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Research Article

Systemic Administration of γ-Hydroxybutyric Acid in Adolescent Rat Impairs Contextual Fear Conditioning, But Not Cued Conditioning

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Abstract γ-hydroxybutyric acid (GHB) causes retrograde amnesia in juveniles and young adults. Earlier, we have reported that in adolescent rat, GHB impairs the hidden platform task performance in the Morris water maze. In the present study, a classical fear conditioning paradigm was used to examine the effects of GHB on the acquisition of contextual conditioning, a hippocampus-dependent associative task, and cued tone conditioning, a hippocampus-independent task, in adolescent rats. Administration of GHB before the presentation of tone-shock pairings dose-dependently disrupted the acquisition of contextual conditioning with no effect on tone conditioning, when conditioned fear was measured 24 h later. Administering GHB prior to testing did not disrupt either contextual or tone conditioning. These results demonstrate that in the adolescent rat exposure to GHB preferentially disrupt hippocampal-dependent learning.

Keywords juvenile; addiction; learning and memory; adolescence; sodium oxybate

1. Introduction

γ-hydroxybutyric acid (GHB) was first synthesized in the early 1960s to study the GABAergic system [28]. GHB, a metabolite and a structural analog of GABA, is found endogenously throughout the mammalian brain in micromolar concentrations [5,11,32]. GHB has been used therapeutically in the treatment of sleep disorders [19,29,60,64], and to treat alcoholism and alcohol-withdrawal symptoms [7]. Illicit use of GHB has been reported in humans [66].

In the mammalian central nervous system, systemically administered GHB has been shown to have affinity for specific GHB binding sites, referred to as the GHB receptor [3,12,14,25,36,54,65]. High affinity GHB binding sites are thought to contribute to the biological effects of GHB [65]. Some behavioral effects of GHB have been attributed to its interaction with the GABAergic system [10,15,21]. GHB has weak affinity for the $GABA_B$ receptor [34]. Recently, it has been shown to bind with high affinity to the α4-containing $GABA_A$ receptor [1].

In humans, low doses of GHB induce short-term anterograde amnesia [24,50,63]. GHB's amnesia-causing effect is associated with its short half-life and rapid clearance rate [2,8,16,61,66]. In experimental animals, GHB has been shown to cause memory deficits [27,42,51,52,53,62]. We have earlier reported that GHB given to adolescent rats cause significant deficits in spatial learning and memory. When tested in the hidden platform task using the Morris water maze for reference memory, compared to control rats GHB-treated rats took significantly longer and swam greater lengths to find the hidden platform, and their performance during the probe trial was significantly compromised. There was little effect on motoric or motivational functions since performance in the visual cued task did not differ between drug-treated and control rats [51,52,53].

Hippocampal damage is known to impair declarative or explicit memory but spares procedural or implicit memory in humans [41,57,58]. In rodents, hippocampal injury impairs spatial and contextual learning but not the ability to learn simple associations between discrete stimuli. In the classical fear conditioning paradigm, lesioning of the hippocampal formation disrupts the association between complex contextual stimuli (conditioning chamber) and foot shock, but does not affect the development of association between an auditory tone and foot shock [17,18,23,40,47]. Animals with hippocampal damage exhibit disruption in conditioned fear to the context in which the conditioning occurred but show normal conditioned fear to the tone [30]. Learning impairment has been reported following exposure to substances of abuse including ethanol. Ethanol causes deficits in the learning of hippocampal-dependent tasks, and spares the learning of hippocampal-independent tasks [37,38], and impairs the acquisition of contextual

fear conditioning [22,38,39] but not the consolidation of conditioning [22].

Although deleterious effects of GHB on learning and memory have been documented in humans and laboratory animals, the specific cognitive processes affected by GHB have not been examined. GHB could have direct effects on learning, consolidation, or sensitivity to the shock stimulus. In addition, the neural substrates underlying GHB-induced cognitive impairments remain unknown. In the present study, the hypothesis tested was that GHB in young animals preferentially disrupts learning of hippocampus-dependent task. The effects of a single injection, as well as repeated administrations of GHB on classical conditioning to contextual stimuli and conditioning to auditory tone were examined in adolescent female rats.

