Altered Cortical Synaptic Morphology and Impaired Memory Consolidation in Forebrain-Specific Dominant-Negative PAK Transgenic Mice

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Summary

Molecular and cellular mechanisms for memory consolidation in the cortex are poorly known. To study the relationships between synaptic structure and function in the cortex and consolidation of long-term memory, we have generated transgenic mice in which catalytic activity of PAK, a critical regulator of actin remodeling, is inhibited in the postnatal forebrain. Cortical neurons in these mice displayed fewer dendritic spines and an increased proportion of larger synapses compared to wild-type controls. These alterations in basal synaptic morphology correlated with enhanced mean synaptic strength and impaired bidirectional synaptic modifiability (enhanced LTP and reduced LTD) in the cortex. By contrast, spine morphology and synaptic plasticity were normal in the hippocampus of these mice. Importantly, these mice exhibited specific deficits in the consolidation phase of hippocampus-dependent memory. Thus, our results provide evidence for critical relationships between synaptic morphology and bidirectional modifiability of synaptic strength in the cortex and consolidation of long-term memory.

Introduction

The storage or consolidation of declarative memories is thought to involve a reorganization of neural circuits in the cortex as memories are “transferred” from the hippocampus to the cortex (Squire and Alvarez, 1995; Bontempi et al., 1999). However, little is known about the cellular mechanisms underlying cortical memory consolidation, though it has been proposed that one such mechanism is cortical synaptic plasticity (Bear, 1998). This proposal has been supported by a recent study on mice that are heterozygous for a null mutation of \( \alpha \)-calcium/calmodulin-dependent protein kinase II (\( \alpha \)-CamKII), which exhibited impaired cortical long-term potentiation (LTP) and deficient long-term memory (Frankland et al., 2001).

While the role of LTP in learning and memory has been the focus of many studies (Kandel, 2001; Martin and Morris, 2002; Tonegawa et al., 2003; Nakazawa et al., 2004), a number of theoretical studies have concluded that bidirectional modifiability of synaptic strength, i.e., both LTP and long-term depression (LTD) capacity, is a crucial feature of an effective memory system (Willshaw and Dayan, 1990; Bear and Abraham, 1996; Paulsen and Sejnowski, 2000). This notion has been supported by empirical studies with several lines of genetically engineered mice in which impaired bidirectional modifiability in the hippocampus has been correlated with poor learning capability (Migaud et al., 1998; Huh et al., 2000; Zeng et al., 2001). However, it remains unknown whether bidirectional modifiability in the cortical networks is required for memory consolidation.

A related unresolved issue is whether morphological alterations of cortical synapses critically underlie the consolidation of hippocampus-dependent memory. Evidence has been obtained that indicates that structural changes of dendritic spines accompany LTP and LTD (Luscher et al., 2000; Yuste and Bonhoeffer, 2001): LTP-inducing stimuli have been shown to increase the proportion of larger spines, while LTD-inducing stimuli have been shown to decrease the proportion of larger spines (Toni et al., 1999; Ostroff et al., 2002; Fukazawa et al., 2003; K. Okamoto and Y. Hayashi, personal communication). However, the relationship between cortical spine or synapse morphology and memory consolidation has rarely been explored.

Changes in synaptic morphology are mediated mainly by the remodeling of actin filaments (Matus, 2000). A critical regulator of actin remodeling, which functions downstream of the small GTPases Rac and Cdc42, is p21-activated kinase (PAK), a family of serine-threonine kinases that are composed of at least three members, PAK1, PAK2, and PAK3 (Bokoch, 2003). In neurons, PAK has been shown to regulate synaptic architecture. For example, studies in the fruit fly Drosophila melanogaster have shown a requirement for PAK in postsynaptic protein localization (Parnas et al., 2001) as well as in axon guidance (Hing et al., 1999). A recent study in cultured rodent neurons also showed that PAK activity contributes to ephrin B-induced spine formation (Penzenes et al., 2003).

The objective of this study is to examine whether an appropriate basal synaptic morphology and bidirec-
tional synaptic modifiability in the cortex are crucial for the consolidation of hippocampus-dependent long-term memory. Toward this end, we produced transgenic mice in which the expression of a dominant-negative PAK (dnPAK) transgene was restricted to the postnatal forebrain. Cortical neurons in these mice had fewer spines and exhibited a shift in the synapse distribution toward synapses of larger size. These alterations in basal synaptic morphology correlated with altered mean synaptic strength and impaired bidirectional modifiability of synaptic strength. Fortunately, spine morphology and synaptic plasticity were normal in the hippocampus of these mice, providing the opportunity to test the relationships between cortical synaptic structure and function and memory consolidation. Indeed, the dnPAK mice were specifically impaired in the consolidation phase of spatial memory and context-dependent fear memory. These results provided critical evidence for crucial relationships between synaptic morphology and bidirectional modifiability of synaptic strength in the cortex and consolidation of hippocampus-dependent memory.

