Associative Memory Formation Increases the Observation of Dendritic Spines in the Hippocampus

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Dendritic spines are sources of synaptic contact that can be altered by experience and, as such, may be involved in memories for that experience. Here we tested whether the acquisition of new memories is associated with changes in the density of dendritic spines. Adult male rats were trained using the trace eyeblink conditioning paradigm, an associative learning task that requires the hippocampus for acquisition. Additional groups were exposed to the same number of stimuli presented in an explicitly unpaired manner or were naive. Twenty-four hours later, the density of dendritic spines was measured using Golgi impregnation. Trace conditioning was associated with an increase in the density of dendritic spines on the pyramidal cells of area CA1 of the hippocampus, an effect that was prevented by blocking acquisition of the learned response with a competitive NMDA receptor antagonist. Training with delay conditioning, a similar task that does not require the hippocampus, also produced an increase in spine density. The learning-induced increase in dendritic spine density was specific to basal dendrites of pyramidal cells in the CA1 region of the hippocampus. Changes did not occur on their apical dendrites or on cells in the dentate gyrus or somatosensory cortex. These results suggest that the formation and expression of associative memories increase the availability of dendritic spines and the potential for synaptic contact.

Key words: **eyeblink conditioning; synaptic plasticity; basal dendrites; synaptogenesis; NMDA; learning**

Introduction

The mechanisms by which memories are acquired and stored in the mammalian brain are assumed to involve modifications in synaptic plasticity (Ramon y Cajal, 1893). The most extensively examined region in which plastic events are thought to occur is the hippocampal formation, a brain region involved in the acquisition of some types of learning (Solomon et al., 1986; Clark and Squire, 1998; Riedel et al., 1999) as well as possessing a remarkable degree of plasticity. Although there are many instances of changes in hippocampal synaptic neurotransmission in response to learning (McNaughton and Morris, 1987; Power et al., 1997), there are few examples of learning-induced changes in structural plasticity that involve either the production of new synapses or a reorganization of existing synapses (Bailey and Kandel, 1993; Moser, 1999). Dendritic spines, small protrusions on the shaft of dendrites in the mammalian brain, represent a means whereby new contacts between cells can be established and existing contacts strengthened. As such, it has long been suggested that dendritic spines are involved in the formation of new memories. Also, because most spines are the location of excitatory synapses in the hippocampus, an increase in their number could translate into a significant increase in excitatory neurotransmission (Andersen et al., 1966; Harris and Kater, 1994), which is often considered an integral step in memory formation.

Although there are reports that environmental experience can affect dendritic spines, especially in cortical regions (Anderson et al., 1996; Kleim et al., 1996; Knafo et al., 2001), evidence distinguishing training-induced effects on dendritic spines from learning itself does not exist. Training on a hippocampal-dependent task of spatial maze learning has been associated with a transient increase in dendritic spine density (O'Malley et al., 2000), although others did not observe a change (Rusakov et al., 1997). There is also indirect evidence associating spines with learning; exposure to a complex spatial environment enhanced spines and, in a separate group of animals, enhanced performance in the water maze (Moser et al., 1994). Training on another hippocampus-dependent task (Solomon et al., 1986; Moyer et al., 1990; Beylin et al., 2001), trace eyeblink conditioning, was associated with changes in synaptic structure, but spine number was not assessed (Geinisman et al., 2000, 2001).

Trace conditioning is an associative learning task in which two stimuli are separated in time. Here we tested whether the acquisition of trace memories alters the density of dendritic spines in the hippocampus using Golgi impregnation. After demonstrating that trace conditioning increased the density of dendritic spines on basal dendrites of CA1 pyramidal neurons, relative to training with unpaired stimuli, we evaluated whether this effect was a result of learning itself. Early acquisition of the classically conditioned eyeblink response is dependent on activation of the NMDA type of glutamate receptor (Servatius and Shors, 1996; Thompson and Disterhoft, 1997). Thus, in the second experiment, the NMDA receptor antagonist was administered to determine whether changes in spine density are evident after the blockade of learning. In the third and final experiment, we evaluated whether learning an association that is not dependent on the hippocampus, but engages its activity, would affect dendritic spine density in hippocampal cell regions.

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Materials and Methods

Experiment 1: the effects of trace conditioning on spine density

Subjects and surgical procedures. Adult male Sprague Dawley rats (300–400 gm; 2–3 months of age) were purchased from Zivic-Miller Laboratories (Zelienople, PA) and maintained in the Department of Psychology at Rutgers University. Rats were individually housed, had *ad libitum* access to laboratory chow and water, and were maintained on a 12 hr light/dark cycle. Rats were acclimated to the colony room for at least 1 week before surgery. They were anesthetized with sodium pentobarbital anesthesia (45 mg/kg) supplemented by isoflurane inhalant and fitted with headstages attached to four electrodes: two recorded electromyographic (EMG) activity for determination of the eyeblink, and two delivered the periorbital stimulation to elicit the eyeblink reflex (Servatius and Shors, 1996). Electrodes consisted of silver wire implanted subcutaneously to emerge through and around the eyelid. One end of the wires was deinsulated, and the other was attached through gold pins to a strip connector that served as a headstage. The headstage was surrounded by a plastic cap and secured to the skull with acrylic. A recovery period of at least 5 d occurred before behavioral testing.

