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Formation of Temporal Memory Requires NMDA Receptors within CA1 Pyramidal Neurons

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Summary

In humans the hippocampus is required for episodic memory, which extends into the spatial and temporal domains. Work on the rodent hippocampus has shown that NMDA receptor (NMDAR) –mediated plasticity is essential for spatial memory. Here, we have examined whether hippocampal NMDARs are also needed for temporal memory. We applied trace fear conditioning to knockout mice lacking NMDARs only in hippocampal CA1 pyramidal cells. This paradigm requires temporal processing because the conditional and unconditional stimuli are separated by 30 s (trace). We found that knockout mice failed to memorize this association but were indistinguishable from normal animals when the trace was removed. Thus, NMDARs in CA1 are crucial for the formation of memories that associate events across time.

Introduction

Human studies have indicated that the hippocampus participates in the acquisition of declarative and/or episodic memory, i.e., the ability to consciously recollect events from everyday experience set in their spatiotemporal context (Cohen and Eichenbaum, 1993; Schacter and Tulving, 1994; Vargha-Khadem et al., 1997; Squire and Kandel, 1999). In rodents, O'Keefe and Nadel (1978) postulated that the hippocampus is responsible for generating spatial maps that guide the animal's navigation, an idea that has received strong support on two grounds: (1) many studies have demonstrated that hippocampal damage leads to deficits on spatial learning tasks (O'Keefe and Nadel, 1978; Morris et al., 1982, 1986; Nadel, 1991; Jarrard, 1993; O'Keefe, 1999), and (2) the existence of "place" cells—that is, hippocampal pyramidal neurons that fire when the animal is in a particular location in its environment (O'Keefe and Nadel, 1978; O'Keefe, 1999).

Is the rodent hippocampus also involved in temporal memory? It has been previously shown that rats with hippocampal lesions or treated with an NMDA receptor (NMDAR) antagonist are deficient in learning tasks that include delays of seconds to minutes between relevant events (Meck et al., 1984; Rawlins, 1985; Lyford et al., 1993; Chiba et al., 1994; Jackson et al., 1998). However, it has been argued that these studies were inconclusive as to the role of the rodent hippocampus in temporal memory because the tasks contained an inadvertent spatial component (Nadel, 1991; O'Keefe, 1999; Wood et al., 1999). More recently, McEchon et al. (1998) reported that rats with hippocampal lesions were impaired in trace fear conditioning. This Pavlovian paradigm has a very clear timing requirement because it demands the association of a conditional stimulus (CS) with an unconditional stimulus (US) across an interval known as a "trace." Learning occurs as the animal links the originally neutral CS with the US, so that later presentation of the CS alone elicits the conditional response. A neural circuit is therefore needed for establishing the CS–US association across the gap imposed by the trace. If the hippocampus participates in this circuit, as the lesion studies suggest, a major question is whether the hippocampal mechanism responsible for temporal memory is different from the one underlying spatial mapping. Conversely, both mechanisms could be part of a more general one.

We recently reported the generation and initial characterization of knockout mice lacking the gene for the NR1 subunit of NMDARs in CA1 pyramidal cells (named NR1-CA1-KO or mutant, henceforth). These mice were shown to be deficient in NMDAR-dependent synaptic plasticity (within CA1 synapses) and spatial memory formation (Tsien et al., 1996). They also displayed degraded coactivation of CA1 place cells during exploration (McHugh et al., 1996). In this study, we have subjected NR1-CA1-KOs to trace fear conditioning. Our purpose was 2-fold: (1) to confirm that hippocampal function is needed for encoding temporal memory by using a more stringent lesion method, namely a cell type–specific gene knockout, and (2) to examine whether NMDAR function within CA1 pyramidal cells is specifically required. We found that mutant mice displayed a memory impairment following trace conditioning, i.e., they were unable to memorize the association between the CS and the US across the trace interval. Conversely, mutant mice could acquire the conditional response when the trace interval was removed in a delay conditioning paradigm.

Results

NR1-CA1-KO Mice Are Slower in the Acquisition of Trace Fear Conditioning

To identify the conditional fear response, we measured the freezing behavior of mice with an automated method. First, we determined that the scores generated by this method were equivalent to the more traditional way of scoring freezing by visual inspection. In order to compare the two methods, mice ($n = 6$) were subjected to "contextual" fear conditioning, in which animals were placed in a conditioning chamber and after 3 min were shocked three times with an interval of 1 min between shocks. The next day, mice were tested in the conditioning chamber for 6 min. As it is well established, mice

