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Brief Communication

Trace eyeblink conditioning requires the hippocampus but not autophosphorylation of \( \alpha \text{CaMKII} \) in mice

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Little is known about signaling mechanisms underlying temporal associative learning. Here, we show that mice with a targeted point mutation that prevents autophosphorylation of \( \alpha \text{CaMKII} \) (\( \alpha \text{CaMKII}^{T286A} \)) learn trace eyeblink conditioning normally. This forms a sharp contrast to the severely impaired spatial learning in the water maze and contextual fear conditioning observed in \( \alpha \text{CaMKII}^{T286A} \) mutants. Importantly, hippocampal lesions impaired trace eyeblink conditioning in \( \alpha \text{CaMKII}^{T286A} \) mice, suggesting a potential role of hippocampal \( \alpha \text{CaMKII} \)-independent mechanisms. These results indicate that hippocampal signaling mechanisms that underlie temporal associative learning as assessed by trace eyeblink conditioning may differ from those of spatial and contextual learning.

The hippocampus plays a key role in the formation of episodic memory—the ability to associate and memorize events from experience set in their spatiotemporal context (Vargha-Khadem et al. 1997; Eichenbaum 2001). Signaling mechanisms underlying hippocampus-dependent spatial and contextual learning have been extensively studied (Abel and Lattal 2001; Matynia et al. 2002; Silva 2003; Tonegawa et al. 2003). For example, recent genetic approaches have demonstrated that spatial/contextual learning and long-term potentiation (LTP) are impaired in a series of calcium/calmodulin-dependent protein kinase II (CaMKII) mutants (Silva et al. 1992a,b; Mayford et al. 1996; Giese et al. 1998; Ohno et al. 2001, 2002; Bejar et al. 2002; Eglerisma et al. 2002; Miller et al. 2002), providing the molecular and cellular basis of this kinase in hippocampal synaptic and behavioral memory (Lisman et al. 2002; Matynia et al. 2002; Eglerisma et al. 2004). However, little is known about what mechanisms could bridge the temporal gap required to learn the association between two events and thereby form a temporal memory, an important aspect of episodic memory.

Autophosphorylation of \( \alpha \text{CaMKII} \) at threonine-286 by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (Silva et al. 1992a,b; Mayford et al. 1996) switches the kinase into a Ca\(^{2+}\)/calmodulin-independent active state (Colbran and Brown 2004; Griffith 2004). A mutation that eliminates phosphorylation of this site (T286A) blocks NMDA receptor-dependent LTP at hippocampal Schaffer collateral-CA1 pyramidal neuron synapses, a cellular mechanism underlying spatial learning and memory (Giese et al. 1998; Ohno et al. 2002). Therefore, knock-in mice with a T286A mutation (\( \alpha \text{CaMKII}^{T286A} \)) provide us with a useful tool to examine the role of \( \alpha \text{CaMKII} \) signaling in hippocampal learning. Here we applied trace eyeblink conditioning for the first time to \( \alpha \text{CaMKII}^{T286A} \) mutant mice. This behavioral paradigm requires the association of events across time, since the conditioned stimuli (CS: tone) and unconditioned stimuli (US: shock) are separated by an empty trace interval. The aim of this study was to disentangle signaling mechanisms underlying temporal memory formation by comparing the behavioral phenotypes of \( \alpha \text{CaMKII}^{T286A} \) mice between temporal associative learning and spatial/contextual learning tasks.