Classical eyeblink conditioning. Headstages were connected to a cable that allowed free movement within the conditioning chamber. Rats were acclimated to the conditioning apparatus for 1 hr. Twenty-four hours later, rats were returned to the conditioning apparatus and spontaneous eyeblinks were recorded. To detect any sensitized response before training, responses to 10 white-noise stimuli [250 msec; 83 dB; intertrial interval (ITI) of 25 ± 5 sec] before training were also recorded. Eyeblinks during the first 100 msec of the white noise were recorded. Rats were then exposed to 300 trials of trace conditioning with paired stimuli ($n = 9$) or unpaired training ($n = 8$). During trace conditioning, an 83 dB, 250 msec burst of white-noise conditioned stimulus (CS) was separated from a 100 msec, 0.7 mA periorbital shock unconditioned stimulus (US) by a 500 msec trace interval (see Fig. 1*A*). These stimulus parameters produce learning that is dependent on an intact hippocampus in rats (Beylin et al., 2001). Each block of trace conditioning consisted of 100 trials with every 10 trial sequence composed of one CS-alone presentation, four paired presentations of the CS and US, one US-alone presentation, and another four paired presentations of the CS and US. The ITI was 25 ± 5 sec. During unpaired training, rats received the same number of CS and US exposures presented in an explicitly unpaired manner. The ITI was 10 ± 3 sec. To detect the occurrence of an eyeblink, the maximum EMG response occurring during a 250 msec prestimulus baseline recording period was added to four times its SD. Responses that exceeded that value and were >3 msec were considered eyeblinks. During trace conditioning, eyeblinks were considered conditioned responses (CRs) if they began 500 msec before US onset. In the unpaired protocol, eyeblinks were recorded during the same time interval as paired training. Eyeblink performance was computed as a percentage of CRs to the CS. Twenty-four hours later, rats that underwent trace conditioning with paired or unpaired stimuli were killed with a group of naive animals ($n = 8$) that did not receive stimulus exposure. Blood samples were collected via cardiac puncture before perfusion for the radioimmunoassay of corticosterone (CORT). Previous studies have demonstrated that paired and unpaired training elevates CORT levels (Shors et al., 1992), a factor that has been implicated in regulating changes in dendritic morphology in the hippocampus (Woolley et al., 1990; Shors et al., 2001).

Golgi method. Rats were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 120 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer and 1.5% picric acid (v/v). Brains were postfixed and stored overnight in the same solution. After postfixation, a modified version of the single-section Golgi impregnation procedure was used to process brains (Gabbott and Somogyi, 1984; Woolley and Gould, 1994; Shors et al., 2001). Serial coronal sections (150 μm) were cut on an oscillating tissue slicer in a bath of 3.0% potassium dichromate in distilled water. The sections were incubated overnight at room temperature in individual wells containing 3.0% potassium dichromate. The following day, the sections were rinsed and mounted onto ungelatinized slides, a coverslip was glued over the sections at the four corners, and the slide assembly was placed in a Coplin jar containing 1.5% silver nitrate in distilled water. After 48 hr, the slide assemblies were dismantled and the sections removed from the slides. The sections were rinsed in distilled water, dehydrated in ethanol, cleared in xylenes, and mounted onto ungelatinized glass slides. Slides were coverslipped with Permount and allowed to dry before quantitative analysis.

Spine density analysis. Spine density analysis was conducted blind to experimental condition. For CA1 pyramidal neurons, spine density was measured on apical dendrites of stratum radiatum and basal dendrites of stratum oriens. Quantitative analysis was conducted on tissue stained dark with Golgi impregnation that was uniform throughout the section. Six Golgi-impregnated pyramidal neurons discernible from nearby impregnated cells were selected. These neurons were located within the CA1 region of the dorsal hippocampal formation and were required to have no breaks in staining along its dendrites. Measurement occurred at least 50 μm away from the soma for apical dendrites and 30 μm for basal dendrites on secondary and tertiary branches. Five segments between 10 and 20 μm in length and in the same plane of focus were chosen. In some cases, the segments were from the same branch. Counting required focusing in and out with the fine adjustment of the microscope (Nikon Eclipse E400; Nikon, Tokyo, Japan) using 1000× magnification and oil immersion. Only spines that were distinct from the dendritic branch were counted. Spine density was calculated by dividing the number of spines on a segment by the length of the segment and was expressed as the number of spines per 10 μm of dendrite. Densities of spines on five segments of a cell were averaged for a cell mean, and the six cells from each animal were averaged for an animal mean. Spine density values using this method are underestimates, because spines protruding either above or beneath the dendritic shaft are not accounted for (Woolley and Gould, 1994).