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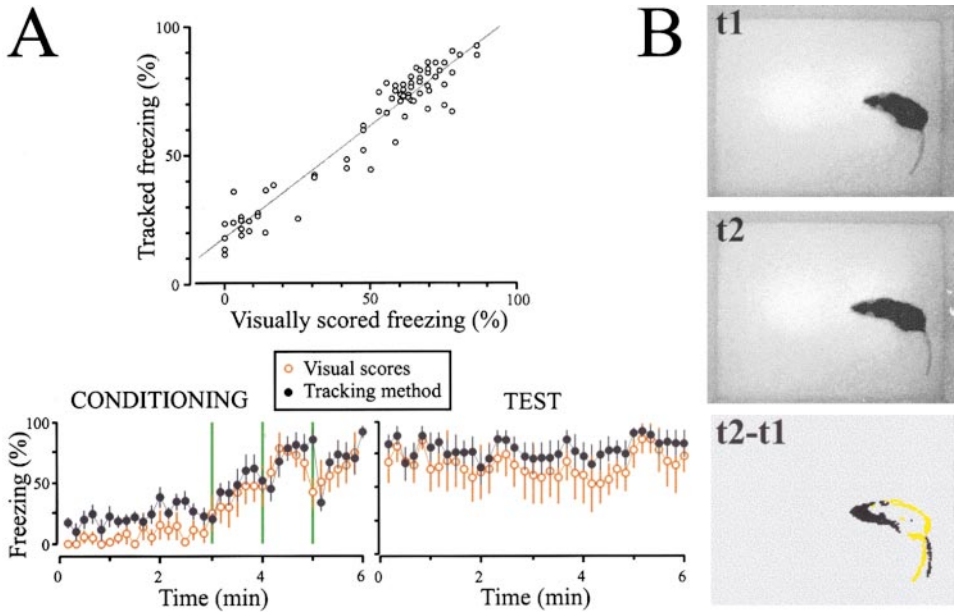

Figure 1. Calibration of the Tracking Method for Measuring Freezing

(A, top) The comparison of visually scored freezing done by blind observers (x axis) to freezing scores generated with the tracking method (y axis). The data were fitted by a linear regression with $y = 18 + 0.87x$, $r = 0.96$.

(A, bottom) The time course of the context conditioning paradigm ($n = 6$ mice) used to generate the visual (open circles) and tracked (closed circles) freezing scores. Details of the paradigm are given in the Experimental Procedures. The green bars during conditioning represent US deliveries.

(B) Sequence of video frames, at times t1 and t2 (200 ms later), showing a mouse exploring the chamber. The video method calculates the difference (t2 − t1). The leading edge of the mouse is represented by black, whereas the trailing edge is represented by yellow.

showed robust freezing during the 24 hr test. Figure 1 shows that the freezing scores generated with the automated method matched well with visually scored freezing.

For trace training, a mouse was placed in a conditioning chamber and, after 60 s, received the CS (white noise, 15 s), followed by a trace period (30 s) and the US (foot shock, 0.5 s). The sequence was repeated ten times with an intertrial interval (ITI) of 210 s. Comparison of the freezing scores revealed that mutants froze significantly less than controls after the initial trial (ITI-1, 105–315 s, mutant, 27.7% ± 2.6%, control, 41.7% ± 2.9%, $p < 0.001$, $F_{1,44} = 13.2$) and the subsequent two trials (ITI-2, 360–570 s, mutant, 41.9% ± 4.5%, control, 60.3% ± 4.6%, $p < 0.01$, $F_{1,44} = 8.1$; ITI-3, 615–825 s, mutant, 52.3% ± 5.0%, control, 68.9% ± 4.8%, $p < 0.05$, $F_{1,44} = 5.7$). However, thereafter the conditioning of mutants quickly caught up with that of controls, and by the end of the training session the scores from the two groups were not significantly different (ITI-10, 2400–2610 s, mutant, 66.6% ± 4.0%, control, 67.8% ± 4.8%, $p = 0.85$, $F_{1,44} = 0.04$). Since mice stayed in the conditioning chamber for over 40 min during the training session, it was quite feasible that habituation also contributed to the increase of immobility. To measure habituation, we placed control mice that received neither CS nor US into the conditioning chamber for 43.5 min (these mice are labeled "naive" hereafter). By the end of the session, the freezing scores of naive animals were only slightly less than those of controls and mutants (ITI-10, naive, 52.2% ± 8.7%; control versus naive, $p = 0.1$, $F_{1,28} = 2.5$; mutant versus naive, $p = 0.1$, $F_{1,28} = 2.8$). This suggests that habituation might be a significant factor contributing to the behavior of control and mutant mice as the conditioning progressed.

Additionally, a group of control mice was subjected to pseudo-conditioning (see Experimental Procedures; these mice are labeled "pseudo" hereafter). Following subsequent deliveries of the US, pseudo mice exhibited increasing levels of freezing (Figure 2C). This effect has been observed previously (Phillips and LeDoux, 1994; McEchon et al., 1998). It has been interpreted as resulting from the association of the US with the background context, i.e., the static stimuli within the conditioning chamber (Phillips and LeDoux, 1994).

