HIPPOCAMPUS 19:1153–1158 (2009)

RAPID COMMUNICATION

CA3 NMDA Receptors Are Required for the Rapid Formation of a Salient Contextual Representation

Thomas J. McHugh* and Susumu Tonegawa

ABSTRACT: The acquisition of Pavlovian fear learning engages the hippocampus when the conditioned stimuli are multimodal or temporally isolated from the unconditioned stimuli. By subjecting CA3-NR1 KO mice to conditioning protocols that incorporate time-dependent components, we found that the loss of plasticity at recurrent CA3 synapses resulted in a deficits in contextual conditioning specifically when the exposure to the context was brief or when the unconditioned stimulus was signaled with a competing, predictive unimodal stimulus. Our results suggest CA3 contributes both speed and salience to contextual processing and support the theory of competition between multimodal and unimodal conditioned stimuli for associative learning. © 2009 Wiley-Liss, Inc.

KEY WORDS: CA3 NMDAR; contextual fear; conjunctive learning

INTRODUCTION

Hippocampus-dependent contextual fear conditioning has properties that make it a useful tool in assessing an animal's ability to form and recall contextual memory. If the foot shock (unconditioned stimulus [US]) is applied immediately after the rodent is placed in a novel context, conditioning is ineffective; at least a 20–40 s placement-to-shock interval (PSI) is needed (Fanselow, 1990). Further, under asymptotic values of 1–2 min, as the PSI is increased the amount of fear is also increased, indicating a strong correlation between the time in the context, the salience of the contextual conditioned stimulus (CS), and the strength of the CS-US association. Although pretraining damage to the hippocampus does allow for some context fear learning via nonhippocampal circuits (Maren et al., 1997; Frankland et al., 1998; Wiltgen et al., 2006), these circuits cannot support one-trial learning with protocols even at moderate PSIs (Wiltgen et al., 2006). Thus, the hippocampus is always required for rapid contextual fear learning and, moreover, there is a minimum time required within the hippocampal circuit to form a conjunctive representation of the multimodal contextual cues that define the CS (Rudy and O'Reilly, 1999).

If a unimodal CS such as a tone is delivered simultaneously with a multimodal CS such as context, the US is associated with both the tone and the context. Theory and data suggest these two distinct CS-US associations engage parallel neuronal circuits that interact in a competitive manner to acquire associative strength; a hippocampal-based circuit supporting multimodal CS-driven learning and another circuit independent of the hippocampus enabling unimodal CS-driven learning (Rescorla and Wagner, 1972; Kim and Fanselow, 1992; Phillips and LeDoux, 1994). However, the nature of this competition remains to be elucidated (Maren et al., 1997; Biedenkapp and Rudy, 2009).

In contrast to posttraining hippocampal lesions that produce profound contextual fear memory deficits (Kim and Fanselow, 1992; Anagnostaras et al., 1999), rodents with pretraining lesions can acquire contextual fear memory under many protocols (Maren et al., 1997; Frankland et al., 1998; Wiltgen et al., 2006), suggesting that animals trained in the absence of hippocampal output can utilize alternative circuits to acquire the memory. However, when intact and available, the conjunctive learning of the hippocampus seems to dominate this presumably less-efficient unimodal system. It is not the case, however, that all interventions made prior to training seem to uncover the alternative compensatory nonhippocampal learning pathways. For example, many pharmacological and genetic disruptions of hippocampal plasticity or transmission resulted in robust contextual conditioning deficits (Young et al., 1994; Frohardt et al., 1999; Rotenberg et al., 2000; Gale et al., 2001; Quinn et al., 2005; Nakashiba et al., 2008) without affecting conditioning to a tone, suggesting the damage to the hippocampus caused by these manipulations did not unmask or activate the alternative learning systems. A parsimonious interpretation of these data would be that these manipulations may have robustly reduced

