Supplementary Methods

Subjects

One hundred nineteen male Sprague Dawley rats obtained from Charles River served as subjects. All animals were housed 4 to a cage in a vivarium maintained on a 12 hr light/dark cycle. Experiments took place during the light portion of the cycle, and food and water were available ad libitum. All procedures were carried out with approval of the Emory University Institutional Animal Care and Use Committee.

Surgery

Before surgery animals were anesthetized with IP injections of sodium pentobarbital (57.5mg/kg). Each rat received an IP injection of Atropine (.04mg/kg) just prior to surgery and a subcutaneous injection of Buprenorphine (.01mg/kg) on the day of surgery and 1 day after. Rats were implanted with 22 gauge bilateral cannulae aimed at the amygdala (AP = -2.8/L = $\pm$ 5.2/V = -8.0). The cannulae were anchored to the skull using stainless steel screws and acrylic cement. Stainless steel obdurators were inserted into the guide cannulae to prevent blockage.

Apparatus

Rats were trained and tested in two identical 9 × 14 × 10 cm (depth × width × height; internal dimensions) Plexiglas and wire-mesh cages, each suspended between compression springs within a steel frame and located, together, within a custom-designed 60 × 79.5 × 59.5 cm sound-attenuating chamber lined with 6.3 mm thick Plexiglas. The floor of each cage consisted of four 6.0 mm diameter stainless steel bars spaced 18 mm apart. Affixed to the bottom of each cage was an Endevco accelerometer (Model 2217E). Cage movement resulted in displacement of the

accelerometer that produced a voltage output proportional to the velocity of cage movement. The accelerometer's output was amplified by an Endevco Model 104 amplifier and digitized on a scale of 0–2500 units by an InstruNET device (GW Instruments, Model 100B) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak–to–peak voltage that occurred during the first 300 msec after onset of the startle-eliciting noise burst.

Startle responses were evoked by 50 msec 95 dB white noise bursts (5 msec rise-decay time, 0–22 kHz) generated by a Macintosh G3 computer sound file, amplified by a Radio Shack amplifier (Model MPA–200), and delivered through high frequency speakers (Radio Shack Supertweeter) located in front of each cage. Background noise (60 dB wideband) was produced by an ACO Pacific white noise generator (Model 3024) and was delivered through the same speakers as those used to provide white noise bursts. Sound level measurements were made with a Brüel & Kjaer model 2235 sound level meter (A scale; random input) with the microphone (Type 4176) located 10 cm from the center of the speaker, which approximates the distance of the rat's ear from the speaker during testing.

The olfactory cue and the means for delivering it have been described in detail elsewhere (Paschall and Davis, 2002). In brief, a continuous flow of air was delivered from a compressed-air cylinder at a rate of 1.0 L/min through a small port (1.3 mm lumen diameter) positioned just above a 12.5 mm diameter opening in the top of each cage. For delivery of the olfactory stimulus, a computer-controlled solenoid (Model H15–03, Coulbourn Instruments) was opened for 4 sec, thereby diverting clean air from the compressed air cylinder into and through a sealed 135 cm^3 glass jar that contained 20 ml of 10% (vol/vol) acetophenone (i.e., the odorant) in propylene glycol solution. The inlet and outlet ports of the glass jar were positioned above the solution such that clean air from the tank mixed with the odor containing vapor. The output was then mixed with

clean air (3:5 ratio) before flowing into the cage. The olfactory stimulus was delivered through Pharmed Tygon tubing (3.2 mm inner diameter) that was routed through the ceiling of the training chamber. The end of the tubing hung approximately 7 cm above the floor–bars.

The chamber (0.284 m^3 total volume) was actively exhausted into the building's ventilation system at a rate of 0.0114 m^3/sec. Thus, a volume of air equal to the chamber's total volume was vented every 25 sec. Previous results with fear conditioned rats indicate that with these procedures startle amplitude returns to baseline levels within 30 sec of solenoid closure (Paschall and Davis, 2002). Cages were cleaned daily with warm tap water and were air dried overnight.

The illumination for the light cue (82 lux) was provided by Med Associates Inc. (Georgia, VT) PHM–258 fluorescent bulbs positioned 10 cm behind each cage. The unconditioned stimulus for all experiments was a 0.5 sec 0.4 mA scrambled shock delivered through the floor bars. Shock intensity was measured with a 1 kW resistor across a differential channel of an oscilloscope in series with a 100 kW resistor connected between adjacent floor bars within each cage. Current was defined as the root–mean–square voltage across the 1 kW resistor where mA = 0.707 × 0.5 × peak–to–peak voltage. Shocks were produced by LeHigh Valley shock generators (SGS 004). The presentation and sequencing of all stimuli was under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc.).

