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The Role of GABA and Anxiety in the Reconsolidation of Conditioned Fear

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The current study examined the effects of systemic administration of a GABA agonist [midazolam (MDZ)] and a GABA antagonist (bicuculline) on fear responding after brief CS exposure, a procedure thought to involve memory reconsolidation. Using a contextual fear-conditioning paradigm, rats were initially given two context-shock training trials, followed 24 hrs later by a 90-s context exposure (reactivation), and 24 hrs later by a 3-min context test. In Experiment 1, MDZ (2 mg/kg, i.p.), whereas in Experiment 2, bicuculline (1 mg/kg, i.p.), was administered immediately after reactivation. MDZ reduced conditioned freezing, whereas bicuculline only marginally potentiated conditioned freezing. The MDZ fear disruption effect did not occur in the absence of reactivation, and was evident 10 days after the initial test. Experiment 3 induced high levels of baseline anxiety using the single prolonged stress paradigm, and replicated the essential procedure of Experiment 1. Results indicated that MDZ fear disruption did not differ between high and low anxiety rats. The data suggest the involvement of GABA receptors in reconsolidation processes, and the possible clinical use of MDZ in fear reduction with brief reexposure.

Keywords: reconsolidation, midazolam, anxiety, single prolonged stress, fear

Memory consolidation has been described as the "postacquisition stabilization of long term memories" (Dudai, 2004, p. 51), whereby newly formed short term memories progressively become more resilient as they are transformed into stable long term memories (McGaugh, 1966, 2000). In contrast to the traditional notion that a memory consolidates only once, gaining in stability and strength over time, empirical work by Lewis (e.g., Misanin, Miller, & Lewis, 1968) and Nader (e.g., Nader, Schafe, & LeDoux, 2000) has challenged this view by showing that stable, long-term memories can be disrupted if the memory is reactivated before the administration of amnestic agents. This process is known as memory reconsolidation and is believed to play an important role in long term memory processing (Dudai, 2006; Nader, 2003; Tronson & Taylor, 2006).

Memory reconsolidation has been reported in a large variety of species, including rodents (Nader et al., 2000), crabs (Pedreira, Perez-Cuesta, & Maldonado, 2002), sea slugs (Child, Epstein, Kuzirian, & Alkon, 2003), snails (Gainutdinova et al., 2005), chicks (Salinska, Bourne, & Rose, 2004), honey bees (Stollhoff, Menzel, & Eisenhardt, 2005), round worms (Rose & Rankin, 2006), and humans (Walker, Brakefield, Hobson, & Stickgold, 2003). Neuroanatomical evidence has implicated the basolateral amygdala (Nader et al., 2000) and hippocampus (Debiec, LeDoux, & Nader, 2002) in reconsolidation. These data, coupled with recent neurochemical evidence (Rose & Rankin, 2006; Valjent et al., 2006), provide convincing evidence for the specificity and evolutionary significance of the reconsolidation process (Nader, 2003).

However, the reconsolidation literature does not lack controversy. Although many behavioral and pharmacological studies have shown that reconsolidation is a crucial process in the maintenance of long term memory (for reviews, see Nader, 2007; and Tronson & Taylor, 2006), other studies have failed to disrupt memory after retrieval (Cammarota, Bevilaqua, Medina, & Izquierdo, 2004) or demonstrated only transient effects (Lattal & Abel, 2004; Prado-Alcala et al., 2006). These negative results suggest that there must be parameters outside of which memories cannot be permanently disrupted. Tronson and Taylor (2006) recently argued that the strength and age of the memory, as well as the length of the reactivation session, influences the extent to which reconsolidation occurs.

Reconsolidation also has potential clinical implications. Pharmacological agents frequently have been investigated in extinction studies to determine their efficacy in fear reduction (Davis, Myers, Chhatwal, & Ressler, 2006; Ledgerwood, Richardson, & Cranney, 2003, 2004, 2005). An alternative method of reducing fear could utilize pharmacological agents to disrupt the reconsolidation of fear. Many disrupting agents such as the protein synthesis inhibitors, hypothermia and NMDA antagonists cannot be used with humans. In contrast, benzodiazepines (BDZs) are currently prescribed for selected disorders and are deemed relatively safe for humans.

BDZs facilitate GABA-mediated neurotransmission in the central nervous system (Mao, Guidotti, & Costa, 1975), and the role of GABA has been widely studied in fear conditioning. GABA antagonists (e.g., bicuculline; Castellano & McGaugh, 1990) have been known to enhance memory consolidation, whereas GABA

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agonists (e.g., muscimol; Akirav, Raizel, & Maroun, 2006) have been shown to interfere with memory consolidation. The data suggest that GABA receptors are functionally involved in the formation and stabilization of memories.

Although many studies have shown evidence for its role in memory consolidation, to our knowledge only one study has examined the role of GABA in memory reconsolidation processes. Bustos, Maldonado, and Molina (2006) demonstrated that the GABA agonist midazolam (MDZ) appears to disrupt reconsolidation processes (i.e., lead to decreased responding) if administered after a brief reactivation session. The induced amnesia was long lasting and specific to the training context.

The current study aimed to partially replicate and extend Bustos et al.'s (2006) study. In Experiment 1, reconsolidation protocols were established by examining the role of MDZ in the reduction of fear following brief CS reexposure (reactivation). Experiment 2 investigated whether bicuculline, a GABA antagonist, would yield results opposite to Experiment 1, presumably by potentiating memory reconsolidation. Finally, Experiment 3 determined whether the MDZ disruption of fear responding would be modulated by preexisting baseline anxiety.

Experiment 1: Effects of MDZ on Memory Reconsolidation

The aim of this experiment was to replicate Bustos et al.'s (2006) MDZ effects, using slightly different parameters in our laboratory conditions. It was hypothesized that following memory reactivation, systemic infusion of MDZ would impair reconsolidation of a contextual fear memory.