The RIKEN-MIT Center for Neural Circuit Genetics, Howard Hughes Medical Institute, The Picower Institute for Learning and Memory, Department of Biology and Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts
Grant sponsor: NIH; Grant numbers: BR01-MH078821, P50-MH58880.
Thomas J. McHugh is currently at Laboratory for Circuit and Behavioral Physiology, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan.
*Correspondence to: Thomas J. McHugh, Laboratory for Circuit and Behavioral Physiology, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan. E-mail: tjmchugh@brain.riken.jp
Accepted for publication 22 June 2009
DOI 10.1002/hipo.20684
Published online 31 July 2009 in Wiley InterScience (www.interscience.wiley.com).

the speed at which the representation of the contextual CS is formed and hence may result in a deficit in hippocampal-dependent contextual fear learning while retaining the ability of the hippocampus to suppress the alternative learning pathway. Further, such manipulations may compromise the saliency of the context representations and may lead to a reduced contextual fear memory under a protocol where the context CS competes with a tone CS for association with the US.

The ability to restrict genetic manipulations to specific cell types has made it possible to address how plasticity or transmission in the individual hippocampal circuits contribute to the formation of spatial or contextual representations (Nakazawa et al., 2002; McHugh et al., 2007; Nakashiba et al., 2008). To further test how the synaptic plasticity in the CA3 subregion contributes to contextual learning, we subjected mice lacking NMDA receptors (NRs) specifically in CA3 pyramidal cells (CA3-NR1 KO mice (Nakazawa et al., 2002) to multiple versions of the conditioned fear task. We first used a protocol in which the PSI was altered; groups of CA3-NR1 KO mice and littermate controls were placed in a novel conditioning chamber and received a single unsignaled foot shock at one of four PSIs (0, 20, 40, and 60 s). Both genotypes demonstrated equal exploration of the novel chamber (Fig. 1a) and reacted similarly to the shock onset (Fig. 1b). The next day we returned the mice to the conditioning chamber and recorded freezing levels, a measure of contextual fear memory. Comparing the genotypes' freezing across PSIs revealed the mutants froze significantly less at the intermediate PSIs (Fig 1c; 2-factor ANOVA (PSI × genotype); significant effect of PSI ($F_{3,197}$(PSI) = 14.71, $P < 0.0001$); significant effect of genotype ($F_{1,197}$(genotype) = 6.40, $P = 0.0122$; Bonferroni post-test of genotypes at each PSI shows a significant deficit in the CA3-NR1 KO mice at the 40" PSI: $P < 0.05$; CA3-NR1 KO 33.6% ± 4.4%; flNR1 49.4% ± 4.7%), although by 60 s of exposure the genotypes exhibited indistinguishable 24 h fear memory. Our ability to detect a deficit only at intermediate PSIs suggests that the loss of NRs in CA3 does not abolish a hippocampal-dependent representation of the contextual CS, but does cause a significant change in the rate at which the context is processed and encoded. The relationship between the amount of time in the context and the saliency of the contextual CS is presumably dependent of many aspects of hippocampal processing; however, through the specificity of our genetic manipulation we reveal a quantifiable contribution of CA3 NR-mediated plasticity to the rapid formation of the representation.

We next addressed the question of the functional saliency of the mutants' contextual CS. Our strategy was to measure the levels of contextual fear memory that was acquired when mice were also conditioned to a tone CS under various protocols (Phillips and LeDoux, 1994). To titrate the ability of the tone to compete with the context for association with the footshock, we manipulated the temporal relationship between the tone and shock, such that the predictive value of the tone would be strong (delay conditioning), moderate (trace conditioning), or poor (backwards trace conditioning) (Fig. 2a) (Quinn et al., 2002). We subjected groups of mice to one of the three protocols, each employing three tone-shock pairings. Similar to a previous report in CA3 lesioned rats (Lee and Kesner, 2004), we observed a small acquisition deficit in mutants during delay training; however, we also observed a slight enhancement of learning in mutants during trace training (Fig. 2b). Despite these differences, genotypes demonstrated equal freezing during the final minute of training (Fig. 2c), indicating all mice could acquire the task.

