained, trained, and

tested in pairs. Subcutaneous injections were administered at times specific to the experiment. Intracranial injections were administered 30 min prior to passive avoidance training. The type of drug given varied according to the nature of the experiment. One or more control group of chickens was employed in most of the experiments. The experimenter was blind to the drug treatment for each experimental group.

Chicks were initially trained to peck a small chrome bead dipped in water. The chrome bead was presented twice to each pair of chicks. Following an interval of approximately 20 min, each pair was presented with a red, and then a blue, bead dipped in water. The number of pecks in each 10-s period and the latency to first peck were recorded on a computer using an electronic hand set. Approximately 30 min later, a red bead similar to the one used in the pretraining trial, but dipped in the chemical aversant methyl anthranilate (MeA), was presented to each pair of chicks for 10 s. Chicks typically show evidence of distaste by shaking their heads and wiping their beaks on the floor of the box immediately after pecking. The number of pecks were recorded, and chicks that failed to peck the red bead within the first 10 s were excluded from later data analysis. Retention was tested at various training-test intervals, depending on the nature of the experiment. Chicks were presented with a dry red bead followed by a dry blue bead, and the number of pecks were recorded. Retention was indexed as a discrimination ratio (DR) between the number of pecks at the nonaversive (blue) bead and the number of pecks at both the aversive (red) and nonaversive beads:

$$\mathrm{DR} = \frac{\text{Number of pecks at blue bead}}{\text{Number of pecks at blue bead + Number of pecks at red bead}}$$

The average of the discrimination ratios for the individual chicks within each group was calculated to yield the mean DR for each group. A DR of 1.0 indicates perfect discriminated memory, while a DR of 0.5 indicates a lack of discriminated memory of the aversive and nonaversive beads. Chicks failing to peck the blue bead were excluded from later analysis, as a failure to discriminate the aversive and nonaversive beads is indicative of a generalized avoidance response rather than discriminated memory, and renders the DR indeterminate (7).

Statistics

Statistical analyses were performed on the DRs of each treatment group using analysis of variance (ANOVA) with appropriate post hoc tests where relevant. As sample sizes were unequal due to factors not associated with the treatments, all analyses were performed on unweighted means (54).

RESULTS

Experiment 1: Diazepam Dose Response

The aim of this first experiment was to establish whether administration of diazepam would interfere with passive-avoidance learning in day-old chicks, and if so, to determine the optimal dose required to yield maximal impairment. Diazepam is a well-known and widely used drug for its sedative, hypnotic, and anxiolytic effects drugs (32,50). It is a central BZD receptor agonist that enhances the action of GABA on its receptor (36), and has a pharmacological profile mediated by an enhancement of chloride conductance.

Chicks received subcutaneous (SC) injections of either 154 mM saline or one dose of diazpam (0.0625, 0.125, 0.25, 0.5, or 1.0 mg/kg) after training with the MeA coated bead. The results are shown in Fig. 1. The doses were chosen using Venault and colleagues' (57) most effective dose of 0.25 mg/kg as a midpoint in the concentration range. Twenty chicks received saline injections, and 20 chicks received each dose of diazepam. All groups were tested at 180 min postlearning, by which time the protein synthesis processes necessary for LTM formation are thought to have already transpired (21).

A one-way ANOVA indicated a significant effect for dose of diazepam, $F(5, 108) = 4.92$, $p < 0.0005$. Post hoc Dunnett's tests revealed a significant difference between diazepam- and saline-injected chicks only with the 0.125-mg/kg dose of diazepam. These results suggest that the dose–effect function is a narrow U-shaped curve, where the optimal dose for achieving maximal deficits is 0.125 mg/kg. As the doses chosen above and below 0.125 mg/kg do not yield a significant deterioration in retention, there appears to be a narrow intervention window. It is unclear why the high doses do not produce an effect. A narrow dose window is not an unusual phenomenon, as noradrenaline has also been found to exhibit a dose-specific effect within this experimental paradigm (7).