Measurement of dendritic length. Dendritic length measurements were conducted on a subset of animals ($n = 5$ per group). To be selected for analysis, three isolated and thoroughly impregnated CA1 pyramidal neurons were chosen from each animal. Images of each cell were taken with a CCD camera mounted to the microscope at 400× magnification. From this image, three secondary and three tertiary dendrites were traced, and the length was measured using Scion Image software (Scion Corporation, Frederick, MD). The mean length of secondary and tertiary dendrites for each cell was calculated, and the cells were averaged for an animal mean.

Radioimmunoassay of corticosterone. As indicated, cardiac blood was collected before perfusion. Samples were added immediately to test tubes containing 0.1 ml of heparin and centrifuged at 3000 rpm for 20 min. Plasma aliquots were stored at −20°C and thawed before analysis. Circulating levels of CORT were measured using a solid phase radioimmunoassay system (Coat-A-Count; Diagnostic Products, Los Angeles, CA). The assay sensitivity was 5.7 ng/ml.

Experiment 2: the effects of associative learning on spine density

To determine whether the effect of trace conditioning on spine density was sensitive to learning itself or whether it was simply the product of training, rats were injected intraperitoneally with the NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphoric acid (CPP) (10 mg/kg; Sigma, St. Louis, MO) or saline vehicle 1 hr before training. As before, rats were exposed to 300 trials of trace conditioning or unpaired training ($n = 6$–9 animals/group) and killed 24 hr later for Golgi impregnation. To confirm that NMDA receptor blockade did indeed prevent learning, separate groups were injected with saline ($n = 7$) or CPP ($n = 5$) and exposed to 300 trials of trace conditioning. Twenty-four hours later and in the absence of the drug, these groups were exposed to 300 additional trials of training.

Experiment 3: the effects of trace versus delay conditioning on spine density

Rats were exposed to 300 trials of conditioning using a trace ($n = 6$), unpaired ($n = 5$), or delay ($n = 6$) paradigm to determine whether the effect of conditioning on spines was specific to hippocampal-dependent learning. In delay conditioning, an 850 msec, 83 dB CS overlapped and coterminated with a 100 msec, 0.7 mA US (see Fig. 1*B*). These stimulus parameters do not require an intact hippocampus for learning (Beylin et al., 2001). As in the previous experiments, rats were killed 24 hr after

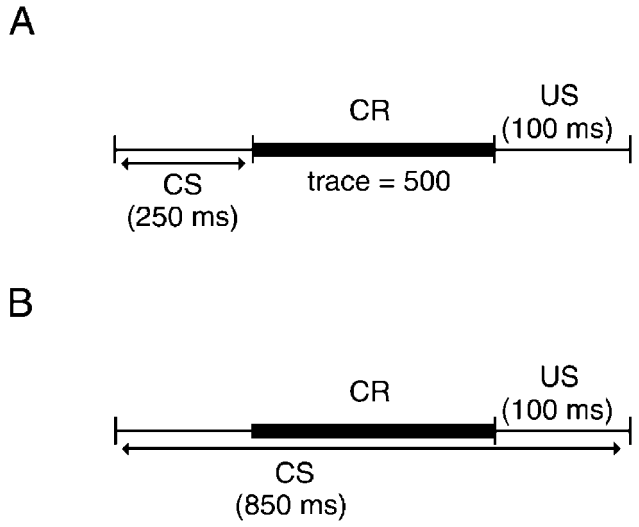

Figure 1. Schematic diagram of trace (*A*) and delay (*B*) conditioning procedures. In trace conditioning, there is a temporal gap (*trace*) between the CS offset and US onset. In delay conditioning, the CS overlaps and coterminates with the US. *Bold lines* represent the interval during which an eyeblink was considered a CR.

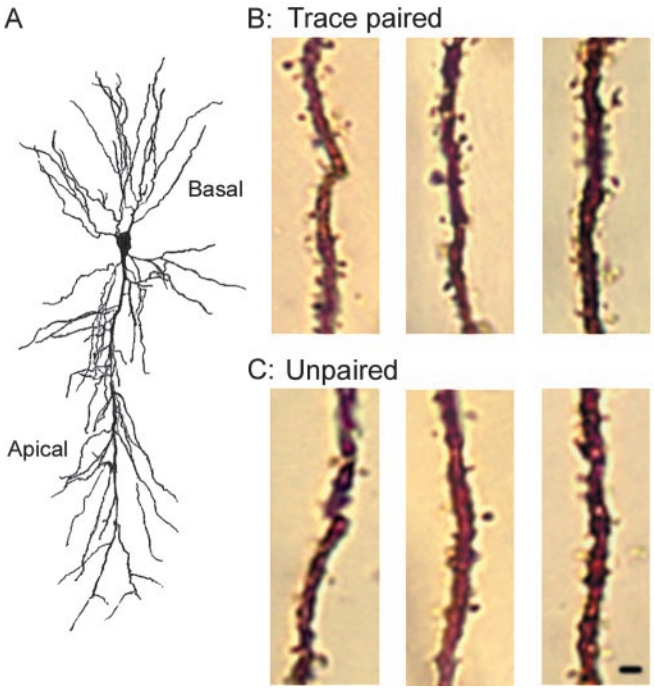

Figure 3. Golgi impregnation of pyramidal cells in area CA1 of the hippocampus. *A*, Photomicrograph (200× magnification) of a Golgi-impregnated CA1 pyramidal cell illustrating the apical and basal dendrites. *B, C*, Representative basal dendritic segments (1000× magnification) from animals exposed to trace conditioning with paired (*B*) versus unpaired (*C*) stimuli. Scale bar, 1 μm.