NR1-CA1-KO Mice Fail to Show the Conditional Response during a Memory Test, after Trace Conditioning

The memory test for trace conditioning was conducted 24 hr after the training session. A mouse was placed in a novel chamber and, after 60 s, was exposed to the CS ten times (ITI of 210 s). Upon introduction into the chamber, mutants and controls showed elevated freezing compared to naive mice (pre-CS period, 0–60 s, mutant, 53.1% ± 3.7%, control, 56.9% ± 2.0%, naive, 24.0% ± 4.1%; mutant versus naive, $p < 0.001$, $F_{1,28} = 17.0$; control versus naive, $p < 0.0001$, $F_{1,28} = 62.0$). This effect has been described as a generalization of the fear response. That is, rodents that have undergone strong fear conditioning are found to freeze upon mere exposure to environments that were not used for conditioning. Unavoidable factors in the experimental environment, such as being manipulated by an experimenter,

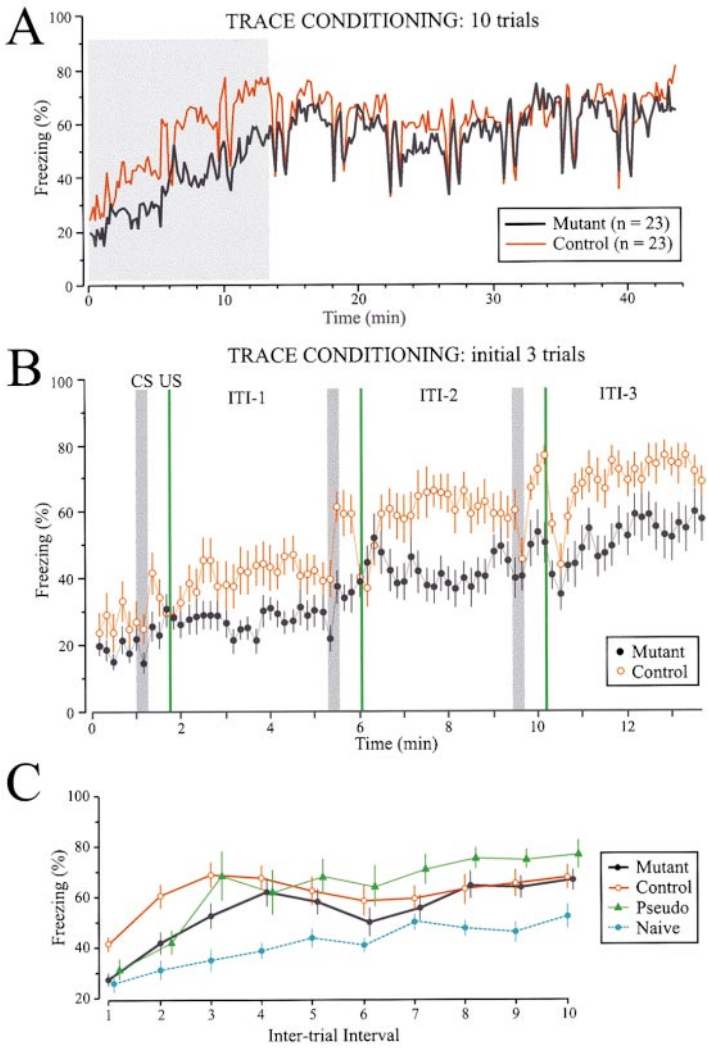

Figure 2. NR1-CA1-KO Mice Are Slower in the Acquisition of Trace Fear Conditioning

(A) Time course of the average percent freezing displayed by mutants (n = 23) and controls (n = 23) during trace conditioning. The gray box, at left, indicates the initial three trials, which are enlarged in (B).

(B) Pooled data showing the average percent freezing on the initial phase of acquisition for NR1-CA1-KOs (closed circles) and controls (open circles). Symbols represent mean freezing (± SEM) on a 10 s epoch. CS presentations are indicated by gray bars and US occurrence by green bars. It is clear that mutants show lower freezing during this initial phase.

(C) Average percent freezing during the ITIs for mutant (n = 23), control (n = 23), pseudo-conditioned (n = 12), and naive (n = 7) mice.

being placed in a box that differs from the home cage, and others, may contribute to the generalization of fear (Fanselow, 1990; Radulovic et al., 1998). During the first ITI (75–285 s), the freezing of NR1-CA1-KOs remained at the same level as that of the pre-CS period, whereas the freezing of controls was elevated to above 80% (Figure 3). The difference in freezing scores between the two groups was highly significant (ITI-1, 75–285 s, mutant, 56.3% ± 2.2%, control, 84.3% ± 2.9%, $p < 0.0001$, $F_{1,44} = 58.3$) and remained as such for the subsequent trials (ITI-2, 300–510 s, mutant, 60.5% ± 2.2%, control, 85.1% ± 2.7%, $p < 0.0001$, $F_{1,44} = 58.3$; ITI-3, 525–735 s, mutant, 62.7% ± 1.6%, control, 83.6% ± 2.3%, $p < 0.0001$, $F_{1,44} = 54.9$).