Materials and Method

Subjects and Design

Thirty-six male Sprague–Dawley rats (220-320 g) were acquired from the Gore Hill Research Laboratories, Sydney, Australia. Rats were housed in groups of eight in plastic boxes (67 cm long × 40 cm wide × 22 cm high) in a colony room maintained on a 12-hr light–dark cycle. Rats were given unlimited access to food and water. Before each experiment, rats were handled for approximately 3 minutes each on at least three separate occasions. A 2 (Drug: MDZ, SAL) × 2 (Condition: R – reactivation, NR – no reactivation) design was employed in this experiment, with rats being randomly assigned to the four groups (n = 9, R-SAL, R-MDZ, NR-SAL, NR-MDZ; see Table 1). The dependent variable was percent freezing during test. All procedures used in all three experiments were in accordance with the ethical guidelines established by the American Psychological Association and approved by the Animal Care and Ethics Committee of the University of New South Wales.

Apparatus

There were four standard conditioning chambers where the rats were conditioned, reactivated, and tested (20 cm long × 12 cm wide × 12 cm high). Each chamber consisted of a Perspex ceiling, and rear wall, stainless steel mesh sides, and a hinged Perspex front door, which shut magnetically. The floor was made of stainless steel rods that were 2 mm in diameter and spaced 13 mm apart (center to center). Each floor was located 8 cm above a stainless steel tray, which was used to collect urine and boli. Unscrambled 50-Hz AC shock from a constant current generator (developed and constructed at the University of New South Wales) was delivered to the floor of each chamber. The chambers were contained in pairs in two sound-attenuating wooden cabinets, and each chamber was separated from the other by a solid wooden timber partition within each cabinet. Each of the chambers could be viewed through a Perspex window located at the front door of each cabinet. To prevent the rats from being distracted by extraneous visual stimuli, a 15-W red light bulb illuminated each chamber and the experimental room was illuminated in red light. In addition, video cameras were mounted at the back of each wooden cabinet so that the rats within each of the four chambers could be simultaneously video-recorded. Before each session, two of the chambers were wiped with 1.0% vanilla (in tap water) and the alternate two with 0.5% acetic acid (in tap water). All programming, timing, and shock stimulus presentations were computer controlled.

Drugs

MDZ (source: Sigma Aldrich, Sydney, Australia) was diluted in sterile isotonic saline (SAL) (0.9% wt/vol) and injected intraperitoneally (i.p.) at a dosage of 2.0 mg/ml/kg. Although 1.0 mg/kg of MDZ was previously shown to induce amnesia (Bustos et al., 2006), pilot studies did not find it to be adequate in our laboratory.

Procedures

Fear conditioning. Fear conditioning took place in the chambers as described above. Rats were placed in the conditioning chamber for 3 minutes. At the end of this period, a 0.8-s, 0.8-mA shock was delivered through the grid floor of the chamber. Thirty

Table 1
Procedure for Each of the Four Groups in Experiment 1

	Procedure				
Group	Day 1	Day 2	(Postinjection)	Day 3	Day 13
R-MDZ	Conditioning	Reactivation	MDZ	Test 1	Test 2
R-SAL	Conditioning	Reactivation	SAL	Test 1	Test 2
NR-MDZ	Conditioning	Handling	MDZ	Test 1	Test 2
NR-SAL	Conditioning	Handling	SAL	Test 1	Test 2

Note. MDZ = midazolam; NR = no reactivation; R = reactivation; SAL = saline.

seconds after the first shock, a second shock of the same duration and intensity was delivered. Rats remained in the chambers for a further 30 seconds before being removed and placed back in their colony box. Placement of rats from the four groups was counterbalanced across the four chambers.

Memory reactivation. Twenty-four hours after conditioning, rats that received reactivation (see Table 1) were put back in the chambers in which they received conditioning for a total of 90 seconds without any delivery of shock. Immediately after reactivation, the rats were injected i.p. with either MDZ (R-MDZ) or SAL (R-SAL). Rats were then placed back in their colony box. During every 2.5-s period (signaled by a beep), the rater sequentially observed and recorded the behavior of two rats, followed by a second 2.5-s period for the remaining two rats (all being tested simultaneously). Behavior was also video-recorded for future reference. Freezing was scored as the absence of movement except for that related to respiration (Fanselow, 1994). Across Experiments 1 to 3, a second observer, who was unaware of the rats' group designation, rescored reactivation and test freezing for a random sample of rats across all groups. The interrater reliability between the two observers for Experiment 1 was $r = .99$, $p < .05$.

Rats that did not receive reactivation instead received handling by the experimenter for a similar amount of time in a room adjacent to that containing the conditioning chambers, where all the colony boxes were held during the testing. Handling consisted of picking up and stroking each animal for approximately 90 seconds. Immediately after handling, rats received i.p. injections of either MDZ (NR-MDZ) or saline (NR-SAL). The rats were then placed back into their colony boxes.

Fear memory testing. Rats were placed back in the training context and freezing behavior was scored and video-recorded for 3 minutes. Testing took place 24 hours after the reactivation procedure (Test 1). In Experiments 1 and 2, an additional test occurred 10 days after the initial test (Test 2; see Table 1).

Results and Discussion

Mean freezing during the reactivation session for all R rats was 63.22% ($SD = 24.52$), and there was no difference between the R–SAL and R–MDZ groups. In general, this experiment found evidence to support the hypothesis that MDZ impairs reconsolidation of contextual fear memories. Figure 1 indicates that for both Test 1 and 2, the R-MDZ group displayed less freezing than all other groups. This pattern of results was confirmed by a two-way ANOVA of freezing scores during Test 1 that yielded an effect of Condition (R vs. NR) [$F(1, 32) = 18.75$, $p < .05$], no Drug effect [$F(1, 32) = .48$, $p > .05$], and a significant Condition $\times$ Drug interaction [$F(1, 32) = 6.5$, $p < .05$] (see Figure 1). Scheffé analyses confirmed that the R–MDZ rats showed significantly lower levels of freezing compared to the R–SAL rats [$F(1, 32) = 5.24$, $p < .05$], while there was no difference between the NR–MDZ and NR–SAL rats. In addition, a Scheffé analysis showed that the tendency for the R–Sal rats to display less freezing than the two NR groups was not significant.