training, and the tissue was processed for Golgi impregnation. In this experiment, we extended the observation of dendritic spines on CA1 pyramidal cells to include granule cells of the dentate gyrus, as well as basal spines on pyramidal cells of the somatosensory cortex. For analysis of granule cells, three neurons located in the dorsal blade of the dentate gyrus were selected. For the analysis of cortical neurons, three neurons located in the somatosensory trunk regions and parietal association cortices (3.3–3.8 mm posterior to bregma; 2–3 mm lateral) (Paxinos and Watson, 1986) were chosen. Both the dentate gyrus and the cortical neurons selected for analysis were required to be uniformly impregnated and easily distinguished from neighboring cells. For each cell, five segments 10–20 μm in length in the same plane of focus were chosen, and counting began at least 25 μm away from the soma on secondary and tertiary dendritic branches.

Statistical analysis. Repeated-measures ANOVA was conducted to evaluate eyeblink performance. The animal means for the density of spines and length of CA1 pyramidal cell apical and basal dendrites, spine density on neurons of the cortex and dentate gyrus, and CORT levels were analyzed using ANOVA. To evaluate group differences, *post hoc* analysis using the Newman–Keuls test was applied to significant main effects and interactions.

Results

Trace conditioning increases dendritic spine density

The group of rats exposed to paired stimuli during trace conditioning (Fig. 1*A*) emitted more CRs over 300 trials than rats exposed to the same number of unpaired stimuli ($F_{(2,30)} = 4.64$; $p < 0.05$) (Fig. 2*A*). Twenty-four hours after the training experience, the group exposed to paired stimuli possessed a greater density of spines on the basal dendrites of pyramidal cells in area CA1 of the hippocampus compared with the group exposed to the same number of unpaired stimuli or naive controls ($F_{(2,18)} = 11.47$; $p < 0.005$) (Fig. 2*B*). The group exposed to trace conditioning had ~27% more spines than the group exposed to unpaired stimuli ($p < 0.005$) and ~39% more than the naive controls ($p < 0.005$) (Fig. 3). The spine density on basal dendrites was not different between animals exposed to unpaired stimuli versus those left in their home cage (naive) ($p = 0.26$). Thus, exposure to the conditioning stimuli themselves or the context associated with the training procedures did not increase spine density, suggesting that the training-induced increase in spine density is not an artifact of stimulus exposure or production of the unconditioned motor response.

To further characterize the change in spine density on basal dendrites, we examined their distribution in the different groups (Moser et al., 1997) (Fig. 4). All segments from all cells within a group were sorted according to the number of spines that were observed and expressed

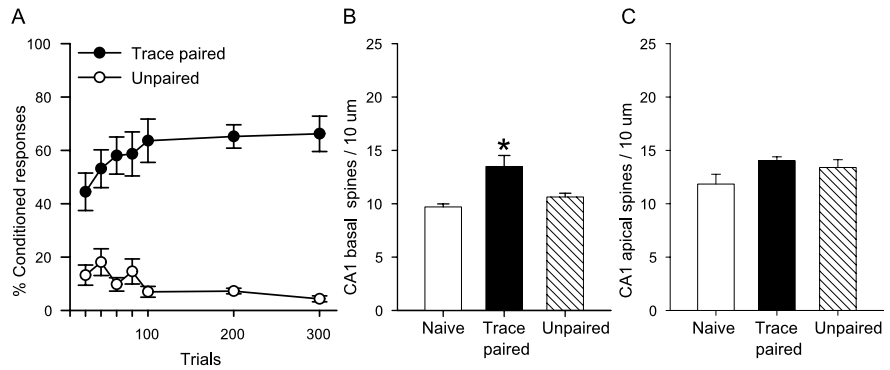

Figure 2. Trace conditioning increases dendritic spine density in area CA1 of the hippocampus. *A*, Animals that underwent trace conditioning with paired stimuli exhibited more CRs than animals trained with unpaired stimuli. The mean density of dendritic spines on basal (*B*) and apical (*C*) dendrites of CA1 pyramidal cells of the hippocampus. Trace conditioning increased the density of spines on basal but not apical dendrites. The *asterisk* indicates a significant difference. Error bars indicate SEM.