These observations are in contrast to the finding of Venault and colleagues (57), who reported significant differences with 0.25 mg/kg diazepam. However, doses lower than 0.25 mg/kg were not used in that study, and it is possible that this dose of diazepam was effective because the chicks appear to have been housed in isolation, a measure that increases levels of corticosterone (29). The narrow nature of the dose–response function suggests that the observed effect is not due to sedation, which would not exhibit a U-shaped curve. Indeed, further work did not reveal significant differences in sedation across doses, nor any robust relationships between the level of sedation and discriminated memory at each dose, and thus supports a dissociation between the memory-impairing effect of diazepam and its effects on sedation. Prior to further investigations, the optimal time for administration of diazepam needed to be determined, and this issue was addressed in Experiment 2.

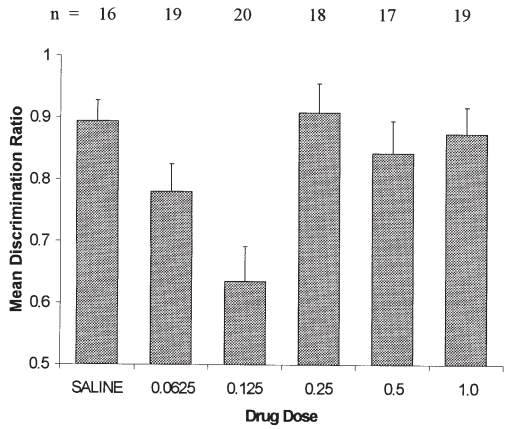

FIG. 1. The effect of various doses of diazepam (mg/kg) on the level of discriminated memory. Chicks were injected immediately after training and assessed immediately after the test of memory functions at 180 min postlearning, as measured by mean discrimination ratio (+ SEM).

Experiment 2: Diazepam Time of Injection Effects

The effect of GABA receptor ligands on memory processes appears to depend on whether they are administered before or after the training experience (27). The evidence suggests that BZD receptor ligands inhibit memory when given before, but not after a learning session. When administered posttraining, BZDs are usually ineffective (28,38). Direct activation of GABA transmission, on the other hand, appears to be effective upon posttraining administration of $GABA_A$ receptor ligands. These findings suggest that GABA is able to modulate memory both at the time of acquisition and later during consolidation, but the BZD receptor only appears to be implicated in the former (27). The aim of this experiment was to determine whether the effects of BZDs on memory are time dependent by comparing the effect of diazepam administered at various times before and after the training experience.

Chicks received a 0.125 mg/kg SC dose of diazepam at various times prior to and following exposure to the aversive stimulus. Chicks given injections prior to training were treated at 30, 15, or 5 min prior to training, and were only injected once the pretraining protocol was completed. Those given posttraining injections were treated immediately after training, or at 15 or 30 min after training. Injections given immediately after training were more precisely defined as Time +0, because there was no temporal delay between training and drug administration. A group of 20 chicks were injected at each time point, and all groups were tested at 180 min after learning.

The results are presented in Fig. 2, and suggest an essentially U-shaped time-of-injection function, with obvious retention losses apparent for administration immediately after training (Time +0). One-way ANOVA revealed a significant effect for time of injection, $F(5, 112) = 2.51$, $p < 0.05$. A post hoc Tukey HSD test indicated that the groups treated with diazepam 30 min prior to training and at Time +0 were significantly different at $\alpha = 0.05$ from those treated at the other times. Treatment with diazepam 30 min before training yielded the highest mean DR (0.81), while treatment at Time +0 yielded the lowest (0.56). Consequently, the effective administration time used for subsequent experiments was immediately after training.

The results of this experiment indicate that the post- but not pretraining mechanisms involved in memory for the passive avoidance task are susceptible to BZD treatment. Diazepam led to deficits in retention only when administered immediately after the training experience; retention did not appear to be affected with greater intervals between training and diazepam administration. This suggests that the effect of diazepam on memory is time dependent, and involves posttraining consolidation processes.