When tested the next day we found no differences between genotypes in freezing to the tone, regardless of training protocol (Fig. 2d) and observed the expected relative drop in the strength of the tone conditioning as the predictive value of the tone decreased (delay > trace > bw-trace) (Marlin, 1981) (Fig. 2e). Mice were tested 24 h later for fear of the conditioning context. The mutants conditioned under forward and backward trace protocols showed as much contextual fear as the controls; however, mutants trained under the delay protocol froze significantly less than controls (Figs. 2f,g; 2-factor ANOVA

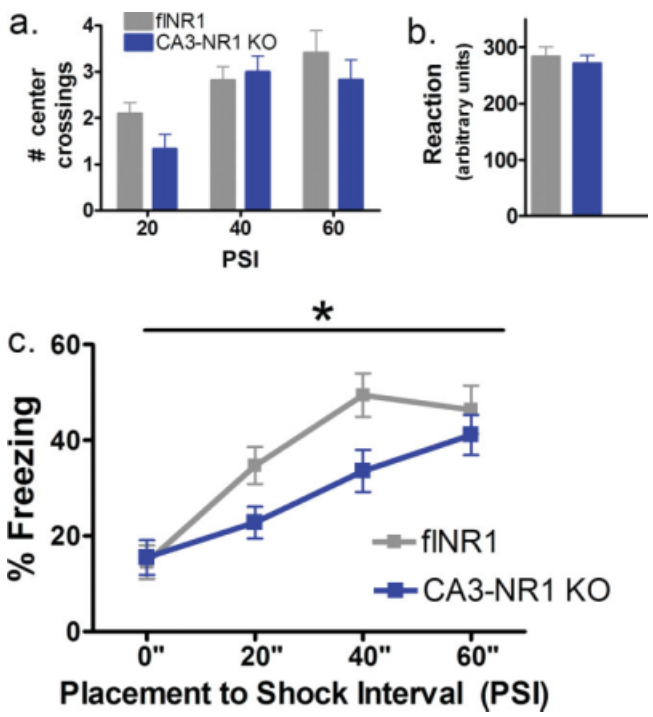

FIGURE 1. CA3-NR1 KO mice exhibit context conditioning deficits at short placement to shock intervals. (a) Pre-shock exploration is similar between genotypes at 20" (CA3-NR1 KO, *n* = 34; flNR1, *n* = 40), 40" (CA3-NR1 KO, *n* = 27; flNR1, *n* = 30), and 60" (CA3-NR1 KO, *n* = 21; flNR1, *n* = 22), PSIs, as is (b) movement immediately following to the foot shock, a measure of shock reactivity(CA3-NR1 KO, *n* = 38; flNR1, *n* = 52). (c) Context freezing assessed 24 h after training. A two-factor ANOVA (PSI × genotype) shows a significant effect of PSI ($F_{3,197}$(PSI) = 14.71, $P < 0.0,001$) and a significant effect of genotype ($F_{1,197}$(genotype) = 6.40, $P = 0.0,122$). Planned comparisons (Bonferroni posttest) of genotypes at each PSI shows a significant deficit in the CA3-NR1 KO mice at the 40" PSI ($P < 0.05$; CA3-NR1 KO 33.6% ± 4.4%; flNR1 49.4% ± 4.7%;). Data shown as mean ± SEM.

