Effects of post-training hippocampal injections of midazolam on fear conditioning

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Benzodiazepines have been useful tools for investigating mechanisms underlying learning and memory. The present set of experiments investigates the role of hippocampal \( \text{GABA}_A \)/benzodiazepine receptors in memory consolidation using Pavlovian fear conditioning. Rats were prepared with cannulae aimed at the dorsal hippocampus and trained with a series of white noise–shock pairings. In the first experiment, animals received intrahippocampal infusion of midazolam or vehicle immediately or 3 h after training. Then, 24 h later, freezing to the training context and the white noise were measured independently. Results show infusion of midazolam immediately, but not 3 h, after training selectively attenuates contextual fear conditioning. In the second experiment, animals received intrahippocampal infusions of an antisense oligodeoxynucleotide (ODN) targeting the \( \alpha_5 \) subunit of the \( \text{GABA}_A \) receptor or a missense control for several days prior to training and testing. Immediately after training, animals received an infusion of either midazolam or vehicle. Western blots conducted after testing showed a significant decrease in \( \alpha_5 \)-containing \( \text{GABA}_A \) receptor protein. This reduction did not alter the effectiveness of midazolam immediately after training at impeding context fear memory. Therefore, \( \alpha_5 \)-containing \( \text{GABA}_A \) receptors may not contribute to the effects of midazolam on context fear conditioning when given immediately post-training.
that α5-containing GABA<sub>A</sub> receptors may play a role in forms of learning that depend on the hippocampus.

Studies have used selective inverse agonists to investigate the role of particular GABA<sub>A</sub> receptor subunits in learning. For instance, Chambers et al. (2003) used a selective GABA<sub>A</sub> α5 receptor inverse agonist (L-655, 708) administered IP before training and found rats performed better than controls in a delayed “matching to place” version of the water maze task. Evidence from our laboratory suggests that the α5 subunit of the GABA<sub>A</sub> receptor has a role in contextual fear conditioning. Bailey et al. (2002) showed that pre-training intrahippocampal injection of the GABA<sub>A</sub> α5 preferring inverse agonist RY024 resulted in dose-dependent effects on contextual fear memory formation. Micro-injection of high doses of RY024 resulted in attenuated freezing to the context in which they were shocked, while lower doses increased freezing to the training context.

A recent study by Collinson et al. (2002) using a genetic knockout of α5-containing GABA<sub>A</sub> receptors in mice showed enhanced learning in the Morris water maze task, a test of spatial learning ability dependent on the hippocampus. Crestani et al. (2002) found that mice with a point mutation resulting in a selective decrease in α5-containing GABA<sub>A</sub> receptors in the hippocampal pyramidal layer demonstrated enhanced trace fear conditioning compared to wild-type controls. The same study also found contextual fear conditioning not affected in the mutant mice. This is surprising because both context and trace fear conditioning have been demonstrated to be dependent on the hippocampus (Kim and Fanselow 1992; Phillips and LeDoux 1992; Quinn et al. 2002). Data from Crestani et al. (2002) and Collinson et al. (2002) seem to suggest that desensitization of the α5-containing receptor to diazepam or disruption of its expression enhances performance in some hippocampal-dependent tasks while not affecting others.

Based on research indicating muscimol (Zarrindast et al. 2002; Rossato et al. 2004) and benzodiazepines (Jensen et al. 1979) impair memory at time points after training, the present experiments sought to test the importance of hippocampal GABA<sub>A</sub> receptors during early and late phases of consolidation. Animals received intrahippocampal infusion of midazolam or DMSO immediately or 3 h after training with pairings of white noise and shock. Results indicated that a significant impairment in contextual fear conditioning was seen when midazolam was infused immediately, but not 3 h, post-training.

The second experiment addressed the role of α5-subunit-containing GABA<sub>A</sub> receptors in memory impairment resulting from midazolam administration. Animals were pretreated with missense or antisense directed at α5-subunit-containing GABA<sub>A</sub> receptors for several days, trained in a fear conditioning task, and administered midazolam or saline into the dorsal hippocampus immediately after conditioning. The following day, animals again received antisense or missense infusions and were tested to the contextual CS. Tissue from these animals was immediately processed for Western blot analysis to determine the effectiveness of the ODN.

Results

No animals were excluded because of misplacement of cannulae. Placements were determined by taking 40-μm slices from the area of the cannulae tracks, staining them with cresyl violet, and identifying the injection sites as within the dorsal hippocampus (Fig. 1).