Drug Preparation & Infusion Procedure

In all cases rats received bilateral infusions into the amygdala. The total volume of the infusion (0.5 µl/side) was given over 2 minutes and the injection cannula remained in place for an additional 90 seconds to ensure diffusion away from the injector tip. The injection cannulae were cut to extend 1 mm beyond the guide cannulae. Zeta

pseudosubstrate inhibitory peptide (ZIP) and a scrambled version of the peptide (SCR ZIP) (Tocris, Ellisville, MO; Anaspec, Fremont, CA) were diluted with bacteriostatic saline to a concentration of 10nmol/µl. Infusions typically occurred 1 week after training and at varying intervals (i.e. 2 hours, 2 days, 12 days, or 15 days) before testing. However, in one experiment (**Supplementary Fig. 3**), the infusions occurred 20 days after training and 2 days before testing.

Behavioral procedures

Baseline Startle

On 2 consecutive days, rats were placed into the test cages and after 5 min, presented with thirty 95 dB startle-eliciting noise bursts (30 second interstimulus interval). Rats were sorted into different treatment groups based on the baseline startle scores such that each treatment group began with equivalent mean baseline startle levels.

Training

The next day after the final baseline session, rats were returned to the same chamber and received five acetophenone–shock pairings. The first pairing occurred 5 min after placement into the startle chamber and successive shocks occurred every 4 min. For each pairing, the 0.5-sec shock (0.4mA) was delivered 3.5 sec after onset of the 4.0 sec odor cue.

Memory Test

For testing, rats were again returned to the same chamber in which they had previously received shocks. After 5 minutes, the first of thirty 95 dB startle-eliciting noise bursts was presented. Successive stimuli occurred every 30 sec. Rats then received 40 test trials consisting of 10 odor test trials each followed by three startle–alone test trials. For odor test trials, the 95 dB noise burst was presented 3.5 sec after onset of the odor cue. For noise–alone test trials, the 95 dB noise burst was presented alone. The ISI for all

stimuli was 30 sec. The memory retest session was identical to the original test session. For the experiments in Figure 3, the initial test sessions were limited 5 trials in order to minimize any extinction that may be occurring as a result of the first test.

Reminder

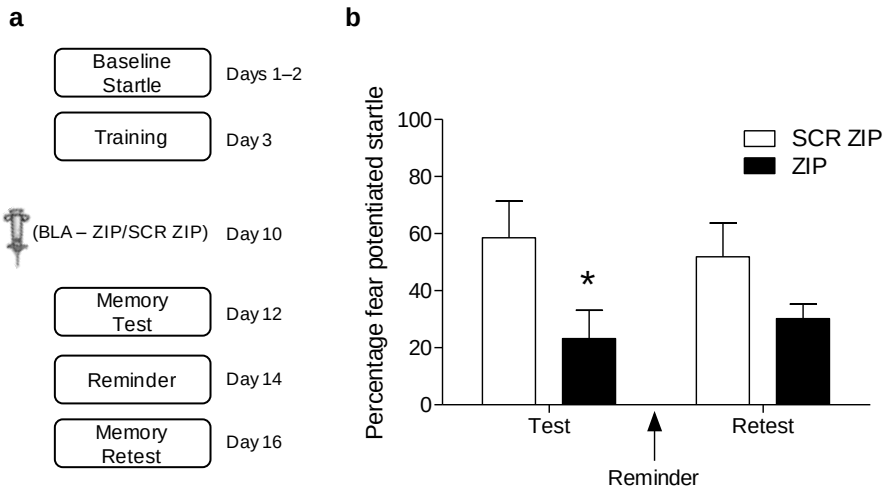
One set of rats were given a single reminder shock 2 days after testing (**Supplementary Fig. 1**). The reminder shock was same duration and intensity as during training and was delivered 5 minutes after placement into the same chamber where rats had been tested. Rats were retested 2 days later in a manner identical to the first test.