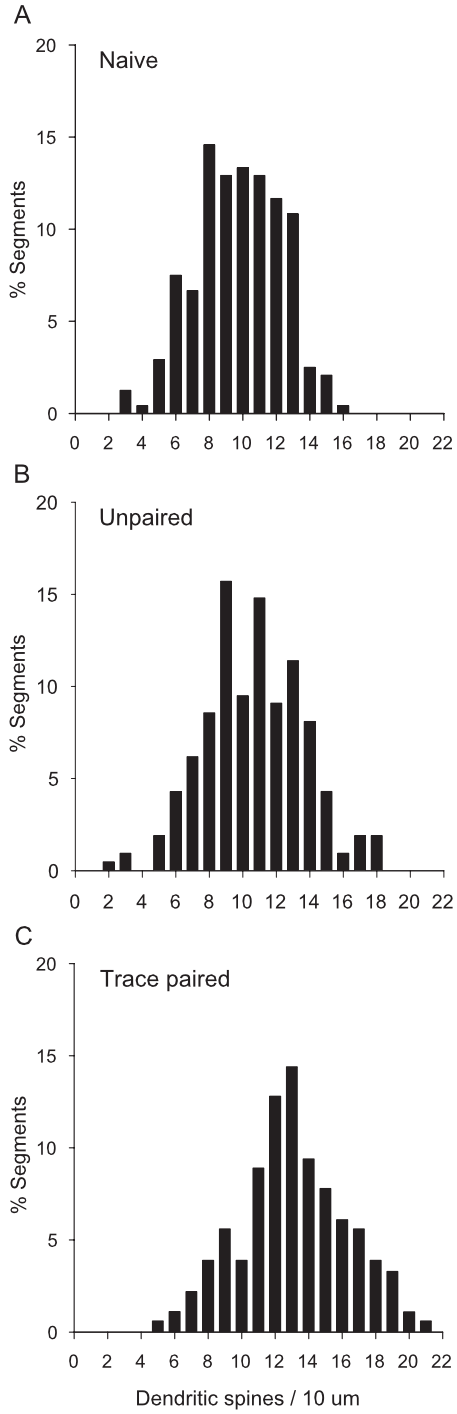

Figure 4. Distribution of spine densities on basal dendrites. Segments within groups of naive animals (*A*) and those exposed to unpaired (*B*) and paired (*C*) stimuli were sorted according to spine number and expressed as the percentage of the total number of segments.

as the percentage of the total number of segments (180 trace-paired segments, 210 trace-unpaired segments, and 240 naive segments). Although the distributions are similar among groups, there are a greater number of segments with high spine density and fewer segments with low spine density from animals exposed to trace conditioning. In contrast, the groups exposed to unpaired stimuli and those left in their home cage have a greater number of segments with low numbers. A Pearson correlation revealed no significant relationship between the number of CRs in individual animals exposed to paired training and the number of spines on their dendrites ($p = 0.71$).

There was no effect of trace conditioning on spines located on the apical dendrites of CA1 pyramidal cells ($F_{(2,19)} = 2.53$; $p = 0.11$) (Fig. 2*C*). In addition, there was no effect of training on the length of secondary or tertiary branches of apical and basal dendrites, suggesting that training did not induce an expansion or retraction of the dendrite (Table 1). Finally, exposure to paired versus unpaired training did not differentially affect CORT levels (paired, 214.91 ± 21.14 ng/ml; unpaired, 221.20 ± 27.70 ng/ml; naive, 245.53 ± 24.87 ng/ml) ($F_{(2,22)} = 0.43$; $p = 0.65$). Note that these levels were obtained from blood drawn 24 hr after training. Therefore, differing levels of glucocorticoids at the time the animals were perfused did not mediate the training-induced changes in spine density. In addition, changes in glucocorticoid levels during training are not likely to mediate this effect, because previous studies have shown that exposure to both paired and unpaired training elevates corticosterone levels to a similar degree (Shors et al., 1992).

NMDA receptor antagonism prevents learning and the increase in spine density

Administration of the NMDA receptor antagonist CPP did not affect the spontaneous blink rate ($F_{(1,27)} = 0.08$; $p = 0.78$) or responding to a white-noise stimulus before training ($F_{(1,27)} = 0.54$; $p = 0.47$). There was a significant three-way interaction between injection with CPP versus saline, exposure to paired versus unpaired stimuli, and trials of training ($F_{(2,54)} = 6.43$; $p < 0.005$); only saline-injected animals exposed to paired stimuli acquired the CR ($p < 0.05$) (Fig. 5*A*). There was also an interaction between injection with CPP versus saline and exposure to paired versus unpaired training on the spine density of basal dendrites in area CA1 ($F_{(1,23)} = 6.50$; $p < 0.05$) (Fig. 5*B*). The density of spines in those that were injected with saline and exposed to paired training was greater than in those injected with saline and exposed to unpaired training ($p < 0.05$). However, spine density in those injected with CPP before training did not differ between those exposed to paired and those exposed to unpaired stimuli ($p > 0.05$). Thus, the training-induced increase in spine density did not occur in those that did not emit CRs. There was no effect of training ($F_{(1,22)} = 0.13$; $p = 0.72$) or drug administration ($F_{(1,22)} = 3.89$; $p = 0.06$), nor was there an interaction between training and drug administration ($F_{(1,22)} = 1.48$; $p = 0.24$) on the spine density of apical dendrites.