For Experiment 1 all data were subjected to One-Way Analysis of Variance (ANOVA) in which the drug (midazolam or DMSO) was the factor and freezing was the dependent measure. An ANOVA revealed normal fear-related behavior during acquisition. No significant differences during training baseline, CS-US pairings, or during the post-shock period (P > 0.05) were found (data not shown).

Figure 2A shows the percentage of freezing behavior during the context test session for all groups. Animals that received midazolam immediately after training froze significantly less than those that received control infusions (F<sub>1,10</sub> = 6.654, P < 0.03). Figure 2A also shows percentage of freezing behavior during the context test session for animals that received midazolam or DMSO 3 h after training. There were no differences between groups with infusion 3 h post-training during the context test
midazolam administration. Data from Experiment 2 were subjected to a two-way ANOVA with midazolam and oligodeoxynucleotide (ODN) as factors and freezing as the dependent variable. For training, no significant differences were seen during the CS-US presentation or post-shock ($P > 0.05$). However, during baseline, animals that received antisense froze significantly more than those that received missense, although freezing behavior was below 1% for all groups ($F_{1,20} = 5.455$, $P > 0.03$). This difference in freezing is likely spurious because freezing during baseline is extremely low (<1%) and animals froze normally during both the shock and post-shock period, thus suggesting normal fear conditioning.

A two-way ANOVA conducted on data from the context test revealed a main effect for midazolam ($F_{1,20} = 6.082$, $P < 0.03$). As shown in Figure 3A, animals that received midazolam froze significantly less than those that received saline, regardless of ODN treatment. There was no main effect during the context test for the group that received ODN and there was also no interaction between ODN and midazolam ($P > 0.05$).

Western blot analysis revealed a significant decrease in the expression of α5 subunit protein between rats that received antisense and those that received missense treatment ($t_{15} = 2.383$, $P < 0.05$). Analysis of antisense-treated animals indicated that there was a 48% decrease in relative optical density of the antisense ODN group signal compared to missense ODN-treated controls (Fig. 3B). Importantly, this result indicates that the antisense administration produced a significant decrease in the number of α5-containing GABA$_A$ receptors in the hippocampus.

**Discussion**

We evaluated the role of intrahippocampal infusion of the GABA$_A$ agonist midazolam when given immediately or 3 h after Pavlovian fear conditioning. Results from the context test show that immediate post-training intrahippocampal infusion of midazolam attenuates freezing. These results are in agreement with data that demonstrate GABA$_A$ receptors are important for memory consolidation (Jensen et al. 1979; Fanselow and Helmstetter 1988; Dickinson-Anson and McCaugh 1993). In addition, Igaz et al. (2002) showed that infusion of an inhibitor of mRNA synthesis revealed a time period around training and another time period 3–6 h after training in which memory is sensitive to disruption. Rosato et al. (2004) have shown intrahippocampal infusion of muscimol impaired one trial inhibitory avoidance when infused immediately but not when infused >30 min post-training. These data, along with our present findings suggest that the contribution that the GABA$_A$ receptor within the hippocampus makes to memory consolidation is during the initial time period after training.

As expected, no differences were found during the auditory cue test for either immediate or 3-h post-training group in either drug condition. This finding supports research showing a crucial role for the hippocampus in memory for the context but not the auditory cue (Kim and Fanselow 1992; Phillips and LeDoux 1992). Animals in the present experiments froze comparably to control animals during the discrete cue presentation. This finding rules out the possibility that impairments seen during the
context test were due to an inability to perform the freezing behavior or to hyperactivity.

Results from Experiment 2 demonstrate that animals that received intrahippocampal midazolam immediately after training, regardless of ODN treatment, showed impaired context fear memory. In particular, the group treated with antisense followed by saline showed learning that was equivalent to missense controls, while antisense followed by midazolam attenuated contextual fear conditioning. These findings suggest the α5-containing GABA$_\text{A}$ receptor does not play a critical role in the midazolam-induced deficit in context fear memory.