2. Materials and methods

2.1. Subjects

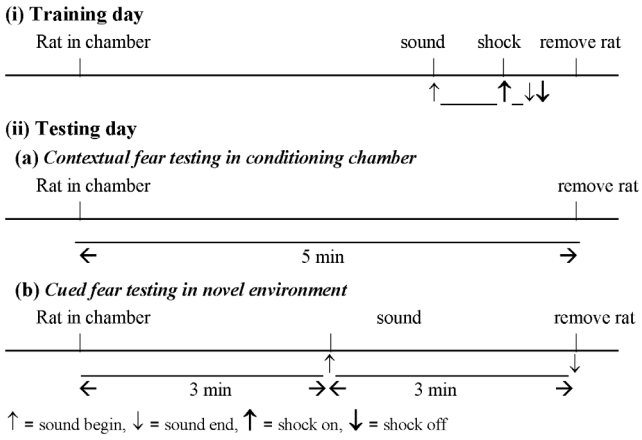
A total of 126 adolescent (postnatal day [PD] 28, weighing 57–120 g, at the time of fear conditioning) female Sprague-Dawley rats (Taconic, Germantown, NY, USA) with no previous drug experience served as subjects. Female adolescent rats were used for the study since our earlier studies showed GHB-induced spatial memory deficits in female adolescent rats [51,52,53]. Rats were group-housed in plastic cages with ad libitum access to food and water in a temperature- and humidity-controlled animal care facility with a 12 h light/12 h dark cycle. Animals were randomly assigned to one of five acute or repeated treatment groups with 12–16 rats in each group. All experimental protocols were approved by the institutional animal review committee and were in compliance with the NRC Guide for the Care and Use of Laboratory Animals [13].

2.2. Drug and drug solution

Gamma-hydroxybutyric acid (GHB) sodium salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sterile saline, which also served as the vehicle control. Drug and saline solutions were injected intraperitoneally (IP) in a volume of 1 mL/kg. Doses of GHB used were based upon our previous studies [51,52,53].

2.3. Apparatus

Fear conditioning was conducted in a Plexiglas rodent conditioning chamber ($28 \times 21 \times 21$ cm) with a metal grid floor, lit with a single house light and enclosed within a sound-attenuating cubicle (Med Associates, St. Albans, VT, USA). The floor grid was connected to a shock generator and scrambler (Med Associates) for the delivery of an electric foot shock, which was used as an unconditioned stimulus (US). The chamber was also equipped with an electronic alarm with a speaker (Mallory Sonalert, Indianapolis, IN, USA) for the delivery of a tone, which served as a conditioned stimulus (CS) for cued fear conditioning. The same conditioning chamber was used for testing of contextual fear memory. In testing for cued fear memory, the chamber was modified with a rectangular partition placed at a diagonal, a wall cover with novel texture, a fitted flat Plexiglas floor cover, and a novel scent.



2.4. Drug administration

2.4.1. Single GHB injection

On PD 28, separate groups of rats were given GHB or saline either 30 min before training (pretraining groups) or 30 min before the testing session (pretesting groups) that took place approximately 24 h after training. (i) Rats in the pretraining injection groups received IP injections of one of two doses of GHB (50 or 100 mg/kg) or saline 30 min before fear conditioning. They were injected with saline 30 min before testing on the following day. (ii) Animals in the pretesting injection groups received saline injections 30 min before training and were given one of the two doses of GHB (50 or 100 mg/kg) 30 min before testing. One group of rats received saline vehicle on both days (i.e., 30 min prior to both training and testing) and served as a control group for both the pretraining and pretesting injection groups.

2.4.2. Multiple GHB injections

To examine the effects of repeated GHB administration on contextual and cued fear conditioning, animals were randomly assigned to one of three groups. Starting on PD 26, separate groups of rats were given GHB daily IP injections (one of two GHB doses 50, 100 mg/kg) on five consecutive days. On the fifth day (PD 30), drug injection took place 30 min before training. All rats were injected with saline vehicle 30 min before testing on the following day. Additional group of animals received daily saline injections on five consecutive days and also 30 min before testing, and served as the control group.