Experiment 3: Retention Time Course of Diazepam

There is evidence to suggest that the deficits in retention associated with BZD usage occur early in the process of memory formation. Assessments of amnesia following BZD use in humans have found that although retention remains unaffected soon after learning, it is susceptible to temporal delays (1,25). Such findings indicate an intact STM, but an effect on LTM processes. In animal studies, administration of the $GABA_A$ receptor agonist muscimol has been found to impair passive avoidance learning in young chicks as early as 10 min after learning (6). These findings suggest that stimulation

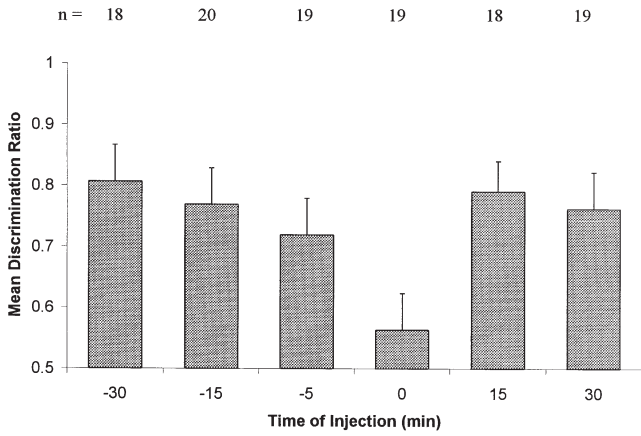

FIG. 2. The effect of diazepam injections at various times before and after training on the level of discriminated memory. Chicks were administered a 0.125-mg/kg dose of diazepam, and tested at 180 min postlearning, as measured by mean discrimination ratio (+ SEM).

of the BZD–$GABA_A$ receptor has an effect on the early neurobiological processes that mediate learning and memory.

To date, there are no conclusive findings on exactly which processes are disrupted by $GABA_A$ receptor activation, nor how BZDs are able to achieve the effects on memory that they do. It was, therefore, of interest to use an established memory paradigm to investigate BZD modulation of memory. The temporally precise Gibbs and Ng (21) three-stage model of memory was used to determine the time at which drug-induced amnesia became apparent.

Chicks were given an SC injection of either 0.125 mg/kg diazepam or 154 mM saline immediately after training, the time found to yield the lowest DR in the previous experiment. Chicks were tested for retention at training-time intervals (TTIs) of 5, 10, 20, 25, 30, 40, 60, and 90 min postlearning. The retention function for diazepam and saline is illustrated in Fig. 3. These times were chosen in accordance with the different stages of memory outlined in the Gibbs and Ng (21) three-stage model of memory formation. At each time point, 20 chicks were administered diazepam and 20 chicks were administered saline, allowing a direct comparison at each TTI.

A two-way ANOVA [drug (2) × TTI (8)] yielded a significant drug, $F(1, 261) = 15.87$, $p < 0.0005$, and a TTI, $F(7, 261) = 5.03$, $p < 0.0005$, main effect. A significant drug by TTI interaction was also obtained, $F(7, 261) = 2.26$, $p < 0.05$. Levene's test of equality of error variances indicated significant differences among the variances, $F(15, 246) = 6.41$, $p < 0.0005$; however, violating the assumption of homogeneity of variances in this instance would have a minimal effect on the Type I error rate because there were approximately equal sample sizes (54). Simple main-effects analysis revealed significant differences at 40, $F(1, 246) = 9.89$, $p < 0.05$, 60, $F(1, 246) = 8.24$, $p < 0.05$, and 90, $F(1, 246) = 11.89$, $p < 0.05$, min postlearning. No significant differences were found at 5, $F(1, 246) = 0.09$, $p = 0.769$, 10, $F(1, 246) = 2.27$, $p = 0.606$, 20, $F(1, 246) = 1.34$, $p = 0.249$, 25, $F(1, 246) = 0.26$, $p = 0.608$, or 30, $F(1, 246) = 0.20$, $p = 0.654$, min postlearning. Furthermore, a significant difference was found for TTIs within diazepam, $F(7, 246) = 6.00$, $p < 0.0005$, but not for TTIs within saline, $F(7, 246) = 0.87$, $p > 0.533$.