To verify that exposure to the NMDA receptor antagonist did prevent learning, additional groups of animals were injected with CPP before training, trained, and then trained again in the absence of the antagonist. During exposure to the first 300 trials, those injected with CPP emitted fewer CRs (<10%) than the group injected with saline ($F_{(1,10)} = 19.98$; $p < 0.005$). On exposure to 300 additional trials, their response rate did not differ from those injected with saline and exposed to the first 300 trials of training ($p = 0.13$) (Fig. 5*C*). Thus, there was no evidence of residual learning in animals injected with CPP and trained, further suggesting that the increase in spine density after trace conditioning is a result of learning and not performance or exposure to the training conditions.

Hippocampal-independent learning also increases spine density

Groups of rats that underwent trace (Fig. 1*A*) and delay (Fig. 1*B*) conditioning emitted more CRs than those exposed to unpaired stimuli ($F_{(2,13)} = 29.58$; $p < 0.005$) (Fig. 6*A*). Those exposed to delay conditioning emitted more CRs than those exposed to trace conditioning, as reported previously ($p < 0.005$) (Beylin et al., 2001). Training on both conditioning tasks increased the spine

Table 1. Effect of trace conditioning on dendritic length of CA1 pyramidal cells

	Naive	Trace paired	Trace unpaired	*p* value
Apical dendritic length (μm)				
Secondary branches	99.3 ± 2.5	110.5 ± 4.0	106.5 ± 3.2	0.09
Tertiary branches	47.8 ± 1.9	47.9 ± 2.1	49.1 ± 1.7	0.86
Basal dendritic length (μm)				
Secondary branches	85.5 ± 4.6	82.5 ± 4.3	87.4 ± 3.1	0.70
Tertiary branches	30.6 ± 1.9	35.4 ± 5.9	32.5 ± 2.3	0.68

Values represent means ± SEM of dendritic length for CA1 pyramidal cells from animals that were naive or underwent trace conditioning with paired or unpaired stimuli ($n = 5$ per group). No significant differences were observed.

density on basal dendrites in area CA1 ($F_{(2,11)} = 7.11$; $p < 0.01$) (Fig. 6*B*) compared with animals exposed to unpaired stimuli ($p < 0.05$). Neither trace nor delay conditioning altered the density of spines on apical dendrites ($F_{(2,9)} = 0.42$; $p = 0.67$). In addition, there was no effect of trace or delay conditioning on the spine density of pyramidal cells in the somatosensory cortex ($F_{(2,11)} = 0.53$; $p = 0.60$) (Fig. 6*C*) or granule cells of the dentate gyrus ($F_{(2,12)} = 0.19$; $p = 0.83$) (Fig. 6*D*).

Discussion

It has long been postulated that dendritic spines are an anatomical substrate involved in memory formation or storage (Ramon y Cajal, 1893). However, there are no reports of a direct and persistent effect of learning itself on dendritic spines in the hippocampus, a brain region critically involved in some types of learning and memory processes (Solomon et al., 1986; Clark and Squire, 1998). From our initial experiment, we present data indicating that associative learning enhances dendritic spine density by ~20% on hippocampal pyramidal cells of area CA1. Although these data suggest that learning increases spine density in the hippocampus, they are inconclusive, because other aspects of the training experience could alter their numbers. Therefore, in a second experiment, we prevented acquisition of the learned response by administering a competitive NMDA receptor antagonist before training (Servatius and Shors, 1996; Thompson and Disterhoft, 1997). Animals that were injected with saline and trace-conditioned possessed a greater density of dendritic spines in area CA1 compared with those exposed to unpaired stimuli, whereas those that were injected with the antagonist did not emit CRs and showed no increase in spine density. To verify that no learning occurred in those that were injected with the NMDA receptor antagonist, a similar group was injected with the antagonist and underwent additional training in the absence of the drug. There was no evidence of residual learning in this group. Thus, the training-induced increase in spine density appears to be specific to learning the association between the CS and the US. Because the increase was observed only in those exposed to the paired stimuli and not in those exposed to explicitly unpaired stimuli, it appears to be specific to learning a positive association between the two conditioning stimuli. To our knowledge, these results are the first demonstration that changes in spine density occur as a result of learning and not a result of training per se.