Figure 1 indicates that the pattern of results found in Test 2 is similar to that in Test 1. As expected, a two-way ANOVA of Test 2 data showed that the R rats displayed less freezing than the NR rats [$F(1, 32) = 6.52$, $p < .05$], and a significant Condition $\times$ Drug interaction [$F(1, 32) = 8.89$, $p < .05$] indicated that this effect was dependent on whether MDZ was administered. This was confirmed by a Scheffé analysis, whereby R-MDZ rats showed significantly lower levels of freezing compared to the R–SAL rats [$F(1, 32) = 7.23$, $p < .05$], whereas there was no difference between the NR–MDZ and NR–SAL rats. In addition, a $2 \times 2 \times 2$ (Condition $\times$ Drug $\times$ Test) ANOVA confirmed that there was no significant change in freezing from Test 1 to Test 2. Overall, this pattern of results is similar to that reported by Bustos et al. (2006) who used slightly different experimental parameters and a dose of 1 mg/kg MDZ.

Experiment 2: Effects of Bicuculline on Memory Reconsolidation

Past studies suggest that while GABA agonists impair memory formation (Degroot & Parent, 2001), GABA antagonists enhance memory consolidation (Helm et al., 2005). In Experiment 1, the systemic administration of the GABA agonist MDZ appears to have blocked the reconsolidation of contextual fear memory. Experiment 2 explored the possibility that systemic administration of the GABA antagonist bicuculline would lead to increased conditioned

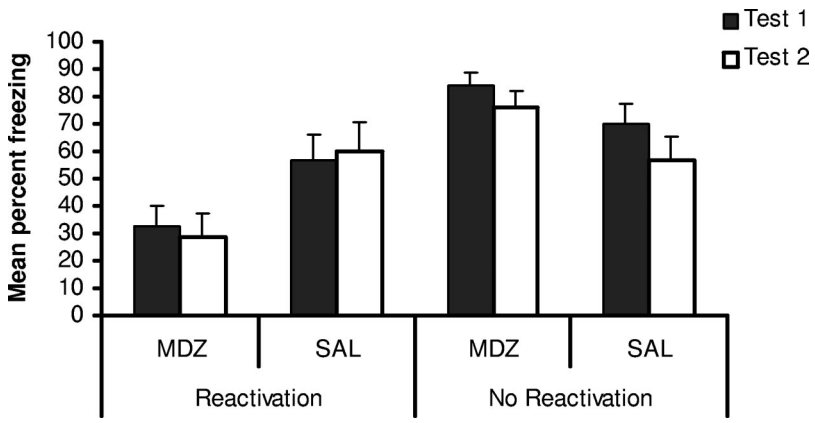

Figure 1. Mean percent test freezing (+*SEM*) as a function of Condition (Reactivation, No Reactivation), Drug (MDZ, SAL), and Time (Test 1, Test 2). (MDZ = Midazolam; SAL = Saline.)

fear and thus appear to potentiate reconsolidation. This effect was expected to last up to 10 days after the first test.

Materials and Method

Subjects

Thirty-two male Sprague–Dawley rats weighing 433 to 768 g were used in this experiment. They were obtained from the same source and at the same time as those rats in Experiment 1, but run 2 months later. They were maintained in the same conditions as described in Experiment 1. A 2 (Drug: bicuculline, SAL) × 2 (Condition: R, NR) design was employed, with rats being randomly assigned to the four groups ($n = 8$, R-BIC, R-SAL, NR-BIC, NR-SAL; see Table 2). The dependent variable was freezing during test.

Drug Administration and Procedure

Bicuculline ([-] – Bicuculline methiodide, Sigma-Aldrich, B6889) was freshly dissolved in sterile isotonic saline (0.9% wt/vol) and injected i.p. at a dosage of 1.0 mg/ml/kg immediately after reactivation (R–BIC group) or handling (NR–BIC group). The dose of bicuculline was chosen on the basis of a dose response curve undertaken in pilot work by Graham (2006) who studied the effect of bicuculline in a conditioned freezing extinction paradigm. Saline rats were injected with isotonic saline in a volume of 1.0 ml/kg. After injections, rats were returned to their home cages. Experimental procedures were identical to those described in Experiment 1. Behavior was scored for freezing and videotaped for future reference. The interrater reliability between the two observers for Experiment 2 was $r = .97$, $p < .05$.

Results and Discussion

Similar analyses to that of Experiment 1 were undertaken. Mean freezing during the reactivation session for all R rats was 77.15% ($SD = 21.88$), with no differences between groups. In general, this experiment found limited evidence to support the hypothesis that bicuculline enhances reconsolidation of contextual fear memories. Figure 2 shows that the R–BIC group displayed more freezing than all other groups at Test 1, but not at Test 2. This pattern of results was only marginally confirmed by a two-way ANOVA of freezing scores that yielded an effect of Condition (R vs. NR) [$F(1, 28) = 6.21$, $p = .02$], no Drug effect [$F(1, 28) = .17$, $p > .05$], and a marginal Condition × Drug interaction [$F(1, 28) = 4.00$, $p = .056$]. Scheffé analysis found no difference between the R–BIC rats and R–SAL rats, or between the NR–BIC and NR–SAL Rats.

There were no significant main or interaction effects for Test 2. A 2 × 2 × 2 (Condition × Drug × Test) ANOVA similar to Experiment 1 yielded no significant effects. Overall, this pattern of results gives little support for the notion that the administration of bicuculline potentiates reconsolidation.