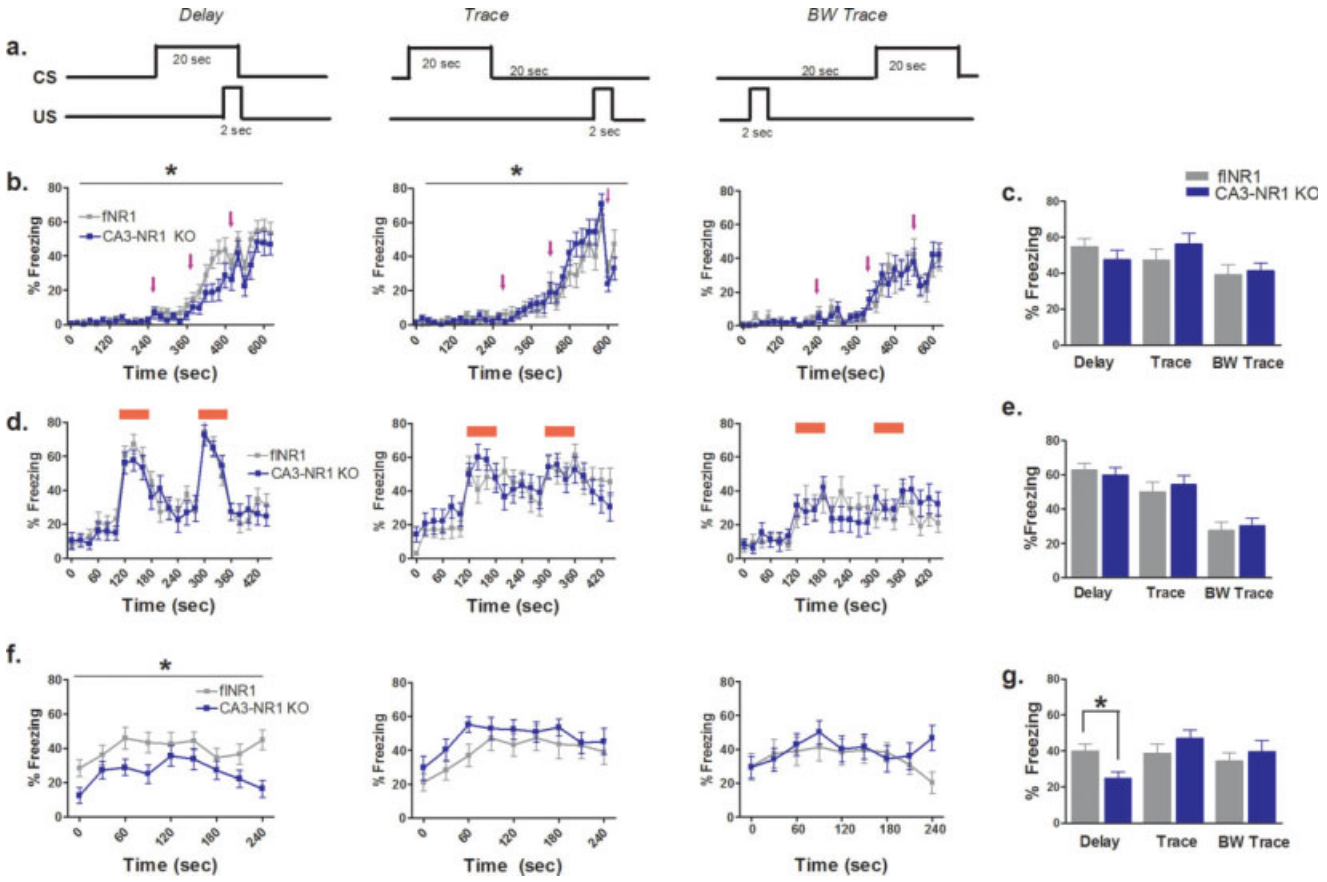

FIGURE 2. Context fear deficit in CA3-NR1 KO mice trained with a delay, but not with a trace or backward trace protocol. After four minutes in a novel chamber mice received three tone-shock pairings under one of three training protocols, delay (left), trace (center) or backward trace (right) (a). (b) Freezing levels measured in 20" bins during training (purple arrows indicate shock delivery times). CA3-NR1 KOs (n = 19) acquired delay fear (left) slightly slower than controls (n = 18; two-factor ANOVA; $F_{31,1,085}$ (time × genotype) = 1.589, P = 0.0219), but acquired trace (center) slightly faster (mutant n = 15; control n = 15; two-factor ANOVA; $F_{34,952}$ (time × genotype) = 1.562, P = 0.0221). Despite these differences freezing during the last minute of training for each protocol was indistinguishable between genotypes (c). Twenty-four hours after training mice were placed in a second chamber and after 2 min were presented with the conditioning tone for 60" followed by 2 min of silence and a second 60" tone. (d) Freezing was similar between genotypes during the tone test (tone presentation indicated by red bars) for delay (left), trace (center), and backward trace (right) conditioning, as was average freezing during the tones (e). On day three, the mice were placed in the conditioning context for a 5′ context test. (f) Comparing freezing over time in 30" bins revealed a significant deficit in the CA3-NR1 KO mice trained with the delay protocol (left; two-factor ANOVA genotype × time; $F_{1,315}$ (genotype) = 7.87, P = 0.0,082), but similar robust freezing in mutants and controls trained with trace (center) and backward trace (right; mutant, n = 14; control, n = 11). (g) A two-factor ANOVA comparing average context freezing across genotype and protocol reveals a significant interaction ($F_{2,86}$ (genotype × protocol) = 4.057, P = 0.0207) and planned comparisons (Bonferroni post-test) between the genotypes under each protocol shows a significant deficit ($P < 0.05$) in mutants trained in delay conditioning. Data shown as mean ± SEM.

comparing average context freezing across genotype and protocol reveals a significant interaction ($F_{2,86}$ (genotype × protocol) = 4.057, P = 0.0207) and planned comparisons (Bonferroni post-test) between the genotypes demonstrates significant deficit ($P < 0.05$) in mutants trained in delay conditioning). These data suggest the salience of the contextual representation formed without CA3 NRs is reduced, such that when, and only when, the tone is a good predictor of the shock (i.e., delay protocol), the unimodal tone CS can overshadow the multimodal contextual CS; however, as the predictive strength of the tone decreases, the contextual fear deficit is alleviated.