Previous studies showed that eyeblink conditioning is both hippocampus- and NMDA receptor-dependent in mice if a trace interval of 250 msec is inserted between the CS and US (Takatsuki et al. 2001; Tseng et al. 2004). Therefore, we used the trace eyeblink conditioning paradigm with a 250-msec trace interval to test the temporal memory function of \( \alpha \text{CaMKII}^{T286A} \) mutants. Interestingly, during training (two sessions of 30 trials per day for 3 d), \( \alpha \text{CaMKII}^{T286A} \) mutant mice as well as wild-type control mice showed a rapid and dramatic increase in the percentage of conditioned responses (CRs) and significant learning across sessions (\( \alpha \text{CaMKII}^{T286A} \), \( F_{(5,48)} = 6.34, P < 0.01 \); wild-type, \( F_{(5,36)} = 5.21, P < 0.01 \); and no difference was found between \( \alpha \text{CaMKII}^{T286A} \) mutants and wild-type littermates (\( F_{(1,14)} = 0.01, P > 0.05 \); Fig. 1A). Importantly, pseudoconditioning, i.e., random presentations of unpaired tone and shock at the same number of times with the same temporal density, did not cause significant learning (\( \alpha \text{CaMKII}^{T286A} \) mutants, \( F_{(5,56)} = 0.70, P > 0.05 \)) or in wild-type mice (\( F_{(5,42)} = 0.45, P > 0.05 \)). Consequently, the trace-conditioned \( \alpha \text{CaMKII}^{T286A} \) and wild-type mice showed significantly higher percent CRs than the respective pseudoconditioned mice during training (\( \alpha \text{CaMKII}^{T286A} \), \( F_{(1,14)} = 20.90, P < 0.01 \); wild-type, \( F_{(1,13)} = 24.13, P < 0.01 \)), suggesting specific learning of trace eyeblink conditioning in both groups. \( \alpha \text{CaMKII}^{T286A} \) mice were also normal in timing characteristics of CRs during the last three sessions of training, including CR onset latency (\( F_{(1,14)} = 0.06, P > 0.05 \)), latency to the peak of the CR (\( F_{(1,14)} = 1.39, P > 0.05 \)), and duration of the CR (\( F_{(1,14)} = 0.20, P > 0.05 \)) (Fig. 1B).

Although our previous study showed that eyeblink conditioning with a 250-msec trace interval is sensitive to hippocampal lesions in mice (Tseng et al. 2004), we confirmed that \( \alpha \text{CaMKII}^{T286A} \) mutants actually acquired this behavior in a hippocampus-dependent manner. Lesions of the hippocampus with injections of 1% ibotenic acid significantly impaired trace eyeblink conditioning in \( \alpha \text{CaMKII}^{T286A} \) mice compared to vehicle-injected mice (\( F_{(1,21)} = 8.85, P < 0.01 \); Fig. 2A), as in C57BL/6 mice (Tseng et al. 2004). Histological examination revealed that...
the mice with lesions had significant bilateral cell body loss in the hippocampus, in contrast to minor needle track and injection pressure damage in vehicle-injected control mice (Fig. 2B–D). Percent CRs during trace eyelid conditioning in the hippocampus-lesioned αCaMKII^T286A mutants were indistinguishable from those during pseudoconditioning in the intact αCaMKII^T286A mice (F_{1,16} = 2.54, P > 0.05), and indicated no significant learning across the training sessions (F_{5,72} = 0.72, P > 0.05). Taken together, these data demonstrate that αCaMKII^T286A mutants have normal temporal associative learning, as assessed by hippocampus-dependent trace eyelid conditioning, suggesting a potential for hippocampal αCaMKII-independent mechanisms underlying the formation of temporal memory.

To clarify the roles of αCaMKII signaling in the different types of hippocampus-dependent learning, we tested the αCaMKII^T286A mice by using Morris water maze and contextual fear conditioning paradigms (Fig. 3). In the hidden platform version of the water maze, animals learn to locate a submerged platform in a pool filled with opaque water; this spatial navigation performance is known to be hippocampus-dependent (Morris et al. 1986; Tsien et al. 1996; Rampon et al. 2002; Colbran and Brown 2004). Previous genetic and pharmacological studies showed that hippocampal NMDA signaling is crucial for spatial learning in the water maze and contextual fear conditioning (Morris et al. 1986; Tsien et al. 1996; Rampon et al. 2000). Consistent with these findings, in the present study the αCaMKII^T286A mutants were severely impaired in spatial and contextual learning.

Next, we tested the αCaMKII^T286A mice with a hippocampus-dependent contextual fear conditioning paradigm, in which mice learn to associate a distinct context (CS) with an aversive foot shock (US) (Fanselow 2000; Rampon et al. 2000). Wild-type control mice exhibited a robust conditioned fear response, as measured by freezing (the absence of all but respiratory movements), when placed back in the same training context 24 h after two CS/US pairings (Fig. 3D). αCaMKII^T286A mutants showed significantly less freezing than wild-type mice in response to the training chamber (F_{1,14} = 68.79, P < 0.01), indicating impaired contextual fear conditioning. Altogether, these results demonstrate that in contrast to normal trace eyelid conditioning, hippocampus-dependent spatial and contextual learning is severely impaired in the αCaMKII^T286A mutants.