Western blot analysis conducted on animals from Experiment 2 showed a significant decrease (−48%) in α5GABA$_\text{A}$ receptor protein levels in animals treated with antisense compared to missense controls. Thus, the null effect found with antisense is not likely due to an ineffective decrease in the target protein. Work from Crestani et al. (2002) using mice with a point mutation that selectively reduced the number of α5-containing GABA$_\text{A}$ receptors in the hippocampus found no effect on unsignaled contextual or delay fear conditioning but enhanced trace fear conditioning. The lack of an effect with antisense suggests the α5GABA$_\text{A}$ receptor may support some forms of hippocampal dependent memory such as trace fear conditioning (Crestani et al. 2002) and Morris water maze (Collinson et al. 2002), but not others.

The present findings show that administration of midazolam immediately post-training impairs contextual fear conditioning. Furthermore, an ODN directed at α5-containing GABA$_\text{A}$ receptors had no effect on contextual fear conditioning and did not contribute to the memory-impairing effect of post-training infusion of midazolam. Later phases of consolidation, on which mRNA and protein synthesis inhibitors were effective, were not influenced by midazolam. These results support the early involvement of the hippocampal GABA$_\text{A}$ receptor in contextual fear memory consolidation.

Materials and Methods

Subjects

A total of 60 naïve male Long Evans rats were obtained from Harlan weighing ∼300–350 g. All animals were individually housed in stainless steel hanging cages in a temperature and light controlled vivarium and maintained on a 14-h light/10-h dark cycle. Experiments took place during the light portion of the cycle. Food and water were available ad libitum. All procedures were carried out with approval of the Animal Care and Use Committee.

Surgery

Rats were implanted with bilateral cannulae aimed at the dorsal hippocampus. Before surgery animals were anesthetized with IP injections of ketamine (100 mg/kg body weight) and sodium pentobarbital (2.5 mg/kg per rat). The animals were mounted into a Kopf stereotaxic frame used to position the 26-gauge stainless steel guide cannulae in the dorsal hippocampus (AP = −3.5, L +/− 2.6, V = −3.0). Coordinates were chosen based on a rat brain atlas ( Paxinos and Watson 1998). The internal cannulae were 0.5 mm longer than guide cannulae. The cannulae were anchored to the skull using stainless steel screws and acrylic cement. Then 33-gauge obdurators were inserted into the guide cannulae to prevent blockage.

Apparatus

Auditory fear conditioning was conducted in a set of four Plexiglas and stainless steel observation chambers (Contex A). The floor was comprised of 18 stainless steel bars 5 mm in diameter spaced 12 mm apart and connected to a shock generator. Ventilation fans produced 62–64 dB of background noise. The shock generator was set to deliver a 1-mA footshock. Each chamber was equipped with a speaker centered in the middle of one end of the chamber. Before and after testing of each animal, Context A was cleaned with a 5% ammonium hydroxide solution.

To increase context discriminability, the floor of Context B was comprised of Plexiglas. Additionally, the walls were a different shape and the chamber had an olfactory cue (5% acetic acid solution) different from that of Context A. Each chamber was enclosed in a sound-attenuating box and illuminated with a white light. The overhead ventilation fans in Context B produced 60–64 dB of background noise.

Drug

For the first experiment, midazolam (2 µg/µL) was dissolved in 100% dimethyl sulfoxide (DMSO). For the second experiment, antisense and missense ODNs were dissolved in sterile saline to a concentration of 5 nm/µL (Midland Certified Reagent Co.). The nucleotide sequence for the α5GABA$_\text{A}$ receptors was provided by Ewa Malatyńska (Dept. of Pharmacology, IUSM). The sequence was located within nucleotide bases 391–408, gene accession number LO8494 (GenBank). The α5GABA$_\text{A}$ antisense ODN sequence was 5′-TGTTCTATCCTTGACG-3′. The missense sequence had the same nucleotide composition as the α5GABA$_\text{A}$ antisense ODN; however, the ODN order was transposed to create a novel sequence, 5′-GGTCTATCCTTGACG-3′. All drugs were prepared on the day of infusion.

Procedure Experiment 1

One week after surgery animals were habituated to the handling and injection procedure. To habituate the animal to the microinjection procedure each rat was restrained in a towel, the obdurators were removed, and the scalp was cleaned. After this was complete, the obdurators were replaced and the animal was returned to its home cage. This was repeated once a day for 4 d.