There are several possible reasons why bicuculline did not have a strong facilitating effect in this experiment. First, although there appears to be an ordinal difference in freezing consistent with bicuculline enhancement of freezing, this effect may have been obscured by a ceiling in freezing responding. Second, behavioral facilitating effects are generally less robust than behavioral disruptive effects. Either higher doses of the facilitating agent, or direct administration of the agents to the likely neural areas involved (e.g., hippocampus and amygdala), could lead to a clearer behavioral facilitation. Third, an inverse agonist such as FG7142 may be more effective in blocking the memory inhibitory effects of GABA. Fourth, the rats in Experiment 2 were significantly larger than those used in Experiment 1, and this may have influenced pharmacokinetics, or aspects of the learning experience. Fifth, with an n of 8 per group, this experiment may have been under powered. In summary, further systematic research utilizing FG7142 with standard-size rats should be undertaken before the concluding that GABA antagonists do not facilitate fear when administered after a short CS reexposure.

Experiment 3: Preexisting Anxiety and MDZ Disruption of Memory Reconsolidation

Experiment 3 aims to combine the reconsolidation procedures of Experiment 1 with a single prolonged stress (SPS) procedure (Khan & Liberzon, 2004) to examine the potential moderating effects of preexisting differences in anxiety in a fear reconsolidation paradigm. If reconsolidation blocking is to be considered as a potential form of therapy, it is important to determine whether high anxiety individuals are as susceptible to MDZ reconsolidation blocking, as are low anxiety individuals.

Extinction-like procedures, called exposure therapy, are a dominant therapeutic approach in the treatment of human anxiety disorders. Extinction and reconsolidation procedures share a similar retrieval phase in that both paradigms require exposure to a nonreinforced presentation of the CS. Thus, it has been suggested that the two processes might have similar molecular networks (Dudai, 2004). However, the exposure period is usually very short

Table 2
Procedure for Each of the Four Groups in Experiment 2

	Procedure				
Group	Day 1	Day 2	(Postinjection)	Day 3	Day 13
R-BIC	Conditioning	Reactivation	BIC	Test 1	Test 2
R-SAL	Conditioning	Reactivation	SAL	Test 1	Test 2
NR-BIC	Conditioning	Handling	BIC	Test 1	Test 2
NR-SAL	Conditioning	Handling	SAL	Test 1	Test 2

Note. BIC = bicuculline; NR = no reactivation; R = reactivation; SAL = saline.

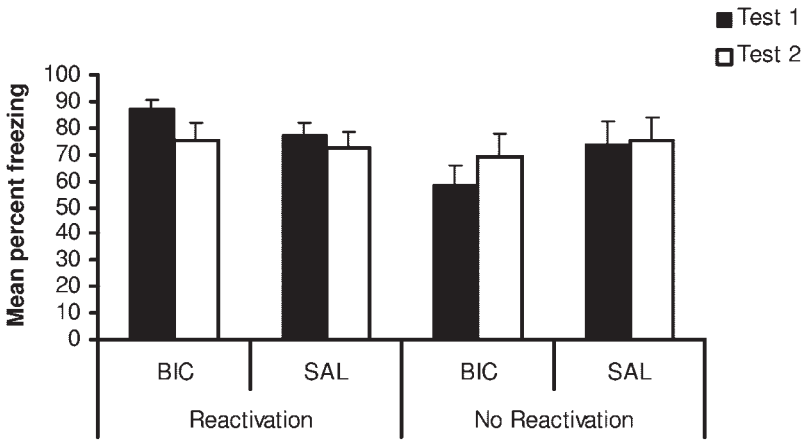

Figure 2. Mean percent test freezing (+*SEM*) as a function of Condition (Reactivation, No Reactivation), Drug (BIC, SAL), and Time (Test 1, Test 2). (BIC = Bicuculline; SAL = Saline.)

during reactivation. If a disrupting agent is administered, it will result in amnesia, which may involve erasure of the original CS-US association. Extinction, on the other hand, requires a longer retrieval session and can consist of more than one presentation of the CS. The reduced freezing seen on test may not be a result of erasure, but the formation of a new inhibitory CS-no US association (Bouton, 1994). Considering that extinction and reconsolidation procedures share certain similarities, the question of interest is whether high anxiety patients, who exhibit higher resistance to extinction-based therapy in comparison to normal patients (Grillon, 2002), would similarly show resistance to reconsolidation blocking.

Panic disorder, generalized anxiety disorder (GAD), and PTSD patients have exhibited reduced levels of $GABA_A$ receptors (Bremner et al., 2000). Rodent studies have similarly found attenuated GABA levels in the hippocampus of SPS rats (Harvey, Oosthuizen, Brand, Wegner, & Stein, 2004). Topiramate, a substance with GABA stimulating properties, has been found to reduce the exaggerated acoustic startle responding in SPS rats (Khan & Liberzon, 2004). These findings strongly suggest that GABA neurons are implicated in SPS and high anxiety. Specifically, high anxiety rats are expected to have less $GABA_A$ receptors for BDZ binding compared to normal rats, thus MDZ will have less effect.

In Experiment 3 we investigated potential differences between high and low anxiety rats in initial fear, reconsolidation, and extinction mechanisms. We expected to replicate Takahashi, Milekic, Monti, and Alberini's (2006) finding that the more anxious SPS rats show more initial contextual freezing than do control rats. Given the possible decreased receptivity to MDZ in SPS rats, we expected that the high anxiety SPS rats would not show as much fear disruption when administered MDZ after the reactivation procedure. These differences should be long lasting as was found in Experiment 1. We then explored the possibility of differences in subsequent extinction training as a function of prior SPS experience, expecting that the high anxiety SPS rats would show resistance to extinction (Lissek et al., 2005). Finally, SPS and control rats were tested in two standard anxiety paradigms to determine whether the SPS experience was still producing the expected anxiety differences at this delayed time. Specifically, we conducted delayed tests of anxiety levels in the Elevated Plus Maze (EPM) and Open field.