The αCaMKII signaling pathway is thought to mediate synaptic plasticity and memory formation triggered by NMDA receptor activation (Ohno et al. 2001, 2002; Matynia et al. 2000). Consistent with these findings, in the present study the αCaMKII^T286A mutants were severely impaired in spatial and contextual fear conditioning.

![Figure 1](https://example.com/figure1.png) **Figure 1.** Trace eyelid conditioning is normal in αCaMKII^T286A mutants. (A) αCaMKII^T286A mice (n = 9) and wild-type littermates (n = 7) were trained for trace eyelid conditioning with 60 trials (two blocks of 30 trials) per day for 3 d. Pseudoconditioned groups of αCaMKII^T286A (n = 7) and wild-type (n = 8) mice received randomly unpaired tones and shocks. The average percent CRs (± SEM) are plotted across training sessions. Before conditioning, all mice were given one habituation session (H) consisting of 30 trials as in the conditioning sessions, except that no CS or US was delivered. Note that percent CRs increase dramatically with trace conditioning and stay low with pseudoconditioning in both αCaMKII^T286A mice and wild-type littermates. (B) Timing characteristics of CRs from αCaMKII^T286A (n = 9) and wild-type (n = 7) mice during the last three sessions of trace eyelid conditioning. There is no difference between mutants and wild-type controls.

![Figure 2](https://example.com/figure2.png) **Figure 2.** Hippocampal lesions impair trace eyelid conditioning in αCaMKII^T286A mutants. (A) αCaMKII^T286A mice received intrahippocampal injections of 1% ibotenic acid (hippocampal lesions, n = 13) or vehicle (sham lesions, n = 10). After one habituation session (H), both groups were trained for trace eyelid conditioning with 60 trials (two blocks of 30 trials) per day for 3 d. The average percent CRs (± SEM) are plotted across training sessions. It is clear that hippocampus-lesioned mutants show lower % CRs than sham-lesioned control mutants during training. (B) The average volume of hippocampal damage (± SEM) is shown. One out of 14 ibotenic acid-treated mice showed damage that was confined to the hippocampus unilaterally, and thus was discarded from all statistical analysis. (C,D) Representative histological sections from mice with a median amount of hippocampal damage from the vehicle-treated (C) and ibotenic acid-treated (D) groups.
Trace eyeblink conditioning in αCaMKII<sup>T286A</sup> mutants

Independent intracellular pathway may mediate temporal memory formation after being triggered by NMDA receptor activation. However, the length of a stimulus-free trace interval between CS and US that is required to render the conditioning hippocampus-dependent is learning task-specific: 250–500 msec and 15–30 sec for trace eyeblink and fear conditioning in mice, respectively (Huerta et al. 2000; Takehara et al. 2003; Tseng et al. 2004; Misane et al. 2005). Since the time intervals in trace fear conditioning are so much longer, we cannot exclude the possibility that the dissociation between hippocampal NMDA-dependent trace fear conditioning and αCaMKII-independent trace eyeblink conditioning may be attributable to their trace interval differences.

In conclusion, our results provide evidence that hippocampal mechanisms underlying temporal associative learning as assessed by trace eyeblink conditioning may not involve αCaMKII signaling and occur differentially from spatial and contextual learning processes. Further experiments with trace eyeblink conditioning applied to genetically targeted mice will contribute to a better understanding of the neural machinery responsible for temporal memory.

Materials and Methods

Mice

The generation of the αCaMKII<sup>T286A</sup> mutant mice was described previously (Giese et al. 1998). The heterozygous αCaMKII<sup>T286A</sup> population was backcrossed more than 10 generations into the C57BL/6N background from the original 129/B6 background. Homozygous αCaMKII<sup>T286A</sup> mutants and control wild-type littermates obtained by intercrosses of heterozygous mutants were used for the experiments. At 3–4 wks post-natally, the mice were weaned and genotyping was performed by PCR analysis of tail DNA. All experiments were done blind with respect to the genotype of the mice and were conducted with the approval of the Northwestern University Animal Care and Use Committee.