The maximal conditional response displayed by controls occurred roughly 30 s after CS delivery (Figure 3B), suggesting that the animals were expecting the shock exactly at the time that it had occurred in the training session. Thus, controls seemed to have developed a strong CS–US association across the trace interval. To demonstrate that the enhanced freezing exhibited by controls was specifically linked to the CS, we compared their behavior to that of pseudo mice. Had the CS elicited a nonspecific fear response on the 24 hr test, then pseudo mice should also respond to the CS with enhanced freezing. However, this was not the case (Figures 3A and 3C). Statistical comparison revealed that the pseudo group froze significantly less than controls (ITI-1, pseudo, 37.7% ± 10.4%, control, 84.3% ± 2.9%, $p < 0.0001$, $F_{1,33} = 65.9$; ITI-2, pseudo, 50.8% ± 6.3%, control, 85.1% ± 2.7%, $p < 0.0001$, $F_{1,33} = 65.9$; ITI-3, pseudo, 59.3% ± 8.8%, control, 83.6% ± 2.3%, $p < 0.001$, $F_{1,33} = 14.9$), implying that only controls had formed the temporal association as a consequence of trace training. Moreover, comparison of the pseudo and mutant groups showed that freezing between the two groups differed statistically after the first trial (ITI-1, mutant versus pseudo, $p < 0.01$, $F_{1,33} = 16.7$) but did not reach statistical significance for the subsequent trials (ITI-2, $p = 0.07$, $F_{1,33} = 3.5$; ITI-3, $p = 0.6$, $F_{1,33} = 0.3$).

In order to more fully characterize the differential conditional response produced by the CS on the 24 hr trace test, we computed the mean change in freezing between the interval prior to (0–60 s) and following (75–135 s) the first CS (Figure 3C). A nonparametric test (Kolmogorov-Smirnov) showed that the mutant score was significantly different from the control score ($p < 0.0001$, $d = 0.8$) but not different from the naive ($p = 0.01$, $d = 0.7$) and

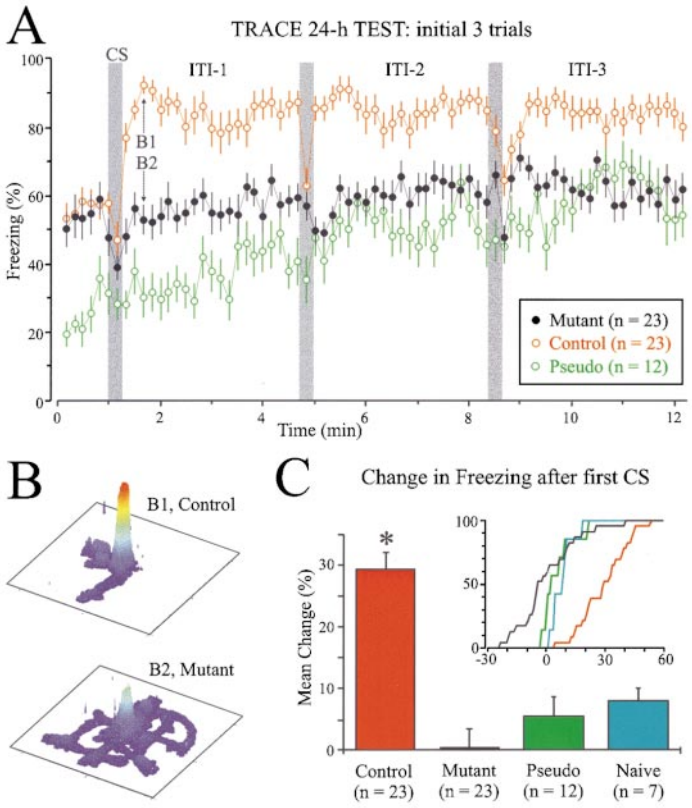

Figure 3. NR1-CA1-KO Mice Do Not Show Enhanced Freezing during the Trace Memory Test

(A) Pooled data showing the average percent freezing on the initial three trials of the 24 hr test after trace training for NR1-CA1-KOs (n = 23, closed circles) and controls (n = 23, open circles). The test was given in a novel chamber that differed from the conditioning chamber. Symbols represent mean freezing (± SEM) on a 10 s epoch. CS presentations are indicated by gray bars. For convenience, pooled data for pseudo-conditioned mice (n = 12, green circles), which received pseudo-training 24 hr earlier, are also plotted.

(B) Occupancy plots of 12 mutants and 12 controls for the 10 s epoch (105–115 s) indicated by "B1" and "B2" in (A). Plots were created by summing frame captures (200 ms in between frames) per mouse. The center of mass of the first frame was defined as the starting location of the mouse, which was then translated to the center of the plot. The x and y axes represent position (total of 300 pixels, which equals 25 cm). The vertical axis is color coded; 100% is red and equals the maximum possible occupancy for that location. High values indicate greater freezing and low values (and wider areas of occupancy) reflect exploration.

(C) Mean change in freezing elicited by the first CS in the memory test of trace fear conditioning. Change in freezing was calculated by computing the difference (F2 − F1) for each mouse, where F1 was freezing for the pre-CS period (0–60 s) and F2 was freezing for an equally long period during the ITI (75–135 s). Scores were averaged across animals, so that each column represents the mean value ± SEM for each mouse group. Asterisk indicates $p < 0.0001$ (one-way ANOVA between controls and mutants). The inset shows the mean change in freezing expressed as normalized cumulative distributions. Colors: control, red; NR1-CA1-KO, black; naive, blue; pseudo-conditioned, green.

the pseudo ($p = 0.02$, $d = 0.6$) scores. Moreover, the scores of controls were significantly higher than those of naive ($p < 0.01$, $d = 0.7$) and pseudo ($p < 0.001$, $d = 0.8$) groups.