Materials and Method

Subjects and Design

Thirty-two male Sprague–Dawley rats weighing 230 to 310 g were used in this experiment. They were obtained from the same source and maintained in the same conditions as described in Experiment 1. A 2 (Anxiety condition: SPS, Control) × 2 (Drug: MDZ, Saline) design was employed in this experiment, with rats being randomly assigned to the four groups (n = 8, SPS–MDZ, SPS–Sal, C–MDZ, C–Sal; see Table 3). The dependent variable was percent freezing during test. All procedures were conducted in accordance with the relevant ethical guidelines.

Apparatus and Drugs

The contextual fear conditioning apparatus, and the drugs (MDZ, SAL), were identical to Experiment 1.

SPS procedure. The restraint apparatus involved clear Perspex cylinders (7.5-cm diameter × 21.5-cm length) obtained from Braintree Scientific, Braintree, MA. The animal was physically restrained to the point that it experienced loss of control over movement in the environment. The forced swim occurred with four rats at a time in a 1.085 m diameter × 0.42 m depth circular plastic pool. The water level was set at a depth of approximately 0.21 m above the pool base, and the water was maintained at room temperature (22 °C).

EPM. The EPM was made out of laminated timbers, and consisted of two open arms (50 cm × 12 cm; [l × w]) and two opposite closed arms that were enclosed with side walls (50 cm × 12 cm × 40 cm; [l × w × h]). The open and closed arms intersected at a neutral central square (10 cm × 10 cm). Elevation to a height of 59 cm meant that the maze would generate anxiogenic effects through fear of heights in the rodent (Treit, Menard, & Royan, 1993). A video camera was positioned at approximately

Table 3
Procedure for Each of the Four Groups in Experiment 3

	Procedure							
	Day 1		Day 9	Day 10	Day 11	Day 15	Day 16	Day 17
Group	SPS	7-day REST	Conditioning	Reactivation and drug	Test 1	Test 2/extinction training	Extinction test	EPM; open field
SPS-MDZ	SPS	Rest	Conditioning	React MDZ	Test 1	Extinct	Ext test	EPM
SPS-SAL	SPS	Rest	Conditioning	React SAL	Test 1	Extinct	Ext test	EPM
Control-MDZ	Handle	Rest	Conditioning	React MDZ	Test 1	Extinct	Ext test	EPM
Control-SAL	Handle	Rest	Conditioning	React SAL	Test 1	Extinct	Ext test	EPM

Note. EPM = elevated plus maze; Extinct = extinction training; Ext test = extinction test; MDZ = midazolam; NR = no reactivation; R = reactivation; SAL = saline; SPS = single prolonged stress.

150 cm above the central arena of the EPM. Animal behavior was recorded for repeated scoring. Experiments were always conducted in a dimly lit and quiet laboratory room. The EPM was cleaned with paper towels and tap water after each rat.

Open field. This apparatus was a roofless open field made of timber (60 cm × 60 cm × 50 cm; [l × w × h]). The gray painted base was divided into 16 squares (14 cm × 14 cm) by white colored stripes that were 1 cm wide. The enclosed walls of the open field were painted black. Any exploratory behavior in the open field was recorded by a video camera that was strategically positioned 200 cm above the central arena of the open field. Experiments were always conducted in a dimly lit and quiet laboratory room. The open field was cleaned with paper towels and tap water after each rat.

Procedures

SPS procedure (Day 1). The SPS procedure was similar to that used in Khan and Liberzon (2004), except that ether anesthesia was not conducted, because of occupational, health, and safety reasons. In squads of four, rats in the SPS group were restrained for 2 hours in the plastic cylindrical restrainers, followed immediately by the 20-min swim. Following the swim, the rats were dried and left in a box to recuperate for 60 minutes. Finally, the rats were returned to their colony boxes. A pilot study indicated that this modified SPS procedure produced potentiated light-enhanced startle, similar to that reported by Khan and Liberzon (2004).

Seven day recovery. After the SPS experience, all rats were left undisturbed in their colony boxes for 7 days. The 7-day undisturbed period has been shown to be necessary for the production of specific neuroendocrine characteristics such as enhanced negative feedback (Liberzon, Krstov, & Young, 1997).

Fear conditioning (Day 9), reactivation and drug administration (Day 10), and test 1 (Day 11). The conditioning, reactivation and test were identical for all groups. The procedures were identical to that described in Experiment 1, except that the NR (no reactivation) conditions were not included (see Table 3). All freezing and behavioral responses were scored and taped for future reference. Interrater reliability for a sample group of rats for reactivation and tests was $r = .975$, $p < .05$.

Test 2 and extinction training (Day 15). Three days after Test 1, rats were placed back in the conditioning chambers and their behavior was observed for a total of 9 minutes. No stimuli were presented to the rats in the conditioning chambers during extinction training. The first 3 minutes of the extinction training session also served as a delayed test for MDZ disruption of fear responding. Three rather than 10 days as in Experiment 1 were chosen because of the need to administer the anxiety tests.

Extinction test (Day 16). Twenty-four hours after extinction training, rats were placed back in the conditioning chambers for 3 minutes. Freezing behavior was scored and videotaped for future reference.

EPM and open field (Day 17). Each rat was placed in the corner of the left closed arm of the EPM such that its head was facing the neutral square. The rat was left to explore the apparatus freely in one single 5-min session. All behavior was videotaped for later scoring. The EPM served as a post hoc test of the effectiveness of the SPS procedure in producing high anxiety rats. It was expected that SPS rats would spend a lower percentage of time and make less entries into the open arms, than would the control rats. After EPM testing, rats were returned to their colony boxes and left to wait outside the laboratory room.

Open field testing occurred 2 hours after completion of the EPM. Each rat was placed in the central arena of the open field with its body in the diagonal line of the apparatus and left to explore freely for 10 minutes. It was expected that high anxiety rats would spend less time in the center squares than would the low anxiety rats.