In a final experiment, we determined that the learning-induced increase in spine density was not specific to hippocampal-dependent learning but was also evident in animals trained on the hippocampal-independent task of delay conditioning. This is perhaps not surprising, because the hippocampus must process stimulus information before any knowledge of the task requirements. Also, it has been demonstrated that neuronal activity in CA1 pyramidal cells increases during the performance of both trace and delay tasks (Berger et al., 1980). *In vitro* studies have attributed the enhanced excitability to a reduction in the afterhyperpolarization and enhanced synaptic responsiveness of CA1 pyramidal neurons (Disterhoft et al., 1986; LoTurco et al., 1988; Moyer et al., 1996). Such heightened activity of hippocampal pyramidal cells could influence the formation or extension of dendritic spines. Indeed, changes in activity have been associated with alterations in synaptic structure on Purkinje cells after classical eyeblink conditioning (Anderson et al., 1999). Other studies indicate that tetanic stimulation enhances the *de novo* appearance of dendritic spines, at least *in vitro* (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). Furthermore, exposure to both trace and delay conditioning increases other measures of synaptic plasticity in area CA1 of the hippocampus, such as the binding affinity of AMPA receptors (Tocco et al., 1992). Note that the hippocampus is more engaged in delay conditioning than was previously thought. Using the parameters of the present experiments, animals with hippocampal lesions can acquire the delay response (Schmaltz and Theios, 1972; Solomon et al., 1986; Beylin et al., 2001), but they are impaired under more difficult training parameters (a very long interstimulus interval) (Clark and Squire, 1998; Beylin et al., 2001). Moreover, animals with hippocampal lesions that have already acquired the associ-

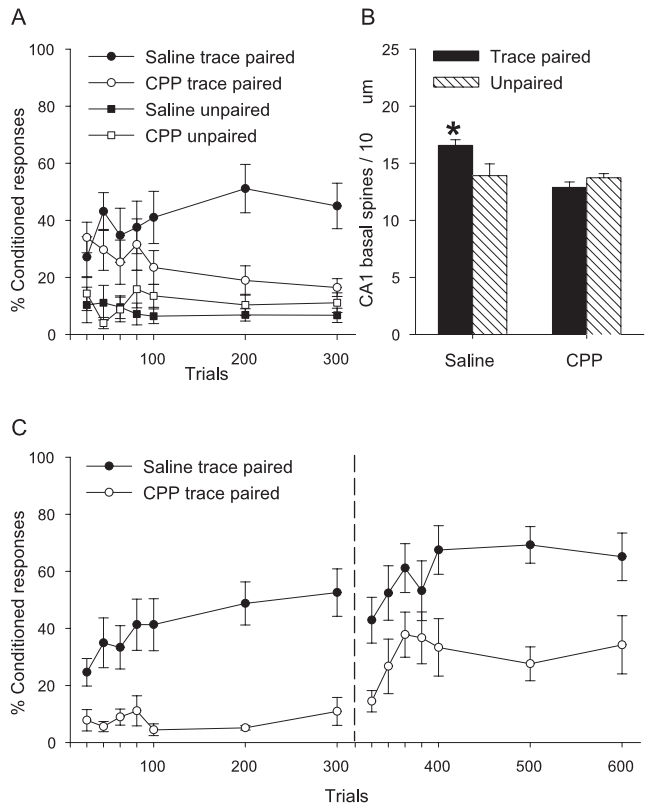

Figure 5. NMDA receptor antagonism prevents learning and the increase in spine density. *A*, Animals injected with the NMDA receptor antagonist CPP displayed significantly fewer CRs than saline-injected animals. *B*, Mean density of spines on basal dendrites of CA1 pyramidal cells of the hippocampus. Blocking acquisition of the learned response with the NMDA receptor antagonist CPP prevented the training-induced increase in spine density on basal dendrites. *C*, An additional group of animals injected with CPP also did not acquire the trace-conditioned response relative to saline-injected controls. Twenty-four hours later and in the absence of the antagonist (as noted by the *dashedline*), these animals acquired the CR and displayed no evidence of residual learning. The *asterisk* indicates a significant difference. Error bars indicate SEM.