Examination of the later segments of the 24 hr trace test (ITI-4 to ITI-10) revealed that controls maintained their high freezing until the end of the test session (data not shown). Comparison of control scores on ITI-10 (84.6% ± 3.1%) versus ITI-1 (84.3% ± 2.9%) showed no statistical difference ($p = 0.9$, $F_{1,44} = 0.006$). This implies that controls did not extinguish their enhanced conditional response even after several exposures to the CS. The freezing of NR1-CA1-KOs increased gradually as the test advanced. By the end of the test, it was only slightly below the freezing level attained by controls (ITI-10, 2100–2310 s, mutant, 75.9% ± 2.3%, control, 84.6% ± 3.1%, $p < 0.01$, $F_{1,44} = 11.6$). Since similar gradual increases in freezing were observed in the naive and the pseudo groups, we attribute these slow rises to habituation to the test chamber.

Finally, it was interesting to investigate whether the trace paradigm could be shortened. To this end we subjected NR1-CA1-KOs (n = 7), controls (n = 7), and pseudo mice (n = 10) to a training session of three CS–trace–US trials, instead of the previous ten trials. NR1-CA1-KOs froze somewhat less than controls during training (data not shown). On the 24 hr test, however, the three groups had similar scores (data not shown). A gradual increment in freezing observed in all groups was consistent with habituation. Thus, three trials of trace training did not seem sufficient for control mice to develop the temporal memory.

NR1-CA1-KO Mice Show Intact Memory in Delay Fear Conditioning

To demonstrate that the lack of enhanced freezing response displayed by the NR1-CA1-KO mice in the trace conditioning paradigm was not due to an impairment in expressing the conditional response per se, we trained a different set of mice in the delay fear conditioning paradigm (McEchon et al., 1998). In this paradigm, the CS onset preceded the US and both stimuli coterminated. The training sequence consisted of ten CS/US presentations with an ITI of 210 s. In this case, the freezing of mutants (n = 13) was only slightly less than controls (n = 15) throughout the training session, without reaching statistical significance (Figures 4A and 4B, overall scores: mutant, 60.8% ± 5.8%, control, 69.0% ± 3.2%, $p = 0.2$, $F_{1,26} = 1.6$; ITI-1, 75–285 s, mutant, 47.8% ± 8.2%, control, 49.4% ± 5.2%, $p = 0.9$, $F_{1,26} = 0.03$; ITI-2, 300–510 s, mutant, 51.4% ± 6.9%, control, 64.5% ± 5.2%, $p = 0.1$, $F_{1,26} = 2.4$; ITI-3, 525–735 s, mutant, 62.7% ± 8.0%, control, 74.1% ± 5.3%, $p = 0.2$, $F_{1,26} = 1.4$). This implies that NR1-CA1-KOs were able to freeze as efficiently as controls and were not exhibiting a generalized fear deficit during trace training.