Scoring

EPM. Behavior in the EPM was videotaped and scored from the monitor during testing. The number of entries into and time spent in the open and closed arms of the maze were scored individually on the basis of the four-paw rule (all four paws had to be in the arm). The percentage of entries into and time spent in the open arms indexes anxiety level (File, 2001). Percentage of open-arm entries was obtained by dividing the number of open arm entries by the total number of open-arm and closed-arm entries, multiplied by 100. Similarly, percentage of time spent in the open arms was obtained by dividing the total amount of time spent in the open arm by time spent in both the open and closed arms (without taking into account central square exploration and risk assessment behavior), multiplied by 100. The total number of entries into any arm was indicative of locomotor activity of the rats (File, 2001). A representative sample of test behavior in the EPM and Open Field

was rescored by an observer who was blind to the experimental conditions, with an interrater reliability of $r = .98$, $p < .05$.

Open field. For the open field, the total amount of time spent in both center (the four central squares) and periphery (the 12 peripheral squares) of the open field were scored individually for each rat on the basis of the four-paw rule (i.e., all four paws had to be in the square). The percentage of time spent in the center squares was calculated by dividing the amount of time in the center by the total amount of time in the center and periphery, multiplied by 100.

Results and Discussion

Reactivation

Data of one rat were excluded on the basis that the rat did not display any freezing during reactivation. A one-way ANOVA showed that there was no difference in percent freezing between the SPS rats (M = 70.82; *SD* = 21.24) and Control rats (M = 65.06, *SD* = 16.18) [$F(1, 27) = .70$, $p > .05$]. Given that this reactivation procedure is equivalent to an acquisition test, we did not replicate Takahashi et al.'s (2006) finding of greater freezing in the SPS group.

Test 1

A two-way ANOVA showed that the MDZ rats were freezing less than the SAL rats [$F(1, 27) = 16.1$, $p < .05$], but there were no main or interaction SPS group effects (see Figure 3). Scheffé analyses showed that the SPS–MDZ rats were freezing less than the SPS–SAL rats [$F(1, 27) = 14.95$, $p < .05$], and that the Control–MDZ were freezing less than the Control–SAL rats [$F(1, 27) = 9.95$, $p < .05$]. Thus, the MDZ effect of Experiment 1 was replicated; however, contrary to expectation, this effect was not also present in rats with prior SPS experience.

Test 2

A two-way ANOVA showed that the MDZ rats were freezing less than the SAL rats [$F(1, 27) = 12.12$, $p < .05$] (see Figure 3). Similar to the findings of Experiment 1, this confirms that the fear reduction produced by MDZ in a reactivation paradigm persists for 4 days after the first test. The lack of SPS main or interaction effects found in Test 1 persisted at Test 2. In a three-way ANOVA, a main effect for Test [$F(1, 27) = 9.4$, $p < .05$] indicated that all groups showed decreased freezing from Test 1 to Test 2, suggestive of extinction. This finding differs from Experiment 1 where there was no decrease in freezing across the Tests.

Extinction Training

To determine if there were any differences between the groups during extinction training, a 2 (MDZ, SAL) × 2 (SPS, Control) × (3) [Time Block: 3, 6, and 9 minutes] repeated measures mixed model ANOVA was conducted (see Figure 4). The main effect for Time Period was not significant; however, the significant Drug × Time Block interaction [$F(1, 27) = 6.62$, $p = .02$] indicated that the SAL rats, but not the MDZ rats, decreased responding over time. This may have reflected the fact that SAL rats started at a higher freezing level in the first 3 minutes, than did MDZ rats. It is also possible that the MDZ rats were at a floor level of responding, preventing any further reduction in freezing from being observed.

Extinction Test

A two-way ANOVA showed that the MDZ rats were freezing less than the SAL rats [$F(1, 27) = 6.68$, $p < .05$] (see Figure 5). The pattern of results are similar to those of Test 1 and Test 2, whereby only a main effect of Drug was found, suggesting that the fear disruptive effects are relatively long lasting at 6 days after reactivation, and that again there are no effects of the SPS experience. Thus, high anxiety rats did not appear to be more resistant to extinction compared to low anxiety rats. Nevertheless, a further analysis of the first 3-min of the Extinction Training and of the Extinction Test showed no significant difference [$F(1, 27) = 1.81$, $p < .05$], suggesting that there was no significant long-term extinction for either SPS or Control rats. Thus, although parameters were chosen that would normally have produced long-term

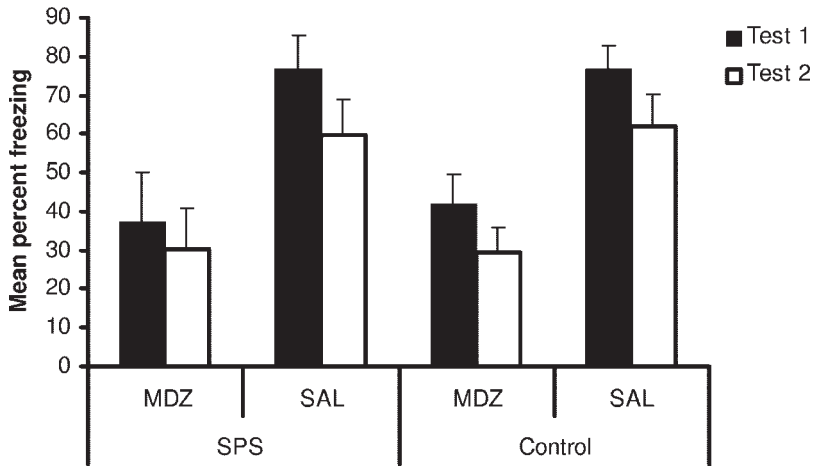

Figure 3. Mean percent test freezing (+*SEM*) as a function of Procedure (SPS, Control), Drug (MDZ, SAL), and Time (Test 1, Test 2). (MDZ = Midazolam; SAL = saline; SPS = Single Prolonged Stress.)