It is apparent that diazepam interfered with the formation of LTM, but not the formation of STM and ITM. Gibbs and

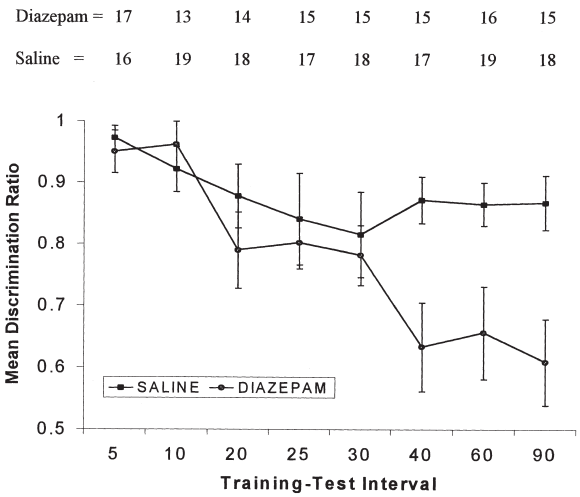

FIG. 3. The effect of diazepam on retention at various times following learning, as measured by mean discrimination ratio (± SEM). All chicks were injected immediately after training and tested at the times specified above.

Ng (23) suggest that ITM consists of two distinct stages, a phase A [ITM(A)] that is energy dependent and susceptible to blockade by the metabolic inhibitor dinitrophenol (DNP), and a phase B [(ITM(B)] that is not DNP sensitive. It is postulated that the neuronal events that trigger the transition from phase A to B give rise to cellular activities that culminate in LTM (23).

As retention levels showed a sharp decline after 30-min postlearning, diazepam did not disrupt the formation of intermediate memory, ITM(A), but interfered with the expression of its second phase, ITM(B). The initiation of this second phase of ITM is purported to be mediated by the actions of stress hormones, such as noradrenaline, ACTH and vasopressin (7,8,10). That diazepam disrupted the formation of ITM(B) suggests the possibility of BZD–GABA involvement in the release of hormones contingent upon the learning experience and the reinforcing effects they provide for memory consolidation.

Experiment 4: Effectiveness of Benzodiazepine- and GABA-Receptor Antagonists on Concentrated Methyl Anthranilate Training

Directly blocking GABAergic neurotransmission through administration of the $GABA_A$ receptor antagonist bicuculline has been shown to enhance memory performance (3,4,6), and overcome the effect of BZDs on learning and memory tasks (18,19). Furthermore, indirectly blocking GABAergic activity through the use of the BZD-receptor antagonist flumazenil also appears to enhance learning (18).

The imidazobenzodiazepine-derivative, flumazenil (Ro15-1788), is a potent and specific antagonist of the central BZD receptor (2). As a competitive antagonist for the BZD receptor (42), flumazenil is able to block the characteristic pharmacological effects of BZDs (2,27,57,58). Pedder and colleagues (49) reported that the anticonvulsant effect of 1.0 mg/kg of diazepam was attenuated by flumazenil at doses of 1.0 mg/kg and higher. Flumazenil has also been found to block the effects of beta-carboline esters (57,58), but is thought to be devoid of intrinsic activity itself (2). However, pretraining administration of low doses of flumazenil has been found to enhance retention on habituation and inhibitory avoidance tasks, and suggests that flumazenil may have an intrinsic effect at the BZD receptor (28).

This experiment attempted to counteract the amnesic effect of diazepam by antagonising GABAergic transmission directly with bicuculline, and indirectly with flumazenil. The dose of bicuculline was chosen in accordance with Clements and Bourne (6), where 0.1 mM was found to significantly enhance memory in young chicks for the passive-avoidance paradigm. As there appears to be a 1:1 dose relationship between diazepam and flumazenil antagonism (49), the dose of flumazenil used was equivalent to the previously determined effective dose of diazepam.

Groups of 40 chicks were pretreated with 0.1 mM bicuculline, 0.1 mg/kg flumazenil, or 154 mM saline 30 min prior to training. Pretreatments were administered intracranially (IC) to the centre of each forebrain in 10 μl vol. Chicks were trained with concentrated MeA, and immediately following training, 20 chicks in each pretreatment group received an SC injection of 0.125 mg/kg diazepam or 154 mM saline. Chicks were tested for retention at 180 min posttraining. The data are shown in Fig. 4.