Following drug/saline injection, order of cages was randomized so that the experimenter while scoring freezing behavior was blind to the treatment received by the animal.

2.5. Behavioral training and testing

2.5.1. Behavioral training

On the training day (PD 29 for acute exposure groups, and PD 30 for repeated exposure groups), 30 min after GHB or saline injection, rats were placed in the conditioning chamber, and the house light was immediately turned on. Ninety seconds later, animals were presented with a continuous tone (2.9 kHz, 80 dB) for 30 s, at the end of which an electric shock (1 mA) was delivered through the floor grid for 2 s and coterminated with the tone. Animals remained in the chamber for another 30 s before being removed to a plastic holding cage. After a 150-second intertrial interval, rats were returned to the conditioning chamber, and the above procedure was repeated once more. Thus, each animal received two tone-shock pairings. Animals were returned to their home cages after the second trial.

2.5.2. Behavioral testing

Approximately 24 h after the training session, rats were tested for contextual fear memory and cued fear memory in two separate sessions. The order of testing for contextual and cued fear memory was counterbalanced within groups to control for possible order effects. The time interval between the two tests was 30 min, during which time the animal was returned to its home cage.

(a) *Contextual testing*

For the test of contextual fear memory, the animal was placed in the same conditioning chamber as had been used for training and observed for freezing behavior in the absence of any shock or tone. Freezing is a species-specific defensive response involving a stereotyped crouching posture with absence of all movements except breathing, and is highly correlated with other measures of conditioned fear [31, 37]. The animal's freezing behavior was manually scored every 10 s on a three-point scale (0: moving; 1: exhibiting head movements only; 2: no movement except for respiration) to determine whether freezing occurred within each 10 s bin. The scores obtained during the observation period were summed up to give freezing sum scores for both contextual and cued fear conditioning.

(b) *Cued testing*

For the test of cued fear memory, the rat was placed in the modified chamber (novel environment) and observed for freezing behavior for 3 min. After 3 min, the animal was presented with the tone CS continuously for 3 min, during which time it was again observed for freezing behavior. The second 3-minute period with the tone presentation constituted testing for cued fear memory.

2.6. Data analysis

Behavioral data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. The level of significance was set at $P < .05$. Statistical analyses were carried out using the Prism software (GraphPad, La Jolla, CA, USA).

3. Results

To assess possible effects of the drug on shock sensitivity, animals were observed throughout the training session. No differences in reactivity to the foot shock were observed among the treatment groups. All rats ran, jumped, and/or vocalized to the shock.

3.1. Experiment 1: Effects of acute administration of GBH on fear conditioning

3.1.1. Experiment 1.1: Acute systemic administration of GHB on contextual fear memory

Figure 1(a) shows the effects of acute pretraining administration of saline or GHB (50 or 100 mg/kg, IP) on contextual fear memory in adolescent female rats. The ANOVA revealed a significant effect for the dose of GHB on freezing ($F_{(2,39)} = 5.498$; $P < .01$). Post hoc comparisons showed that the group injected with 100 mg/kg of GHB prior to training froze significantly less to the context than the control group treated with saline ($P < .01$), indicating that GHB at this dose attenuated the acquisition of contextual fear memory. In contrast, as depicted in Figure 1(b), acute treatment with GHB 30 min prior to testing had no significant effect on freezing to the context ($F_{(2,39)} = 2.090$; $P > .05$), suggesting that GHB did not affect the expression of previously learned contextual fear memory.

3.1.2. Experiment 1.2: Acute systemic administration of GHB on cued fear memory

As shown in Figure 2(a), when rats were tested for auditory cued fear memory in the novel environment, acute pretraining treatment with GHB (50 or 100 mg/kg, IP) did not significantly change freezing behavior to the tone CS ($F_{(2,39)} = 1.892$; $P > .05$), suggesting that in the dose range used GHB did not affect the acquisition of cued fear memory. As can be seen in Figure 2(b), acute pretesting GHB injections also did not affect freezing to the tone CS ($F_{(2,39)} = 0.9969$; $P > .05$), indicating that GHB did not affect the expression of previously acquired cued fear memory.