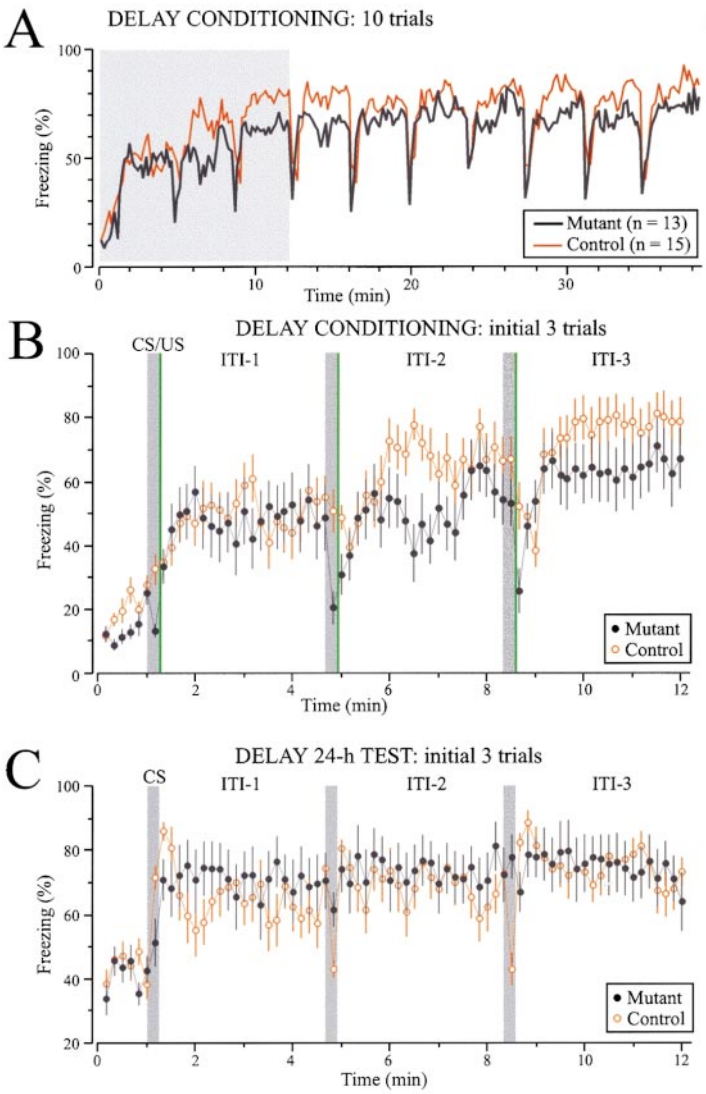

Figure 4. NR1-CA1-KO Mice Are Intact on Delay Fear Conditioning

(A) Time course of the average percent freezing displayed by mutants (n = 13) and controls (n = 15) during the acquisition of delay conditioning. The gray box, at left, indicates the initial three trials, which are enlarged in (B).

(B) Pooled data showing the average percent freezing on the initial phase of acquisition for NR1-CA1-KOs (closed circles) and controls (open circles). Symbols represent mean freezing (± SEM) on a 10 s epoch. CS presentations are indicated by gray bars and US occurrence by green bars.

(C) Pooled data showing the average percent freezing on the initial three trials of the 24 hr test after delay training for NR1-CA1-KOs (closed circles) and controls (open circles).

Additionally, we examined the behavior of delay-trained mice during a 24 hr memory test (Figure 4C). Had the lack of enhanced freezing after the CS of NR1-CA1-KOs in the trace 24 hr test been due to a sensory–motor deficiency that blocked the reaction to the CS (for instance, mutants could have a hearing deficit), it would be expected that mutants show lowered freezing during the delay 24 hr test. However, we found that the freezing responses of mutants did not differ from controls during the ITI periods (Figure 4C; ITI-1, 75–285 s, mutant, 70.8% ± 7.8%, control, 65.3% ± 5.3%, $p = 0.5$, $F_{1,26} = 0.4$; ITI-2, 300–510 s, mutant, 73.6% ± 7.1%, control, 67.7% ± 4.9%, $p = 0.5$, $F_{1,26} = 0.5$; ITI-3, 525–735 s, mutant, 74.8% ± 6.5%, control, 73.9% ± 3.9%, $p = 0.9$, $F_{1,26} = 0.02$). This implies that the white noise used as a CS was able to elicit a robust conditional response in mutants, but only when the appropriate CS–US contingency was met during training. During the presentation of the CS, control mice displayed a reduction in freezing, whereas mutants did not. This could mean that controls were able to learn that it was safe to move during the delivery of the CS, while mutants did not. However, we did not address this point further.

Our results point to a behavioral dissociation in the mutants; that is, they show impaired trace but intact delay conditioning. Since it is accepted that the neural substrate for delay fear conditioning involves the amygdala (LeDoux, 1993; Davis et al., 1994; Rogan et al., 1997; Fendt and Fanselow, 1999), it was reassuring that mutants lacking NMDARs only in CA1 cells were normal in this variant of Pavlovian learning.

Discussion

Our work provides a demonstration that NMDA receptors within CA1 pyramidal neurons are crucial for the formation of a memory based on the association of events across time. What mechanisms involving NMDARs in CA1 could bridge the temporal gap required to learn the association? In what follows we offer two potential mechanisms, the first based on the activity of single hippocampal cells and the second on hippocampal ensembles.

A fundamental assumption regarding the substrate of conditioning is that it requires temporal overlap of the

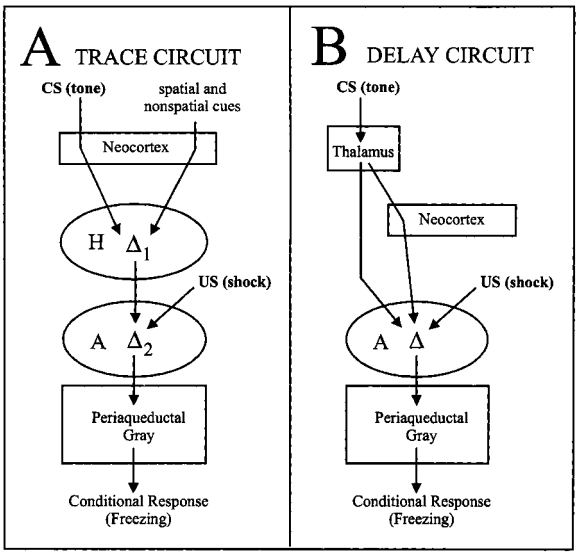

Figure 5. Putative Neural Substrate for Trace Fear Conditioning

(A) The trace circuit shows the brain regions underlying the association of the CS (tone) with the US (shock) across the trace interval. The CS, spatial, and nonspatial cues are processed in neocortex and converge into the hippocampus (H). We hypothesize that NMDAR-dependent synaptic plasticity (indicated by Δ_1) is needed to associate the spatial and nonspatial cues with the CS. This relational representation is fed into the amygdala (A), which associates it with the US via synaptic plasticity (Δ_2). The trace circuit terminates in the periaqueductal gray, which executes the conditional response (freezing).