These experiments were designed to address the properties of a hippocampal contextual representation formed in the absence of CA3 NMDA receptors. However, given that pretraining hippocampal lesions do not disrupt contextual fear learning under many protocols, the possibility that the CA3-NR1 KO mice are using nonhippocampal circuits to acquire the memory described here must be considered. There are several pieces of data that suggest the contextual fear memory we have tested is hippocampal-dependent. First, in our initial experiment, which used a single US, we only observed deficits at PSIs less than 60 s. These data are at odds with those obtained with animals

given hippocampal lesions which showed attenuated freezing at PSIs up to and over 5 min (Wiltgen et al., 2006). Next, the nonhippocampal system that can support contextual fear learning in the lesioned animals was poorer at discrimination compared to the control animals, leading to increased generalized fear (Frankland et al., 1998). In contrast, in our second experiment 24 h after training we observed no difference between genotypes in baseline freezing prior to the tone (Fig. 2d), a measure of context generalization. Previous experiments reported CA3-NR1 KO mice do show a transient increase in generalization 3 h after training, but it is completely absent 24 h after training (Cravens et al., 2006); a pattern not predicted by the lesion data (Frankland et al., 1998). Finally, mice lacking the CA3 NRs can acquire other hippocampal dependent task and exhibit largely normal hippocampal physiology, both in vitro and in vivo (Nakazawa et al., 2002, 2003; Cravens et al., 2006; Rajji et al., 2006) suggesting that the unmasking of nonhippocampal learning circuits seen following neuro- or excitotoxic lesions, but not seen after pharmacological or genetic manipulations, is unlikely to occur in these mice. Hence, we are confident that the contextual fear learning we observed in the CA3-NR1 KO mice is still hippocampal in nature and provides a valuable tool with which we can probe the contribution of CA3 recurrent plasticity to the speed and salience of the formation of a contextual representation in the circuit.