3.2. Experiment 2: Effects of repeated GHB on fear conditioning

Since acute pretraining injection of GHB impaired fear conditioning, in this series of experiments effects of multiple GHB injections, given over several days before training, on fear conditioning were determined.

3.2.1. Experiment 2.1: Repeated systemic administration of GHB on contextual fear memory

Data from repeated administration of GHB (50 or 100 mg/kg, IP) on contextual fear memory in adolescent

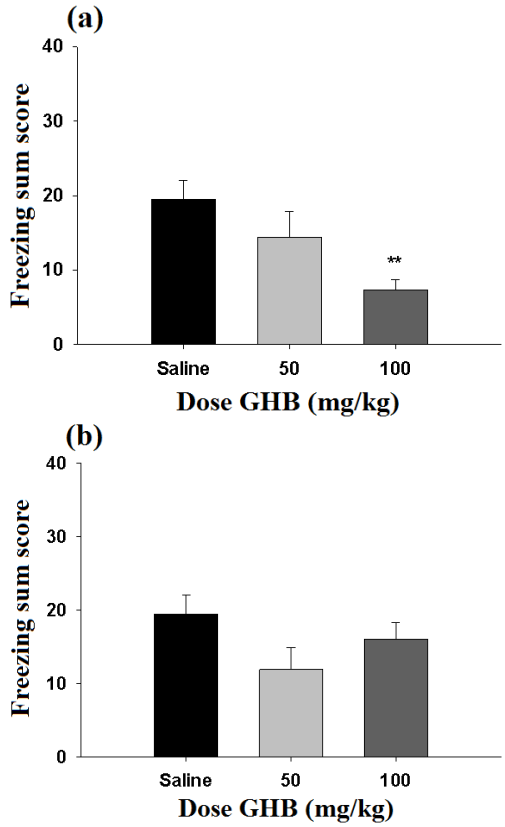


Figure 1: Effects of acute systemic administration of GHB (50 and 100 mg/kg, IP) on contextual fear memory in adolescent female rats. Mean (±sem) freezing sum scores during the observation period are shown (n = 12–14 per group). All behavioral testings were done on the test day, 24 h after training. (a) Effects of *pretraining* injections of GHB (GHB + saline group) or saline (saline + saline group) on freezing for contextual fear memory. All rats received saline injections 30 min before testing. Freezing behavior scores were obtained on the test day for animals pretreated with GHB or saline prior to conditioning. (b) Effects of *pretesting* injections of GHB on freezing for contextual fear memory. All rats received saline injection before training, and either saline or GHB injection prior to testing. Freezing behavior scores were obtained on the test day for all animals. $^{**}P < .01$ versus saline control.