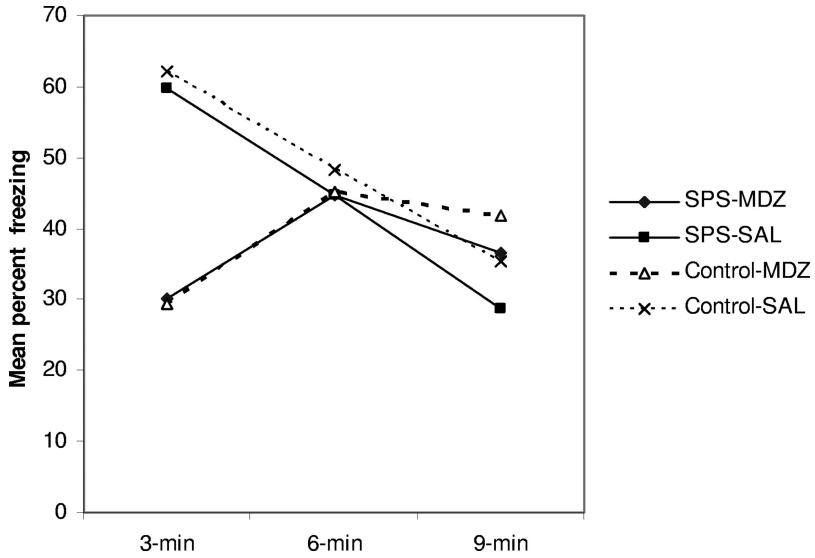

Figure 4. Mean percent freezing during extinction training as a function of time block (3, 6, or 9 minutes) and group. The 3-min time block also serves as a second test for Reconsolidation (Test 2 in Figure 3; MDZ = Midazolam; SAL = Saline; SPS = Single Prolonged Stress).

extinction in our laboratory, this was not effective in this experiment, and so further research is required to adequately test the original hypothesis.

EPM and Open Field

Table 4 presents the mean EPM and Open Field data for the SPS and Control groups. As expected, relative to the control rats, SPS rats spent a lower percentage of time engaged in EPM open arm exploration [$F(1, 30) = 17.62$, $p < .01$], and spent a lower percentage of time in the central squares of the Open field [$F(1, 30) = 4.3$, $p = .048$]. Analyses of additional EPM measures, controlling for number of contrasts, demonstrated a robust SPS effect on the EPM (see Table 4). Overall, then, it appears that 16 days after the initial experience, the SPS rats are showing behavioral evidence of higher anxiety than the Control rats. As expected, at this period following the initial shock and the subsequent drug administration, additional analyses demonstrated no drug effects for these tests.

In summary, Experiment 3 did not find a difference between SPS and Control rats in MDZ disruption of fear responding, suggesting this treatment could work equally well for both high and low anxiety individuals in reducing fear. Reliable anxiety differences were found on the EPM but not the Open Field measures. However, the SPS group did not show greater fear acquisition (Takahashi et al., 2006), which raises the possibility that the modified SPS procedure was not as effective as the standard SPS procedure.

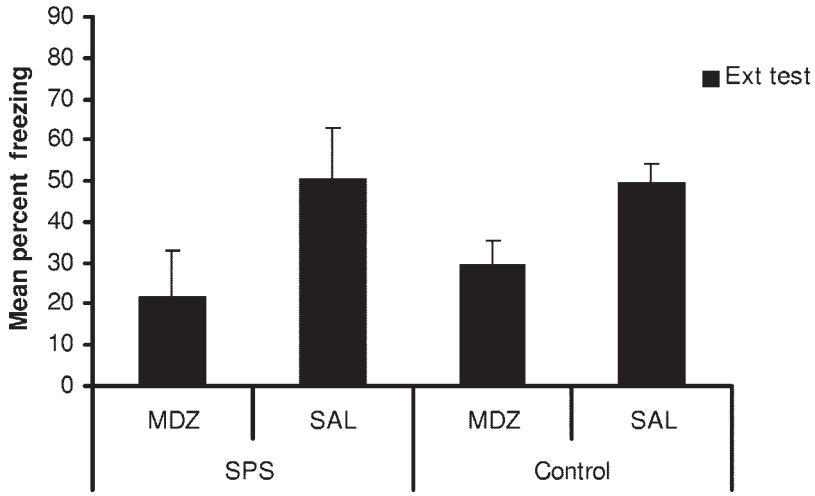

Figure 5. Mean percent freezing (+*SEM*) during extinction test, Experiment 3, as a function of group (MDZ = Midazolam; SAL = Saline; SPS = Single Prolonged Stress).

Table 4
Comparison of Elevated Plus Maze (EPM) and Open Field Measures for SPS and Control Rats (SD in Parentheses)

Behavioral procedure	SPS	Group control	*F*
EPM			
Mean open-arm entries	1.69 (1.3)	3.13 (1.54)	11.15*
Mean total number of entries	6.13 (3.54)	10.94 (6.4)	10.15*
% open-arm entry	23.82 (18.46)	30.70 (11.17)	17.4*
% neutral square exploration	6.23 (5.32)	10.83 (6.55)	8.34*
Open field			
% time spent central squares	9.8 (8.65)	12.56 (7.0)	4.64

Note. * Significant at $p = .05/5 = .01$.

General Discussion

In the current study, the long-lasting reduced freezing following GABA agonist systemic administration suggests robust disruption of reconsolidation of fear memories. In contrast, GABA antagonist systemic administration did not lead to significant enhancement of reconsolidation. Furthermore, both SPS and Control rats appear to be equally susceptible to MDZ fear disruption.