The shift we observed in the PSI/freezing curve (Fig. 1c) in the CA3-NR1 KO mice is very similar to a deficit previously reported in female C57BL/6 mice relative to male mice tested under the same variable PSI conditions (Wiltgen et al., 2001). We concur with Wiltgen et al.'s conclusion that deficits only at intermediate PSIs strongly suggest a difference in the rate at which the context is processed. Here we can extend that assertion by attributing this change in rate in our mice to the loss of NRs in the CA3 pyramidal cells. NMDA-mediate plasticity in the CA3 region is thought to contribute to the storage of information in the recurrent CA3-CA3 synapses and underlie the ability of this auto-associative network to rapidly encode information (McNaughton and Morris, 1987; Zalutsky and Nicoll, 1990; Nakazawa et al., 2002; Rolls and Kesner, 2006). Our behavioral data are in agreement with this hypothesis and support earlier electrophysiological evidence suggesting the loss of associative plasticity at CA3 recurrent synapses compromises mutants' ability to quickly form a conjunctive and associative representation of a novel environment (Nakazawa et al., 2003). Moreover, our experiments have defined a behaviorally relevant timeline for this process that can be explored further in future. At the level of physiology little is known about how hippocampal contextual representations are associated with a US such as the footshock. A comparison of hippocampal output during the first minute of exploration between CA3-NR1 KO and control mice may reveal the requirements for a representation that can be associated with a US and lead to a better understanding of the locus and mechanism of this association.

The use of genetic tools that allow the cell-type specific blockade of plasticity without impacting baseline transmission have been useful in dissecting the hippocampal circuit (Nakazawa et al., 2004). Given the ability of compensatory learning circuits to supersede hippocampal function when output is lost, this type of intervention is needed to create situations in which "bad" information in the circuit may be more harmful than no information at all. The contextual fear deficits displayed by the mutants specifically during delay conditioning (Fig. 2g) support the model of competition between hippocampal and nonhippocampal CS representations for association and suggest that while the loss of CA3 recurrent plasticity does not prevent the formation of a hippocampal-dependent contextual CS, it does result in a decrease of the salience of this representation. Recent work has suggested that the competition between the multimodal hippocampal learning system and the unimodal nonhippocampal circuit occurs in the amygdala and requires hippocampal output via the subiculum (Biedenkapp and Rudy, 2009). Understanding how the loss of NR1 in CA3 impacts the context representation present in the subiculum may shed light on how context and cue salience is represented and compared.

Finally, several experiments have suggested that hippocampal NRs are required for the acquisition of tone fear under a trace conditioning protocol (Quinn et al., 2005), including data showing that a genetic deletion of NR1 in CA1 pyramidal cells leads to deficits in this task (Huerta et al., 2000). Here we report no deficits in the acquisition or recall of trace fear in the absence of CA3 NRs. Plasticity in the CA3 network is therefore dispensable for acquiring temporally discontiguous CS-US associations on the time scale of tens of seconds, but required for the rapid formation of a robust contextual representation.

DETAILED METHODS

Animals

All experiments were carried out using male CA3-NR1 KO mice on a C57BL/6 background of 18–26 weeks of age and their floxed-NR1 (fNR1; control) male littermates. These mice have been previously described, and at these ages the NR1 protein has been lost in the CA3 pyramidal cells of the mutant animals (Nakazawa et al., 2002). Two to four mice were housed per cage under the conditions of a 12 h light/dark cycle and ad libitum access to food and water. All mice were individually handled for several minutes a day for 3 days prior to fear conditioning. All the experiments and analyses were conducted blind to the genotypes of the mice used. All procedures relating to animal care and treatment conformed to the Institutional and NIH guidelines.

Fear Conditioning

Apparatus

Training and context testing was conducted in a dedicated behavioral training room located in the animal facility. The training room was brightly lit and contained four conditioning

chambers. The chambers consisting of a plexiglass front and back and aluminum walls on each side, measured 30 cm × 25 cm × 21 cm (Med Assocaites ENV-008; Georgia, VT). The floor of the chamber consisted of 36 stainless steel rods of 3.2 mm diameter and spaced 7.9 mm apart and was connect via a cable harness to a shock generator (Med Associates ENV-414; Georgia, VT). The chambers were cleaned between animals with 70% ethanol, and a solution of 1% acetic acid was placed beneath the chambers during the experiment to provide an olfactory cue.