(B) The delay circuit is simpler and requires the conjunction of the CS (tone) with the US (shock) in the amygdala. Notice that the CS representation reaches the amygdala from the auditory thalamus as well as from the auditory cortex. The delay circuit finishes in the periaqueductal gray, which executes the conditional response (freezing).

neural activities representing the CS and the US. These activities would converge and be associated within neural structures such as the amygdala for delay fear conditioning (Figure 5; LeDoux, 1993; Davis et al., 1994; Rogan et al., 1997; Fendt and Fanselow, 1999) or the cerebellum for motor conditioning (Kim and Thompson, 1997). In the case of trace fear conditioning, in which the CS and the US are temporally dissociated, a straightforward possibility is that individual CA1 cells could respond selectively to the CS and sustain their activity after CS removal, allowing for the ongoing activity to be associated with the US. Hippocampal recordings during trace eye blink conditioning, in which the trace interval is usually 1 s, have shown that certain CS-responsive cells maintain their firing for about 1 s after CS cessation (Berger et al., 1976; McEchon and Disterhoft, 1997). Thus, it is possible that on trace fear conditioning there might be CA1 cells that respond to the CS and fire for up to 30 s upon CS removal, although such activity has not yet been observed.

A different possibility stems from the observation that ensembles of CA1 cells can encode regularities present in the animal's experience, which include spatial and nonspatial cues as well as behavioral actions. In particular, it is well established that pyramidal neurons in CA1 can exhibit place cell activity; that is, they can fire in a location-specific manner (O'Keefe and Nadel, 1978; Wilson and McNaughton, 1993; O'Keefe, 1999). It is also well known that CA1 cells can fire in response to nonspatial stimuli in rodents performing tasks in which these stimuli occur in a regular fashion (Olton, 1989; Sakurai, 1996; Eichenbaum et al., 1999; Wood et al., 1999). A recent proposal that we shall call the *episodic encoding* model (Eichenbaum et al., 1999) posed that individual hippocampal cells that fire at about the same time, in response to both spatial and nonspatial features, could be organized into an ensemble that represents an event. Moreover, event ensembles could be organized along a temporal sequence, comprising an episode.

Applying these notions to trace fear conditioning, we hypothesize that ensembles of CA1 cells play an intermediary role for the association of the temporally dissociated CS and US. As a mouse is introduced into the conditioning chamber, distinct CA1 cell ensembles would be activated in a continuous manner. These ensembles would be composed of place cells as well as cells encoding nonspatial features. It follows that each US delivery will be paired with an active ensemble. The temporal overlap of CA1 ensemble activity and the US satisfies one of the requirements for conditional association. What remains is the need to incorporate CS-specific information into the activity of CA1 cell ensembles. In fact, it has been documented that sensory-related factors can strongly influence place cell activity (O'Keefe and Speakman, 1987). Also, given the regular occurrence of the CS, it is feasible that distinct CA1 cells become activated by it. We hypothesize that the CS representation is *entrained* into CA1 cell ensembles through NMDAR-dependent synaptic plasticity in CA1 (Figure 5A), possibly leading to enhanced covariance of ensemble responses, which has been shown to be NMDAR dependent (McHugh et al., 1996; Tonegawa et al., 1996). Repeated CS delivery during training would progressively pair the CS with multiple hippocampal ensembles. Subsequent US delivery would overlap with these CS-entrained ensembles, forming hippocampally dependent associations in downstream structures such as the amygdala (Figure 5A). Notice that our description can easily be phrased in terms of the episodic encoding model. Namely, CS-paired and US-paired ensembles represent events that are organized into a repetitive sequence, i.e., the conditioning episode.

Our hypothesis must also explain how the association can be recalled during the memory test in a novel environment. What is required is that, upon presentation of the CS only, the CS-entrained hippocampal ensembles (established on training) become *reactivated* and then trigger downstream circuits responsible for the conditional response. Support for this idea comes from the observation that transient cue delivery can reactivate hippocampal ensembles established through prior association with those cues (Muller and Kubie, 1987; Quirk et al., 1990). This has led to suggestions that the hippocampus is capable of carrying out pattern completion, in which subsets, or even single cues, are capable of reestablishing an original pattern of activity (Marr, 1971; McNaughton and Morris, 1987; Recce and Harris, 1996). In the present study, mice were introduced into a novel

environment during the memory test, presumably activating CA1 cell ensembles that differed from those present in the previous conditioning chamber. Upon CS delivery, ensembles related to the conditioning chamber would be strongly reactivated, driving downstream circuits to execute the freezing response. Such reactivation has not been experimentally tested but would be predicted based on our proposal.

In conclusion, our results point to a novel function of NMDARs within CA1 neurons by showing that they are essential in the formation of temporal memory. The fact that hippocampus-dependent memories in rodents extend into the spatiotemporal domain makes it feasible that they are conceptually close to the episodic memories described in humans.