Eyeblink conditioning

The basic protocols for the surgery and trace eyelink conditioning have been described (Tseng et al. 2004). Under anesthesia with Avertin, four Teflon-coated stainless steel wires were implanted; two wires were subcutaneously passed through the upper eyelid of the right eye to record EMG activity of the orbicularis oculi muscle, and the other two wires were subcutaneously passed through the periocular region caudal to the eye to deliver the shock. One bare stainless steel wire was secured to skull screws to serve as an electrical ground. All wires were soldered to connector pins, and the connector was cemented to the skull with dental acrylic. After recovery from surgery, the mice were placed in Plexiglas cylinders in a sound-attenuated chamber and received one habituation session consisting of 30 stimulus-free trials at 30–60 sec intertrial intervals; each trial was the same duration as a conditioning trial. The next day, trace eyelink conditioning started with the delivery of the tone CS (250 msec, 2 kHz, 85 dB) and the shock US (100 msec). The shock intensity was adjusted daily for each mouse to elicit a head-movement response. Mice were trained by pairing the tone and shock with a 250-msec empty trace interval and received two blocks of 30 trials per day for 3 d (30–60-sec intertrial intervals, 3–4-h interblock intervals). The control group was given randomly unpaired tones and shocks with 15–30-sec intertrial intervals and 60 trials per session (pseudoconditioning). EMG activity during each trial was collected, rectified, and integrated using specially designed Labview routines on a computer.

Lesions and histology

The procedures for hippocampal lesions have been described (Tseng et al. 2004). Under anesthesia with Avertin, a 2-µl Hamil-
ton syringe with a 25-gauge needle was lowered stereotaxically (0.25 mm/min) to the injection site in the mouse hippocampus. The coordinates [anteroposterior (AP), mediolateral (ML), dorso-ventral (DV)] relative to bregma were as follows: –2.0, ±1.2, –1.6, –2.5, ±2.2, –1.6, –3.0, ±3.2, –3.0, and –3.0, ±3.2, –4.0. Each injection site received 100 nl of 1.0% ibotenic acid or vehicle (PBS; pH 7.4) at a rate of 10 nl/min. After the injections were completed, each mouse underwent the surgical procedures for eyetracking conditioning experiments as described above.

After behavioral testing, histological examination of hippocampal lesions was performed as described (Tseng et al. 2004). After brains were fixed in 10% formalin, they were frozen, sectioned coronally (50 µm) on a microtome, and stained with cresyl violet on gelatin-subbed slides. The overall volume of damage in an animal was estimated by converting the section to bitmap images using a Leica DMLB light microscope with a Canon Powershot G5 camera (5 Megapixels).

Water maze
The basic water maze protocol and apparatus have been described (Bourtchuladze et al. 1994). Before training, each mouse was handled for 2 min everyday for 7–10 d. The pool was 1.2 m in diameter and was made of white metal. The water was maintained at 25 ± 1°C and was made opaque with nontoxic white paint to hide the square, white escape platform (10 cm × 10 cm). During training, the platform was submerged (1 cm) below the water surface and remained in the same position in the pool for a particular mouse, but groups of animals were trained with different platform positions to avoid quadrant biases. The mice received six trials per day for 4 d (three blocks of two trials; 1-min intertrial intervals, 1-h interblock intervals). The mouse was placed into the water facing the wall of the pool and was allowed to search for the platform. The starting position varied among four locations in a pseudorandom manner for each trial. The trial ended either when an animal climbed onto the platform or when a maximum of 60 sec elapsed. The mouse was placed on the platform for 60 sec before and after each trial. At the end of training, all mice were given a probe test with the platform removed from the pool. The data collection and analysis were performed using a digital tracking device (HVS Image).

Fear conditioning
Contextual fear conditioning was tested as described (Ohno et al. 2001). During training, mice were placed in the conditioning chamber for 5 min and were then exposed to two foot-shocks (0.65 mA, 1 sec) at a 1-min interval. After the second shock delivery, mice were left in the chamber for another 1 min. The mice were tested for contextual fear conditioning by scoring freezing behavior with automated procedures when the mice were placed back into the same conditioning chamber 24 h after training. The mouse’s moment-to-moment position in the chamber was sampled at 4 Hz, and the absence of all but respiratory movement through four consecutive frames (1 sec) was scored as freezing.

Statistical analysis
A two-way ANOVA with repeated measures was used to analyze the acquisition data from the trace eyetracking conditioning and water maze experiments. Comparisons for the other measures were done by a one-way ANOVA, and post-hoc Fisher’s LSD tests were performed when appropriate.

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