A two-way ANOVA [drug (2) × pretreatment (3)] yielded significant drug, $F(1, 94) = 10.70$, $p = 0.002$, and pretreatment, $F(2, 94) = 3.71$, $p < 0.05$, main effects. The drug by pretreatment interaction was not significant, $F(2, 94) = 2.27$, $p = 0.109$. Levene's test of equality of error variances indicated that the assumption of homogeneity of variances was not met, $F(5, 89) = 3.88$, $p = 0.003$, but as there were approximately equal sample sizes, the effect of this violation on the Type I error rate was considered minimal (54). Analysis of the main effects indicated a significant difference between diazepam and saline, $F(1, 94) = 9.20$, $p < 0.05$, and a significant difference between pretreatments, $F(2, 94) = 3.41$, $p < 0.05$. Tests of the main effects both indicated equality of error variances: drug, $F(1, 93) = 1.04$, $p > 0.05$, pretreatment, $F(2, 92) = 0.57$, $p > 0.05$. A post hoc Student–Newman–Keuls test indicated that the groups pretreated with bicuculline and flumazenil were significantly different at $\alpha = 0.05$ from the saline group.

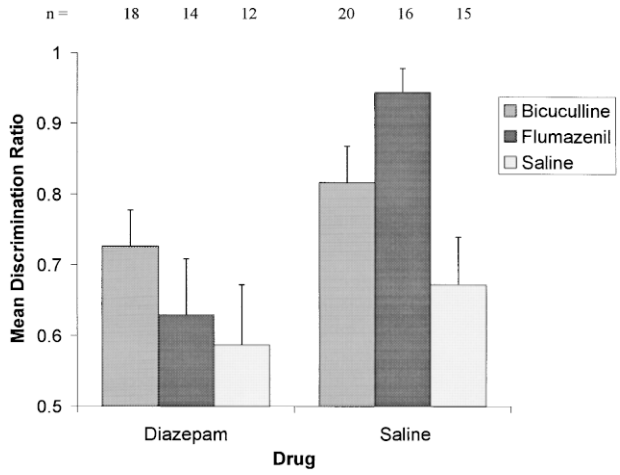

FIG. 4. The effect of subcutaneous administration of diazepam or saline immediately after training on subjects trained with concentrated MeA pretreated with bicuculline, flumazenil, or saline. All subjects were tested 180 min after training, as measured by mean discrimination ratio (+ SEM).

Pretreatment with specific antagonists of the BZD and $GABA_A$ receptors attenuated the diazepam-induced memory deficits and enhanced memory for the task. Chicks given flumazenil and bicuculline pretreatments and injected with saline after training exhibited significantly higher retention levels than those pretreated with saline, and those given diazepam after training. These findings corroborate previous findings that bicuculline enhances memory (3,6). Furthermore, they indicate that flumazenil not only attenuates the effects of systemic diazepam, but has an intrinsic effect on memory processes as well. This finding supports the contention that flumazenil has an independent effect on memory (27,28), but is inconsistent with animal studies that found flumazenil to be devoid of intrinsic activity (2), and human studies that found that flumazenil blocked the sedative and psychomotor effects of BZDs but not their amnesic effects (1,13,26).

Of particular interest is the finding that the group pretreated with saline and injected with saline posttraining displayed an unusually low level of discriminated memory. The mean DR for this group is inconsistent with those obtained under the same conditions in previous studies (11,12), and appears to be an aberrant control group.

Although this may raise concerns about the validity of the overall results, the bicuculline and flumazenil pretreatments clearly attenuated the effects of diazepam on memory and enhanced memory in saline controls. If the responsible variable(s) operated on all groups and the data were contaminated, then, contrary to our observations, the experimental groups should have exhibited poorer memory performance than they did.

The time course of diazepam suggests that it interferes with the transition of memory from phase A of ITM to phase B. That both antagonists were able to enhance retention suggests involvement in this transition, and the consequent triggering of long-term consolidation. Studies have found that the transition from ITM(B) to LTM is associated with the release of stress hormones contingent upon the learning experience (7,8,10). It is possible that modulation of the GABA receptor affects memory processes by influencing the release of stress hormones in the brain. The involvement of GABA receptor ligands in the memory processes underlying long-term consolidation was explored further in Experiment 5 using weakly reinforced training.