Training involved a 6-min baseline followed by four white noise (72 dB, 10 sec)-shock (1 mA/1 sec) pairings separated by a 90-s intertrial interval. After a 4-min post-shock period, animals were removed from the training context (Context A). Immediately or 3 h after removal from the training context, animals received a 0.5-µL/side infusion of midazolam (N = 14) or DMSO (N = 14) into the hippocampus. Prior to infusion, drugs were coded to ensure that the experimenter was blind to the groups. The volume of the infusion was given over 60 sec. The infusion cannula remained in place for 90 additional seconds to ensure diffusion away from the tip. Animals were then returned to their home cage.

Approximately 24 h later, animals were placed into Context A as well as a novel context never paired with shock (Context B), in a counterbalanced order. The context test involved exposing animals to Context A for 15 min. During the white noise test, animals were placed in Context B for 15 min in which a 6-min baseline was followed by 5 min of continuous exposure to the white noise. Rats were removed from the chamber 4 min after the white noise ended and taken to their home cage.

To verify that any action produced by the drug administered was temporary, and not due to toxicity, the animals were retrained and rested 1 wk after original training with all the same parameters as original training and testing.

After completion of testing, animals were euthanized with an IP injection of sodium pentobarbital (100 mg/kg). Animals were transcardially perfused with saline followed by 10% buffered formalin solution. Heads, with cannulae intact, were placed in 10% formalin solution for at least 24 h. The brains were then extracted from the skull and placed in a 30% sucrose formalin solution until they were ready to section.

Procedure Experiment 2

Animals were habituated to the handling procedure after surgery as in Experiment 1. One week after surgery animals received 0.5-µL/side microinjections of antisense (N = 13) or missense (N = 11) for 6 d. Prior to infusion, drugs were coded to ensure that the experimenter was blind to the groups. The volume of the
infusion was given over 60 sec. The infusion cannula remained in place for 90 sec to ensure diffusion away from the tip. Animals were then returned to their home cage. On the fifth day, animals were trained in Context A as in Experiment 1. Immediately after training, animals were removed from the chamber and taken to an injection room different from that used for antisense or missense infusions. The antisense and missense groups were further divided into four groups. The groups included: antisense/saline (N = 7), antisense/midazolam (N = 6), missense/saline (N = 5), and missense/midazolam (N = 6). Either midazolam or saline was then administered (0.5 µL/side) with the same parameters as the antisense/missense infusions, and all animals were returned to their home cage. About 5 h prior to testing, animals were given a final infusion of antisense or missense. Testing involved 15 min of re-exposure to Context A. After testing, animals were given an overdose of ETOH/pentobarbital (100 mg/kg, IP) solution and decapitated. Brains were immediately removed and frozen.

Data analysis
All sessions for Experiment 1 and 2 were videotaped for subsequent behavior analysis. Animals were scored as active or freezing once every 4 sec for the entirety of the session by an experimenter blind to the groups. The percentage of time spent freezing acted as an indicator of conditioning strength. Freezing was defined as lack of all movement except that related to breathing (Fanselow 1980). All behavior other than freezing was scored as generally active. During training and white noise tests, behavior was separated into baseline, CS-US pairings, or CS presentation, and post-shock or post-CS groups were compared using ANOVAs. For the context test, freezing behavior was averaged across the entire session, and group differences were analyzed using ANOVA.

Western blots were prepared from protein samples from a subset of animals in Experiment 2. Data from the antisense (N = 9) and missense (N = 6) groups were quantified using relative optical density (ROD). For both the missense and antisense groups, a “percentage of control” score was derived for each rat by dividing each animal’s ROD score by the missense control group mean. This percentage of control score was then compared using an Independent Samples t-test.

Western blot procedure
Hippocampi from animals in Experiment 2 were dissected out, homogenized in buffer (1.096 g of sucrose, 0.0076 g of EDTA, 86.2 µg of PMSF, 10 µg of leupeptin, and 1 mL of 10% SDS), and immediately placed on dry ice. Samples were stored at −80°C until needed. Samples were thawed and then centrifuged at 4000 rpm for 20 min. A Lowry protein assay was performed on the supernatant to quantify the total amount of protein in each sample. Following primary antibody exposure, the membranes were exposed to secondary antibody (Santa Cruz Biotechnology: 1:150 concentration) for 4 h at room temperature. Following primary antibody exposure, the membranes were exposed to secondary antibody (Santa Cruz Biotechnology: 1:1500 concentration) for 90 min. Membranes were washed thoroughly, exposed to a chemiluminescence solution (Santa Cruz Biotechnology), and exposed to autoradiographic film (Hyperfilm MP). The bands were quantified using densitometry software (MCID, M4).

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