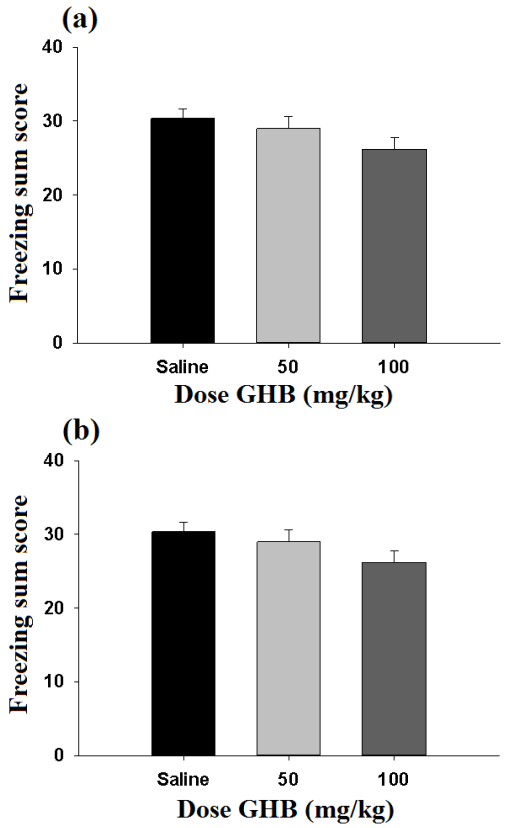


Figure 2: Effects of acute systemic administration of GHB (50 and 100 mg/kg, IP) or saline on auditory cued fear memory in adolescent female rats. Mean (±sem) freezing sum scores during the observation period are shown (n = 12–14 per group). All behavioral testings were done on the test day, 24 h after training. (a) Effects of *pretraining* injections of GHB (GHB + saline group) or saline (saline + saline group) on freezing for cued fear memory. (b) Effects of *pretesting* injections of GHB or saline on freezing for cued fear memory. Drug/saline treatment regime was as in Figure 1.

female rats is shown in Figure 3. There was a significant effect for dose of GHB on contextual fear memory ($F_{(2,37)} = 4.11$; $P < .05$), and *post hoc* comparisons showed a significant difference between the saline vehicle-treated and 100 mg/kg of GHB-treated rats ($P < .05$).

3.2.2. Experiment 2.2: Repeated systemic administration of GHB on cued fear memory

As shown in Figure 4, when rats were tested for auditory cued fear memory in the novel environment, repeated treatment with GHB (50 or 100 mg/kg, IP) did not change freezing behavior to the tone CS indicating that repeated GHB did not affect cued fear memory.

3.3. Experiment 3: Effect of single and repeated GHB treatment on freezing behavior

Adolescent rats treated with GHB or saline were introduced in the novel modified fear conditioning chamber, and freezing behavior was scored every 10 s for 3 min. No sound stimulus or foot shock was applied. There was no statistically significant difference in summed freezing score between drug-treated animals and saline-treated controls either following single acute GHB administration ($F_{(2,40)} = 1.96$; $P = .15$) or repeated GHB administration ($F_{(2,51)} = 0.49$; $P = .62$). Data are presented in Figure 5.

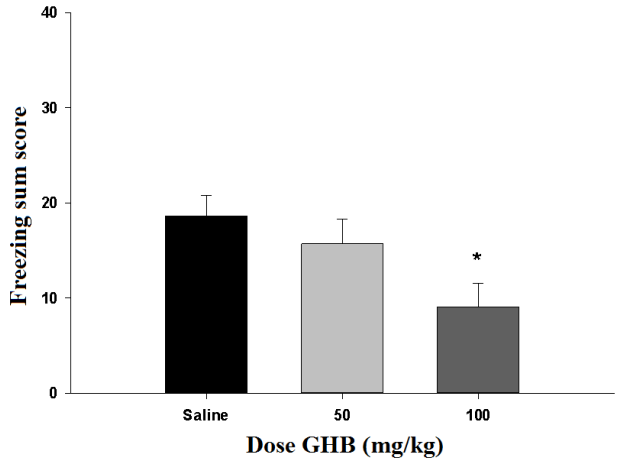


Figure 3: Effects of repeated administration of GHB on the acquisition of contextual fear conditioning. Rats were injected with one of two doses of GHB (50 or 100 mg/kg) or saline vehicle for five consecutive days. They received their last injections 30 min before training. Control rats received daily saline injections on the same days. All rats received saline injections prior to testing. Behavioral testings were done on the test day, 24 h after training. There was a significant effect for dose ($F_{(2,37)} = 4.11$; $P < .05$), and post hoc comparisons showed a significant difference between the saline vehicle and 100 mg/kg of GHB ($P < .05$). Values indicate mean ±sem with 13–14 rats in each group. $^{*}P < .05$ compared to saline control.