Statistical analyses

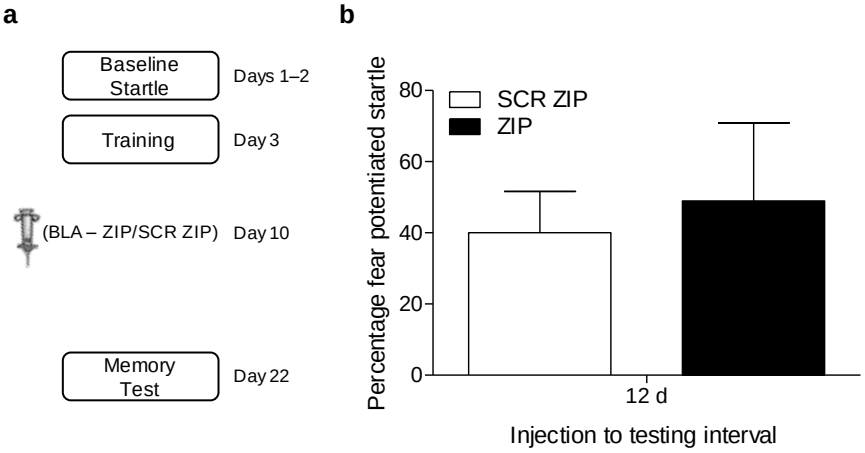
For each rat the mean startle amplitude on startle–alone and on odor test trials was determined and a percent change score was calculated. Percent change scores were used (i.e., vs. absolute difference scores) because previous work from our lab found that they remain stable across large variations in baseline startle amplitude (Walker and Davis 2002). These scores were then analyzed using student's t–test to examine differences between groups. In all tests, the criterion for significance was $P < 0.05$.

Histology

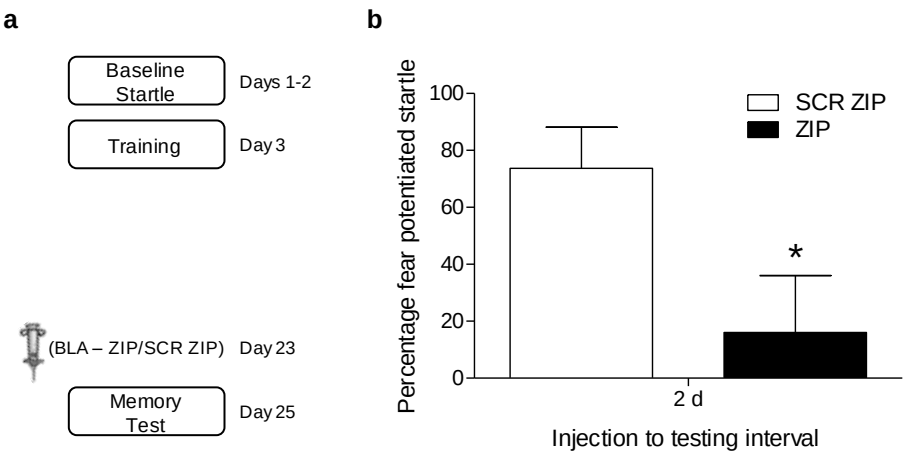
Rats were sacrificed by chloral hydrate overdose and perfused intracardially with 0.9% saline followed by 10% formalin. The brains were removed and immersed in a 30% sucrose-formalin solution for at least 5 days, after which 50 µm coronal sections were cut through the area of interest. Cannulae placements were determined with the aid of a rat brain atlas (Paxinos and Watson, 1998) and were judged by a scorer blind to the animal's group assignment and behavioral data. To be scored as an accurate placement, cannulae tips needed to be within or no further than 0.5 mm from the basolateral amygdala.



Supplementary Figure 1. Reminder shock does not recover ZIP–induced memory deficits. (a) Rats were infused with ZIP (n=11) or SCR ZIP (n=13) into the amygdala 1 week after training and 2 days before testing. (b) Blockade of PKMζ in the amygdala disrupted memory when tested 2 days later. There was no evidence of recovery after reminder shock in ZIP treated rats during retesting 2 days after a single reminder shock.



Supplementary Figure 2. Intact memory in ZIP–treated rats 12 days after infusion. (a) Rats were infused with ZIP (n=6) or SCR ZIP (n=5) into the amygdala 1 week after training and 12 days before testing. (b) There was no difference between groups when the animals were tested 12 days after blockade of PKMζ.



Supplementary Figure 3. PKMζ inhibition disrupts expression of older fear memories when applied closer to the time of testing. (c) Rats were infused with SCR ZIP (n=7) or ZIP (n=7) into the amygdala 20 days after training and tested 2 days later. (d) Animals infused with ZIP showed significantly less ($p < .05$, t–test) fear potentiated startle compared to the control group.

Reference List

Paxinos, G., & Watson, C. (1998). (New York: Academic Press).

Walker, D.L. & Davis, M. *Psychopharmacology (Berl)* **164**, 318–328 (2002).