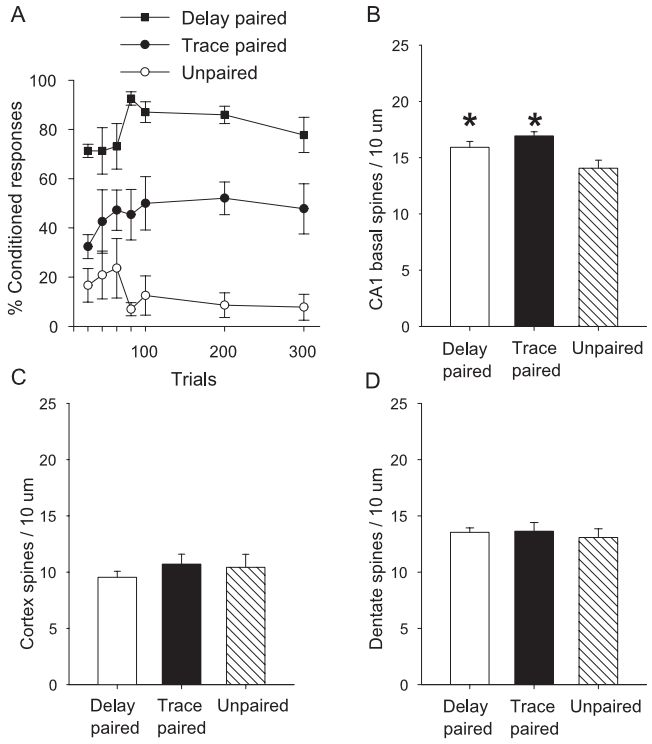

Figure 6. Trace and delay conditioning increase dendritic spine density in the hippocampus. *A*, Animals that underwent delay or trace conditioning exhibited significantly more conditioned responses than animals trained with unpaired stimuli. *B–D*, The mean density of spines on basal dendrites of CA1 pyramidal cells (*B*), pyramidal cells of the cortex (*C*), and granule cells of the dentate gyrus (*D*). Both trace and delay conditioning increased the density of spines in area CA1 but not in the dentate gyrus or cortex. Significant differences are noted with *asterisks*. Error bars indicate SEM.

ation under the typical delay conditions rapidly acquire the learned response using a trace paradigm (Beylin et al., 2001). Thus, once the association has been established, the hippocampus is no longer necessary for learning or expressing the trace memory. The present data suggest that the initial acquisition of these associations affects the density of dendritic spines regardless of whether the hippocampus is necessary.

The length of branches on apical and basal dendrites did not change as a function of training, suggesting that the learning-induced increase in spine density was not attributable to expansion or shrinkage of the dendritic tree. The learning-induced increase in spine density was also regionally specific to area CA1 and not the dentate gyrus. Moreover, the effects were located on basal and not apical dendrites. Interestingly, others have found that experiences such as environmental enrichment and stimulus exposure increase spine density on basal and not apical dendrites; these experiences were associated with enhanced spatial learning ability (Moser et al., 1994, 1997). Basal dendrites receive more contralateral input than apical dendrites (Swanson et al., 1978; Ishizuka et al., 1990; Li et al., 1994; Amaral and Witter, 1995), as well as fewer inhibitory inputs from interneurons (Toth and Freund, 1992). There are also physiological differences between these regions, at least to the extent that the magnitude of long-term potentiation is greater in basal dendrites (Kaibara and Leung, 1993). Together, these data suggest that basal dendrites in CA1 have a high capacity for synaptic plasticity. An examination of the distribution of the spine densities on the basal dendrites demonstrates additional specificity. There are a greater number of segments with high spine density and fewer segments with low spine density in the trace-conditioned group, whereas the unpaired and naive groups show the opposite pattern of results. It should be noted that we examined dendritic spines 24 hr after training; therefore, changes in spine density could occur in other cell regions at earlier or later time points and at different stages in the learning process.

Dendritic spines are the primary source of synaptic contact in the mammalian brain. We cannot verify that the learning-related increase in spine density translates into an increase in synaptic contact, but such a consequence is likely. Others have reported increases in synaptogenesis after training (Wenzel et al., 1980; Van Reempts et al., 1992; Stewart and Rusakov, 1995; Ramirez-Amaya et al., 1999; Kleim et al., 2002) and similarly, use-dependent measures of synaptic plasticity such as long-term potentiation are associated with changes in hippocampal synapse number and/or structure (Desmond and Levy, 1986; Geinisman, 2000; Yuste and Bonhoeffer, 2001). More recently, it was reported that trace conditioning did not alter the number of axospinous synapses in the hippocampus but did increase the number of multiple synapse boutons, a condition under which one presynaptic bouton synapses with two or more dendritic spines (Geinisman et al., 2001). This effect was evident on apical dendrites, but basal dendrites were not examined. The present findings concur, because we also did not observe an increase in spine number on apical dendrites after learning. It has been proposed that existing spines may relocate from nonactivated boutons and synapse with those activated by conditioning, at least on apical dendrites (Geinisman et al., 2001).

These data indicate that an increase in spine density accompanies associative memory formation. They do not indicate that an increase is necessary for learning to occur, especially because their presence was enhanced after delay conditioning, which under the present training conditions does not require the hippocampus (Beylin et al., 2001). The increase in spine density does not appear to be a result of enhanced arousal or the stress of training. In previous studies, we found that exposure to an acute stressful event (30 brief intermittent tail shocks) also increased spine density in area CA1 of the hippocampus, but the effect was less localized and less evident on basal dendrites (Shors et al., 2001). In the present experiment, levels of the stress hormone corticosterone were not elevated at the time of tissue preparation (24 hr after training), but would have been elevated equally during exposure to paired versus unpaired stimuli (Shors et al., 1992). Because only exposure to paired training was associated with an increase in the presence of spines, these data are consistent with learning-related phenomena.

Dendritic spines exist on most excitatory neurons in the hippocampal formation as well as in cortical structures (Harris and Kater, 1994). Although identified over 100 years ago, their functional significance remains unknown. The present results indicate that they are affected by the formation of simple associative memories and are thus consistent with a long-held belief that these spines are sensitive to new experience and memories for experience.

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