Experiment 5: Effectiveness of Benzodiazepine- and GABA-Receptor Antagonists on Weak Training

The retention time course of diazepam indicated that diazepam-induced amnesia only became apparent at 40 min postlearning, the stage in the Gibbs and Ng (21) model of memory formation that corresponds to ITM(B). This phase of ITM is not energy dependent, but is, instead, associated with the release of stress hormones such as noradrenaline in response to the learning experience (7,8,10). The aim of this experiment was to investigate GABAergic involvement in the mechanisms underlying the formation of ITM(B) through the use of weak training. If there is a relationship between the intensity of the training stimulus and GABAergic activity, then diazepam- and saline-treated chicks would not be expected to differ in their retention levels; both should show little or no evidence of memory for the weak aversant. Furthermore, decreasing GABAergic activity through bicuculline and flumazenil pretreatments can provide further support for the role of the GABA system in memory consolidation if the pretreatments are able to overcome the retention deficits due to weak training in both diazepam and saline-treated chicks.

Groups of 40 chicks were pretreated with 0.1 mM bicuculline, 0.1 mg/kg flumazenil, or 154 mM saline 30 min prior to training. Pretreatments were administered IC, to the centre of each forebrain in 10 μl vol. Chicks were trained with a 20% concentration of MeA made up in ethanol. Immediately following training, 20 chicks in each pretreatment group received an *sc* injection of 0.125 mg/kg diazepam or 154 mM saline. Chicks were tested for retention at 180 min posttraining. The data are shown in Fig. 5.

A two-way ANOVA [drug (2) × pretreatment (3)] yielded a significant main effect for pretreatment, $F(2, 99) = 28.93$, $p < 0.0005$, but not for drug, $F(1, 99) = 1.33$, $p = 0.251$. The drug by pretreatment interaction was also not significant, $F(2, 99) = 1.27$, $p = 0.285$. Levene's test of equality of error variances indicated the assumption of homogeneity of variances was not met, $F(5, 94) = 9.52$, $p < 0.0005$, but, as there were approximately equal sample sizes, the effect of this violation on the Type I error rate is minimal (54). Analysis of the main effect for pretreatment indicated a significant difference in mean DRs between pretreatment effects, $F(2, 99) = 29.34$, $p < 0.0005$. A post hoc Student–Newman–Keuls test indicated that the groups pretreated with bicuculline and flumazenil were significantly different at $\alpha = 0.05$ from the saline group.

The data from this experiment provide strong support for GABAergic activity in the modulation of memory for the passive avoidance task. No significant differences in retention were observed between the diazepam- and saline-injected chicks, indicating that the level of memory impairment following weak training is not different from that due to diazepam treatment. The reduction in GABAergic activity through flumazenil or bicuculline pretreatment overcame the effects of the weakened stimulus on retention in both the diazepam and control groups. It thus appears that decreasing GABAergic activity facilitates the transfer from ITM(A) to ITM(B)—the stage normally disrupted in weak learning (7–9)—and, consequently, triggers the processes required for

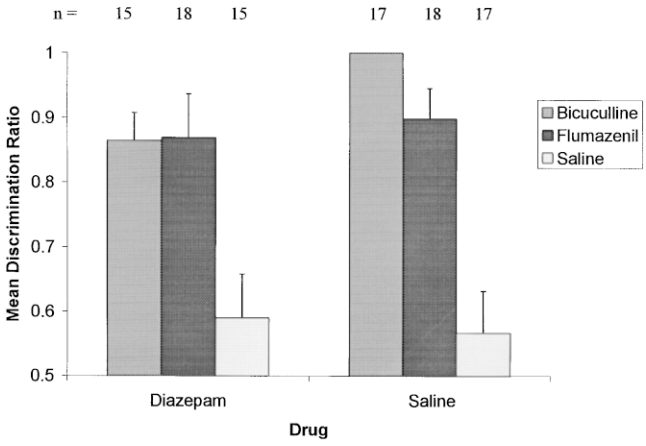

FIG. 5. The effect of subcutaneous administration of diazepam or saline immediately after training on subjects trained with diluted 20% MeA and pretreated with bicuculline, flumazenil, or saline. All subjects were tested 180 min after training, as measured by mean discrimination ratio (+ SEM).

long-term consolidation. There seems to be little, if any difference between direct and indirect $GABA_A$ receptor antagonism, suggesting that it is the net reduction in GABA neurotransmission, rather than the means for achieving this reduction that is critical to an enhancement of memory.