Postreactivation Administration of MDZ

The primary aim of the study was to reduce fear by systemically administering MDZ after a brief memory reactivation procedure. Based on a previous study by Bustos et al. (2006), it was expected that rats administered MDZ after memory reactivation would subsequently display lower levels of contextual freezing at test, compared to saline-administered rats. This pattern of results was found in both Experiments 1 and 3, and was long lasting. However, it should be noted that these experiments used a within-subjects design, whereby there is the opportunity for further learning in Test 1, which may have affected responding in Test 2. Future research should utilize a between-subjects design to test alternative explanations to that of long lasting fear-disruptive MDZ effects.

Our findings are consistent with Nader et al.'s (2000) assertion that disruptive agents such as MDZ are effective only after memory reactivation. In particular, in groups that received no reactivation, no disruption by midazolam was evident. The pattern of findings suggests that memory was in a labile state during the reactivation process. The MDZ findings of this study are also consistent with the notion that GABA normally plays an adaptive role in forgetting (Kim, Richardson, & McNally, 2006). That is, GABA agonists lead to reduced consolidation and reconsolidation of memory. Given the limits of our information processing systems, we cannot remember all the information that enters working memory, nor would we want to, given that much of the information is not important. The administration of GABA agonists in this study, then, leads to the "forgetting" of information that would otherwise be "adaptively" remembered (i.e., the context where painful stimuli were experienced).

Reconsolidation and Extinction

Procedurally, extinction and reconsolidation are similar, as they both require nonreinforced presentation of the CS. Thus, some researchers have argued that in contrast to blockade of reconsolidation, facilitated extinction is a viable alternative explanation for the reduced conditioned responding (Fischer, Sananbensi, Schrick, Spiess, & Radulovic, 2004; Lattal & Abel, 2004). However, GABA agonists usually retard extinction (Akirav, 2007; Hart & Westbrook, 2007; cf. Akirav et al., 2006), and GABA antagonists usually enhance extinction (Berlau & McGaugh, 2006; cf. Harris & Westbrook, 1998). Accordingly, if the reactivation procedure in the current study was effectively an extinction trial, the administration of MDZ should have impaired extinction, leading to enhanced fear responding. Indeed, a recent experiment in our laboratory (Cranney & Makkar, 2008) has shown that administering MDZ after a 2-min CS reexposure leads to less freezing, whereas administering MDZ after a 10-min CS reexposure tends to produce increased freezing. These findings with varying CS reexposure durations are similar to the findings of Lee, Milton, and Everitt (2006) with MK-801, an *N*-methyl D-aspartate antagonist, and Suzuki et al.'s (2004) findings with anisomycin.

Boundary Conditions of Reconsolidation

There is no universal 'reactivation protocol' that satisfactorily induces the lability of consolidated memory (Nader, 2007). It has been suggested that slight parametric differences can influence the successful disruption of reconsolidation processes (Tronson & Taylor, 2006). The present study was an extension of a previous study by Bustos et al. (2006), and supports MDZ impairment of fear memory reconsolidation. Although many of the parameters used in this study were equivalent (e.g., length of reactivation session), initial pilot studies revealed that Bustos et al.'s (2006) conditioning parameters were not effective in the current laboratory. Instead, this study used two shocks at a higher intensity but shorter duration (0.8 mA, 0.8 seconds), in comparison to Bustos et al.'s (2006) three shocks (0.7 mA, 3 seconds). Such findings, suggesting that slight differences in parametric protocols can affect successful disruption, might also explain the conflicting results in the reconsolidation literature. Therefore, it seems that to fully understand this complex phenomenon, a clearer delineation of the boundary conditions is needed, as they seem to differ between laboratories, species, and learning paradigms. Currently, memory age, memory strength, and the duration of the reactivation trial are considered important determinants of whether reconsolidation occurs after a reactivation trial (Tronson & Taylor, 2006). This issue is further complicated by the interaction of these boundary conditions. For example, Suzuki et al. (2004) found that if three shocks were used, no reconsolidation was evident if the reactivation period lasted only 3 or 5 minutes. A reactivation of 10 minutes, however, yielded evidence for reconsolidation. Not only is the nature of the retrieval session important, but the current study also suggests that the dose of the amnestic agent can lead to varying disruptive effects (1 mg/kg in Bustos et al., 2006, vs. 2 mg/kg in the current study). Clearly, more research is required to specify boundary conditions as well as the molecular mechanisms underlying these boundary conditions.

The Modified SPS Procedure and Reconsolidation Blocking

Our modified SPS paradigm resulted in anxious rats as supported by the EPM findings. It appears that the nonpharmacological SPS

manipulation resulted in sustained neural changes that led to increased baseline anxiety that was long lasting. However, this study did not replicate Takahashi et al.'s (2006) finding of increased contextual conditioning in SPS rats, and does not support Grillon's (2002) hypothesis that high anxiety individuals are more sensitive to the context and develop greater contextual fear relative to low anxiety individuals. It is possible that a much greater divergence in anxiety levels, as indicated by the EPM behavior, is needed to produce increased contextual conditioning. Future studies could aim to produce this divergence by adding some form of anesthesia (e.g., halothane) to the SPS procedure, as halothane by itself has been shown to produce neuroendocrinological and physiological responses in HPA axis function (Liberzon et al., 1997; Liberzon, Lopez, Flagel, Vázquez, & Young, 1999). In addition, no-reactivation control groups could be included to check whether, like the Saline rats in Experiment 1, the SPS rats do not show any change with MDZ (and no reactivation). Developmental models that involve stressing rats at a young age could also be tested with the reconsolidation paradigm. Furthermore, avoidance behaviors, a key component in the maintenance of anxiety disorders, should be addressed in future studies.

In summary, the present study provides evidence that MDZ administered immediately after reactivation of a contextual fear memory disrupts reconsolidation, and that this disruption is not reduced in high anxiety SPS rats. Thus, establishing whether the MDZ fear disruptive effect could be generalized to humans would potentially provide a new approach in the treatment of anxiety disorders, whereby the reconsolidation of a traumatic memory could be blocked by subsequent administration of memory disruptors (e.g., McGaugh, Cahill, & Roozendaal, 1996).

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