Experimental Procedures

Subjects

The generation of knockout mice is detailed elsewhere (Tsien et al., 1996). NR1-CA1-KO male mice (49–78 days of age) were heterozygous for the viral *Cre* recombinase gene and homozygous for the floxed *NR1* gene. Mice in the control, pseudo-conditioned, and naive groups were male littermates, homozygous for the floxed *NR1* gene. Their age range was 49–85 days of age. Mice were handled for 5 min per day during a week before testing. They were kept in isolation only during conditioning.

Apparatus

Training occurred in chamber A (25 cm $\times$ 30 cm, height 35 cm; two adjacent walls were painted white, while the others were red and yellow). The floor was a removable foot-shocking grid (Coulbourn, Allentown, PA). Illumination was provided by seven white 1 W bulbs placed onto a transparent plexiglass ceiling. Chamber A was inside a sound-attenuating enclosure. For the 24 hr test we used chamber B (round basket; diameters: 30 cm bottom, 40 cm top, height 60 cm; bedding at the floor, gray walls) and chamber C (25 cm $\times$ 25 cm, height 60 cm; white PVC walls and floor). Chambers B and C were illuminated by three orange 25 W bulbs. A CCD camera (Phillips, LDH 351, b/w) was mounted above them. Chambers were manufactured by Mike's Machine Company (Attleboro, MA).

Conditioning

Stimuli were US (scrambled foot-shock, 0.5 s, 0.7 mA intensity) controlled by a shocker (Coulbourn) and CS (white noise, 15 s, 80 db volume) delivered through a speaker (Med Associates, St. Albans, VT) located outside the chambers. Presentations of CS and US were controlled by routines (L. D. S.) within *ad* software (M. A. W. and L. Frank) running in a DOS PC. For trace training, a mouse was placed in chamber A and, after 60 s, was subjected to ten CS–trace–US–ITI trials (trace of 30 s, ITI of 210 s). The next day, mice were exposed to the memory test (in chamber B or chamber C, located in a different room), which consisted of a 60 s introduction followed by ten CS–ITI trials (ITI of 210 s). Delay training occurred in chamber A and consisted of ten CS/US–ITI trials (CS and US coterminated). The next day, mice received the memory test (ten CS–ITI trials). Mice in the pseudo-conditioned group underwent a training session of unpaired stimuli with the CS and the US separated by 120 s. The sequence of delivery was CS–US–CS–CS–US–CS–CS–US–CS–US–CS–US–US–CS–US–CS–US–CS–US–US. The next day animals were given ten CS–ITI trials in chamber B. Mice in the naive group were placed into chamber A for 43.5 min on the first day and, 24 hr later, were given ten CS–ITI trials in chamber B.

Data Collection and Analysis

We used two independent methods for data collection. For the "tracking" method, the CCD camera provided the signal to a contrast tracker (Dragon, SA-3) with a pixel resolution of 327 horizontal by 243 vertical. Dark objects (i.e., the mouse coat) were tracked on a light background. The x/y coordinates of the tracked points stored in the tracker's cache were transmitted to PC through a 16 bit i/o card (CIO-CTR10, Computer Boards) into the *ad* software (60 Hz sampling rate; each time instance consisted of 50–150 tracked points, maximum of 253) for storage. Data was then transferred to a Linux PC and analyzed with C routines (I. H. Chan and P. T. H.). A masking filter was first applied to those cases that presented streaks of noise. Noise was common in chamber A because of the reflections of the shock bars onto the floor. Then, 12 consecutive frames were collapsed (5 Hz data binning) and the center of mass was calculated. Freezing was defined as a distance of <2 pixels between centers of mass of two consecutive frames. Freezing was summed over a 10 s epoch and expressed as a percentage (where 100% is a value of 50, given the 5 Hz binning) for each mouse. Time course plots were generated by averaging the percentage values across all mice in the group. Statistical analysis consisted of one-way analysis of variance (ANOVA) performed between subjects of two appropriate groups. The tracking method was calibrated by comparing the freezing scores to visually scored freezing done by blind observers (Figure 1). Briefly, six observers scored freezing for six mice subjected to contextual fear conditioning. Mice were placed in chamber A and after 3 min were shocked three times with an interval of 1 min between shocks. The next day, mice were placed in chamber A for 6 min. The observers scored freezing every 10 s (1 s observations on a video monitor).

For the "video" method, the CCD camera provided the video signal to a VCR (taping in SVHS-SP mode). Taped data were replayed into MATLAB (The MathWorks, Natick, MA) video acquisition software (5 Hz sampling rate) through a video card (Matrox Meteor) running in a Linux PC. Each frame was an 8 bit grayscale image (320 $\times$ 240 pixels). Routines in MATLAB (B. Fedeles and L. D. S.) first applied a filter (a bitmapped image of a mouse subtracted from the environment) and then calculated the pixel difference between frames (Figure 1B), which ranged between 0 and 2500 pixels. A difference of <50 pixels was defined as freezing. The freezing scores produced with this method confirmed the results obtained with the tracking method.

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