DISCUSSION

This series of experiments using chicks trained on a single-trial passive-avoidance task has demonstrated that diazepam inhibits the acquisition of memory by 40 min postlearning, the stage corresponding to ITM(B) of the Gibbs and Ng (21) model of memory formation. Blocking GABAergic neurotransmission through direct or indirect antagonism of the $GABA_A$ receptor attenuated diazepam-induced amnesia and enhanced retention levels. Bicuculline and flumazenil successfully overcame diazepam-induced amnesia and enhanced memory in controls, regardless of the intensity of the training stimulus.

Following treatment with diazepam, chicks showed evidence of STM and the initial stage of intermediate memory, ITM(A). Beyond ITM(A), there was little evidence of memory. The transition from phase A to B of ITM is linked to successful long-term consolidation, such that the absence of this phase leads to impairments in retention (23). Previous work in this area suggests that this transition, and the subsequent consolidation of the event, appear to depend on the presence of stress hormones such as noradrenaline (NA) (7,8,10).

The difference in the effective dose of diazepam found in the present study and the previous study by Venault and colleagues' (57) supports a possible relationship between BZD-mediated anxiolysis and amnesia. In the present study, the effective dose of diazepam found to disrupt memory (0.125 mg/kg) was half the effective dose used by Venault and colleagues (0.25 mg/kg). It is possible that this difference is the result of higher treatment-related stress in that study due to isolation of the chicks. Housing the chicks in isolation increases fear and anxiety responses, and has been found to delay or counteract drug-induced amnesia (16,29). This counteractive effect of isolation may extend some phase of memory prior to protein synthesis-dependent LTM. The implication of these findings is that the level of arousal appears to mediate the effects of BZDs on memory such that when arousal is high, a higher dose of BZD is needed to interfere with memory functions than when arousal is low.

An adequate release of stress hormones as a consequence of reinforcement at a critical time following learning is important to long-term consolidation (44). When the strength of a negative reinforcer is reduced, such as in weak training, there is an absence of ITM(B) and long-term retention (7,8), and this loss is associated with decreased levels of forebrain NA (9). These impairments can be ameliorated by the administration of NA itself, or salbutamol, a β2-adrenergic agonist (12). That diazepam was able to interfere with long-term consolidation through the disruption of ITM(B), suggests the possibility of BZD–GABA involvement in stress hormone activity critical to the establishment of LTM.

Anxiolytics such as the BZDs appear to inhibit the release of hormones in the brain by interfering with the activity of the hypothalamic–pituitary–adrenocortical (HPA) axis. The HPA axis is activated in response to stress, and mediates the release of stress hormones such as NA and corticosterone in the brain (35). Inhibition of HPA axis activity prevents the influx of these hormones into the brain, and has been found to affect learning (35,37). Benzodiazepines suppress the stress-induced response of the HPA axis, and also appear to interfere with basal HPA axis activity (15), an effect mediated in part by hypothalamic and pituitary GABA receptors (40).

Given the evidence for GABAergic involvement in the release of stress hormones in the brain, the anxiolytic and memory effects of BZDs appear to be related. Much research has shown that GABAergic involvement in the amygdala interferes with memory for affectively influenced stimuli (17,19, 41,55). Animal studies, particularly data from rats, have implicated the amygdala as the key brain region in the processing of affectively influenced stimuli (32,33,41). Benzodiazepines, by potentiating GABA, are thought to interfere with the processing of memory in the amygdala by attenuating their emotional significance through inhibition of hormonal reinforcement crucial to long-term consolidation (41). However, the notion that BZDs by potentiating GABA interfere with the processing of emotion-based memory in the amygdala is not consistent with the nature of the memory deficits reported in human studies. Human studies indicate that BZDs induce a global, anterograde amnesia in explicit memory processes (39), rather than a specific processing deficit.