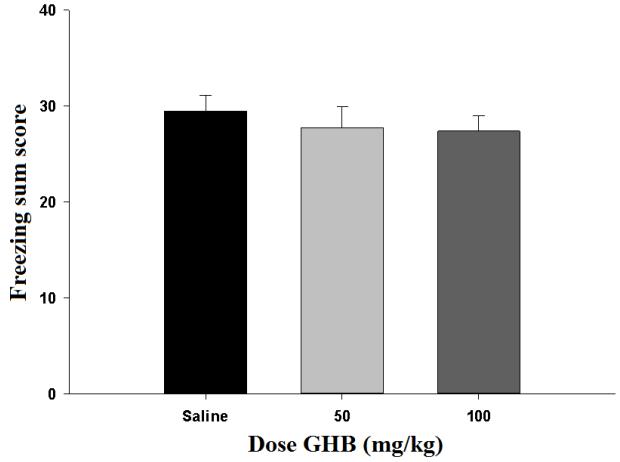


Figure 4: Effects of repeated administration of GHB on the acquisition of cued fear conditioning. Rats were injected with one of two doses of GHB (50 or 100 mg/kg) or saline vehicle for five consecutive days. They received the last injection 30 min before training. All rats received saline injections prior to testing. Behavioral testings were done on the test day, 24 h after training. There was no significant effect of GHB on cued fear memory. Values indicate mean ±sem ($n = 13$–14 rats).

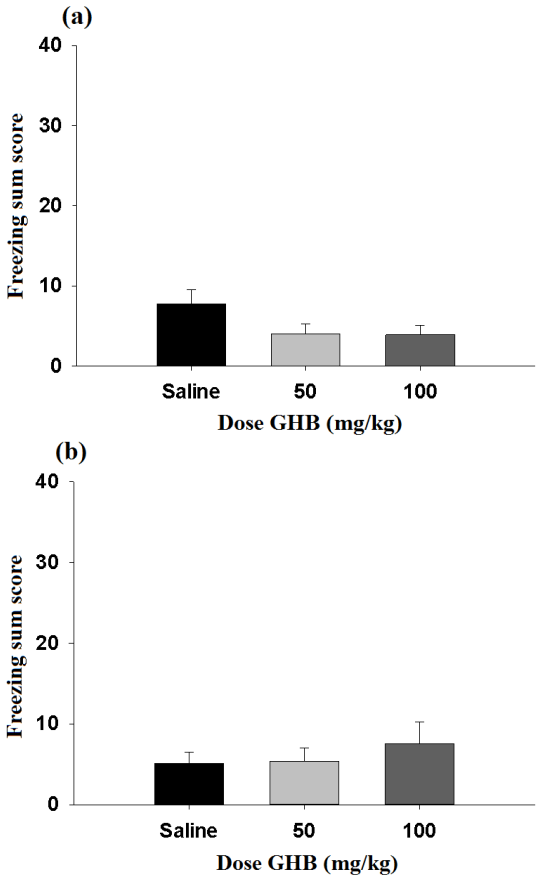


Figure 5: Effect of acute and repeated GHB on freezing behavior. Rats were injected with one of two doses of GHB (50 or 100 mg/kg) or saline vehicle: (a) on one day (acute GHB group) or (b) on five consecutive days (repeated GHB group) and trained for contextual fear conditioning. Twenty-four hours later, rats were placed in the novel chamber with no sound or foot shock, and scored for freezing behavior. There was no significant effect for GHB on freezing. Values indicate mean ±sem ($n = 12$–16 rats).

4. Discussion

The current series of experiments examined the impact of GHB exposure on contextual and cued learning and memory in adolescent female rats. Acute GHB exposure impaired the acquisition of contextual fear memory. Post-training GHB did not alter contextual fear conditioning suggesting that the expression of previously conditioned responses to the context (i.e., consolidation of memory) was not affected. Acute treatment with GHB did not affect the acquisition or expression of auditory cued fear responses. Consistent with the findings of the acute administration experiment, repeated treatment with GHB in adolescent female rats disrupted the acquisition of contextual fear memory but not that of auditory cued fear memory. One possible explanation for impaired learning in GHB-treated rats could be due to

alterations in sensitivity to shock stimulus. The present study did not specifically look into this, but if GHB had caused deficit in memory acquisition by altering sensitivity to the shock, then both contextual and cued conditioning would have shown the same level of impairment in response to GHB treatment.