Tone tests were in a neighboring training room dimly lit with red light. The room contained four chambers similar to the conditioning context, but distinguished by a triangular roof insert, a smooth plastic floor and a unique 0.25% benzaldehyde odor placed beneath the chambers during the experiment to provide an olfactory cue.

Variable placement to shock interval fear conditioning

On both days of the experiment, all mice were transported from the colony to a holding room adjacent to the behavioral room containing the fear conditioning chambers where they sat undisturbed for 30 min prior to the experiment. Mice were transported between the holding room and the conditioning room in their home cages. Training consisted of a single shock (0.75 mA, 2 s) delivered at one of four time intervals following placement of the mouse into the conditioning chamber: 0, 20, 40, and 60 s. Following the shock, the mice remained in the chamber for an additional 30 s and were then moved back to their home cage. On the next day, mice were returned to the original conditioning chamber and freezing was monitored for 5 min. During all sessions, the animal's activity in the chamber was recorded using FreezeFrame software (Actimetrics; Wilmette, IL). Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 2 s (Actimetrics; Wilmette, IL). FreezView was also used to measure the reaction of the mice to the footshock. Movement (distance) was calculated for the 2 s period following shock termination and as the values are meant solely for across treatment comparisons, arbitrary units are used.

Tone signaled conditioning

On all days of the experiment, mice were transported from the colony to a holding room adjacent to the behavioral room containing the fear conditioning chambers where they sat undisturbed for 30 min prior to the experiment. Mice were transported between the holding room and the conditioning room in their home cages and were randomly assigned to one of three training groups: delay, trace, or backward trace conditioning. Mice in the delay group were placed into a novel chamber and a 20 s tone (2 kHz; 75 dB) sounded after 4 min. The tone coterminated with a 2 s, 0.75 mA foot shock. Following a 2-min intertrial interval (ITI), the tone-shock pairing was repeated, followed by a second 2-min ITI and a third tone-shock pairing. Mice were removed from the chamber 100 s after the final shock. Animals in the trace group were trained in a similar manner, the key distinction being that a 20 s trace interval was inserted between the termination of the tone and the delivery of the foot shock. Backward trace conditioning was again very similar; however, in this protocol mice placed into a novel chamber and after 4 min they received a 2 s, 0.75 mA foot shock, followed by a 20 s trace interval and then a 20 s tone. Following a 2-min intertrial interval (ITI), the shock-trace-tone sequence was repeated, followed by a second 2 min ITI and a third repetition. Mice were removed from the chamber 100 s after the final shock.

All the mice were placed in the distinct tone-test chamber 24 h after training (see Section "Apparatus"), and baseline freezing was recorded for 2 min, after which mice were presented with the conditioning tone for 60", followed by 2 min of silence and a second 60" tone. Mice were removed from the chamber 2 min after the second tone.

On day three, mice were returned to the original conditioning chamber and freezing was monitored for 5 min. During all sessions, the animal's activity in the chamber was recorded using FreezeFrame software (Actimetrics, Wilmette, IL). Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 2 s (Actimetrics, Wilmette, IL).

Data analysis

All values are reported as mean ± SEM. Data analysis was performed with GraphPad PRISM software (GraphPad, San Diego, CA). Statistical significance was determined by two-factor analysis of variance (ANOVA) with planned multiple comparisons performed with Bonferroni posttests, one-way ANOVA or two-tailed unpaired Student's *t*-tests; $P < 0.05$ was considered significant.

Acknowledgments

The authors would like to thank Frank Bushard, Lorene Leiter, Candy Carr, and Sean Perry for technical assistance and Drs. Jennie Young, Derek Buhl, and Joe Biedenkapp for comments on the manuscript.

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