Rather than playing a specific role in the modulation of emotional-based memory, stress hormones may simply serve to regulate the importance of stimuli by providing reinforcement in terms of emotional valence. Activation of the BZD–GABA system rather than interfering with the processing of affective stimuli, may simply act as a brake on the hormonal release required for the consolidation of memory. The increase in arousal associated with the release of stress hormones thus appears to play an important role in memory consolidation by allowing the influx of compounds such as NA and corticosterone into the brain. The importance of NA in memory is attributed to its involvement in providing energy for the neuronal processes underlying memory consolidation (44). The energy demands of the different stages of memory appear to be met by different sources, and NA is implicated in the energy processes needed for the continuation of memory processes beyond ITM(A) (46,47). The possible BZD–GABA inhibition of NA release could thus impinge upon memory formation by interfering with the energy levels needed for memory processing beyond ITM(A), consequently inhibiting the transition to ITM(B) and long-term consolidation.

The present findings suggest a possible relationship between BZD-mediated anxiolysis and amnesia. Given the weight of the evidence supporting a relationship between BZD–GABA activity and the stress-hormone mediation of memory, this is an hypothesis that clearly warrants further experimental investigation. To further examine and clarify the possible relationship between GABA and NAergic activity in memory formation, it would be useful to study the effects of NA or β-adrenergic agonists in chicks rendered amnesic through diazepam treatment. The recovery of memory formation in this instance would provide direct evidence that BZDs, through GABA activity, affect the release of NA and memory consolidation. Alternative BZDs such as midazolam should also be used to assess the relative potencies of BZDs on retention deficits. Biochemical assays of NA levels, and turnover rates following BZD treatment would provide corroborative correlational data. Beta-carboline esters are inverse agonists at the BZD receptor, and produce pharmacologically opposite effects (57). It would be of interest to determine whether β-carbolines are able to enhance retention on weak training, and whether β-adrenergic blockers inhibit the effects of β-carbolines on memory.

It would also be of interest to determine whether the dose of diazepam found to effectively induce amnesia also effectively alleviates anxiety in a similar animal model. An anxiolytic dose of diazepam that produces deficits in memory would suggest that these effects are mediated by the same mechanisms, and also provide insights into the mechanisms that underlie memory formation. If it were the case that BZD effects on memory are due to their results on arousal, then their clinical benefits such as sedation and the alleviation of convulsions, muscular tension, and particularly, anxiety, would be offset by their adverse effects on memory.

In the present study, the effects of diazepam on memory were ameliorated by pretreatment with a BZD-receptor antagonist and a specific $GABA_A$-receptor antagonist, and demonstrates that the amnesic effects can be blocked through the net reduction in GABAergic neurotransmission. This suggests that successful memory consolidation is dependent on the net reduction in GABA neurotransmission, and that the effects of antagonising BZD- and $GABA_A$-receptor activity on memory processes are not dissociable. Although decreasing GABAergic activity is able to ameliorate the memory-impairing effects of diazepam, this may not be an appropriate treatment strategy in humans if, as suggested, the anxiolytic and amnesic effects of these drugs are linked. The use of GABA-receptor ligands to inhibit the memory deficits could possibly antagonize the desired psychotropic effects of these drugs as well. Appropriate treatment strategies would, thus, need to be developed to reduce the effects of BZDs on memory, but retain the psychotropic properties of these drugs.

In conclusion, these findings indicate that modulation of the $GABA_A$-receptor can influence memory for the passive-avoidance task. It has been shown that diazepam does, in fact, impair memory storage, and this adverse effect can be blocked by decreasing GABAergic activity directly or indirectly through the BZD receptor. Furthermore, decreasing GABAergic activity led to enhanced memory performance in control animals. Diazepam appears to exert its effects on memory by interfering with the transition in memory processes from ITM(A) to ITM(B). These findings, together with evidence from previous studies that the $GABA_A$ receptor modulates memory for the passive-avoidance task (6) and the fact that the receptor density is at its greatest at birth (52) suggest that the BZD–GABA system plays a crucial role in learning and memory processes in the young chick.

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