In the present experiments, freezing to the context and tone were measured sequentially. The order of testing was randomized so that some rats were tested for contextual conditioning before cued conditioning and in others the order was reversed, that is, cued conditioning was measured before contextual conditioning. Contextual conditioning did not influence freezing to tone, and therefore, did not unduly bias the present results or conclusions. Other studies have also shown that the influence of contextual conditioning on freezing to tone is not robust [43,44,56]. The potential confound of GHB exerting any effect on habituation needs further investigation.

Exposure to relatively low doses of GHB acutely in adolescent rats disrupted contextual fear conditioning when administered prior to training. In rats that had learned the task, GHB did not affect contextual conditioning. In addition, GHB did not alter cued conditioning either when administered before training or before testing. Thus, GHB-induced disruption was specific to the acquisition of contextual memory without affecting acquisition of cued conditioning or expression of contextual or cued memory. The present findings are in line with our previous findings using the water maze where it was shown that repeated administration of GHB (100 mg/kg) in adolescent female rats disrupted the acquisition of spatial memory, but not its expression [51,52,53].

We selected a task that had both hippocampal-dependent and hippocampal-independent components. GHB preferentially affected contextual conditioning without any effect on tone conditioning. The fact that GHB had a more profound effect on the acquisition of a hippocampal-dependent task (contextual conditioning) than on a hippocampal-independent task (cued conditioning) supports the hypothesis that GHB preferentially disrupts information processing at the level of the hippocampus. Contextual fear acquisition involves configural or spatial learning [47], and is mediated by the hippocampus [43]. It is well established that fear conditioning to a target tone is mediated by the amygdala [30,33], and conditioning to context is primarily mediated by the hippocampus [44,45, 46,49,67]. Thus, the present data supports the hypothesis that GHB in adolescent rats disrupts hippocampal functions. Alcohol has been reported to disrupt contextual freezing in young animals, and this alcohol-induced memory deficit is associated with significant CA1 pyramidal neuronal loss [6, 35,59]. Some other brain areas that are thought to support contextual fear conditioning include parahippocampal structures [18,46], medial prefrontal cortex [20], medial geniculate nucleus [31], and cerebellum [48].

The effect of GHB on context conditioning does not appear to be state-dependent learning where the normal transfer of learning from training to the testing situation fails to occur due to changes in drug state between the two experimental sessions. When GHB was administered before training, it is possible that GHB's disruption of context conditioning could have been secondary to changes in state between training and testing. The fact that GHB decreased contextual conditioning but had no effect on tone conditioning in the same animal, and not vice versa (i.e., GHB impairing tone conditioning but not contextual conditioning) argues against this possibility. To examine the issue of state dependency more directly, animals were administered with GHB or saline after training, and then tested for contextual and cued conditioning. No measurable effect of GHB was observed on the expression of freezing to either the context or tone.

GHB binds to the specific GHB receptor as well as the GABA receptor. It acts indirectly through GHB-derived GABA at the high-affinity $GABA_A$ receptor and as a partial agonist at the $GABA_B$ receptor [1,55]. While many of the behavioral effects of high doses (> 200 mg/kg) of GHB are attributed to its effects on the $GABA_B$ receptor [9], it is still unclear which receptor mediates its relatively low dose effects on learning and memory. GHB receptors are particularly abundant in the hippocampus [26], and it is possible that GHB will exert its effects on contextual fear conditioning via these receptors. Therefore, it would be of interest to examine the effects of selective antagonists of the GHB receptor (e.g., NCS-382) and those of the $GABA_B$ receptor (e.g., CGP35348) and $GABA_A$ receptor (picrotoxin, bicucullin) on the GHB-induced reduction of contextual fear responses.

In conclusion, the present experiments demonstrate that both acute and repeated GHB treatment in adolescent female rat markedly disrupted the acquisition of hippocampal-dependent learning while sparing the acquisition of hippocampal-independent learning. GHB failed to alter both the acquisition as well as the expression of amygdala-based memory. These results support the hypothesis that GHB acts preferentially at the level of the hippocampus to inhibit information processing. The present results may help explain why individuals intoxicated with GHB may not remember specific experiences due to disruptions in hippocampal processing but have intact emotional memories [4,68]. Whether GHB impairs hippocampus-based cognitive functioning in male adolescent rat needs to be investigated.

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