Results

Association of Active PAK with the PSD

To investigate the potential role of PAK in synaptic structure and function, we first determined the localization of PAK in the adult brain. As previously shown, all three PAKs are expressed in multiple brain regions, including the cortex and hippocampus (Manser et al., 1995; Allen et al., 1998) (data not shown). In humans, loss-of-function mutations of the PAK3 gene are associated with nonsyndromic X-linked mental retardation (Allen et al., 1998; Bienvenu et al., 2000). Since activation of PAK catalytic activity requires the liberation of the catalytic domain from the autoinhibitory domain and autophosphorylation at Thr 423 (Lei et al., 2000), we determined the subcellular localization of the active, Thr 423 auto-phosphorylated form of PAK (p-PAK) by immunostaining with the p-PAK T423 antibody that recognizes phosphorylated forms of all three PAK isoforms. In 2- to 3-week-old cultures of cortical neurons, p-PAK was distributed throughout the cell soma and the dendritic shafts and was largely absent in the axons (Figure 1A, top panel). Double immunostaining showed little colocalization of p-PAK with neurofilament-M (an axon marker; Figure 1A, top panel) or with synaptophysin (a bouton marker; Figure 1A, middle panel) but revealed extensive colocalization with PSD95, a predominant PSD protein, in the dendritic spines (Figure 1A, bottom panel). To determine whether p-PAK was associated with the PSD, a major site of actin remodeling in spines (Colicos et al., 2001), we performed Western blot analyses on three fractions that were prepared from forebrain homogenates. We isolated synaptosomes and separated them into membrane and PSD fractions (Cho et al., 1992) (for details, see the Experimental Procedures). Phosphorylated forms of all three PAKs were present in the PSD fraction as detected by the p-PAK T423 antibody as well as by PAK1 and PAK3 antibodies (Figure 1B). Interestingly, the phosphorylated (but very little unphosphorylated) PAK was detected in the PSD fraction (Figure 1B). Thus, the majority of PAK in the PSD fraction is the phosphorylated PAK, with the phosphorylated PAK3 particularly enriched in this fraction.

To investigate whether PAK activity was regulated by neuronal activity, we performed Western blot analysis to determine the amount of p-PAK following a brief (5 min) treatment with glycine, a protocol that is known to induce LTP in cultured neurons (Lu et al., 2001). In the synaptoneurosome fraction, the level of p-PAK1 increased by ~62% at 10 min and returned to baseline at 30 min following the glycine treatment (Figure 1C). This transient increase in the active PAK level was blocked by the NMDAR antagonist 2-amino-5-phosphonovaleric acid (APV) (Figure 1C), indicating that the p-PAK increase required activation of NMDAR. Since active PAK was associated with the PSD, these results suggest that NMDAR activation elevates PAK activity in the PSD, which could in turn alter spine morphology through PAK's function in actin remodeling.

Generation of Forebrain-Specific dnPAK Transgenic Mice

To examine in vivo whether inhibition of PAK catalytic activity results in impairments in spine morphogenesis, synaptic function, and memory, we generated transgenic mice that expressed dnPAK specifically in the postnatal forebrain (Figure 2A). The dnPAK consists of the PAK autoinhibitory domain (AID-PAK), which binds to the catalytic domain of all three PAKs to block their catalytic activity requiring the liberation of the catalytic subunit from the autoinhibitory domain and autophosphorylation at Thr 423 (Lei et al., 2000), we determined the subcellular localization of the active, Thr 423 autophosphorylated form of PAK (p-PAK) by immunostaining with the p-PAK T423 antibody that recognizes phosphorylated forms of all three PAK isoforms. In 2- to 3-week-old cultures of cortical neurons, p-PAK was distributed throughout the cell soma and the dendritic shafts and was largely absent in the axons (Figure 1A, top panel). Double immunostaining showed little colocalization of p-PAK with neurofilament-M (an axon marker; Figure 1A, top panel) or with synaptophysin (a bouton marker; Figure 1A, middle panel) but revealed extensive colocalization with PSD95, a predominant PSD protein, in the dendritic spines (Figure 1A, bottom panel). To determine whether p-PAK was associated with the PSD, a major site of actin remodeling in spines (Colicos et al., 2001), we performed Western blot analyses on three fractions that were prepared from forebrain homogenates. We isolated synaptosomes and separated them into membrane and PSD fractions (Cho et al., 1992) (for details, see the Experimental Procedures). Phosphorylated forms of all three PAKs were present in the PSD fraction as detected by the p-PAK T423 antibody as well as by PAK1 and PAK3 antibodies (Figure 1B). Interestingly, the phosphorylated (but very little unphosphorylated) PAK was detected in the PSD fraction (Figure 1B). Thus, the majority of PAK in the PSD fraction is the phosphorylated PAK, with the phosphorylated PAK3 particularly enriched in this fraction.

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Cortical Synaptic Morphology and Long-Term Memory

Figure 1. Active PAK Is Associated with the PSD and Elevated by NMDAR Activation

(A) Double staining of cultured cortical neurons for p-PAK T423 (red) and neurofilament-M (NF-M; green in the top panel) or synaptophysin (SYP; green in the middle panel) or PSD95 (green in the bottom panel). p-PAK predominantly localizes in the cell soma, dendrites, and dendritic spines while it is largely excluded from the axons and boutons of mature cortical neurons.

(B) Biochemical fractionation followed by Western blot analysis reveals the presence of active PAK in the PSD fraction. Phosphorylated forms of PAK1, PAK2, and PAK3 (p-PAK1/2/3) were all present in the PSD fraction, while unphosphorylated PAK1/3 (Up-PAK1/3) were nearly absent from the PSD fraction. As detected by the p-PAK antibody, the amount of p-PAK1 was much higher than that of p-PAK2 and p-PAK3. Thus, the film was exposed for a longer time to show the presence of p-PAK2/3 in the PSD fraction. The quality of biochemical fractionation was verified by PSD95 and synaptophysin (SYP) antibodies that detect PSD95 and SYP localizing predominantly in the PSD and membrane fractions, respectively. Lane SYN, synaptic preparation; MEM, membrane fraction. Each lane contains 5 μg of total protein.

(C) Western blot analysis reveals that PAK activity is elevated upon NMDAR activation. The gel shows a representative example. The graph depicts the averaged result from five experiments. Tubulin and actin served as internal controls for protein loading. The relative levels of p-PAK1 in each treatment condition are 1 for untreated culture (CON), 1.62 ± 0.13 for culture harvested at 10 min after glycine treatment (GLY 10'), 0.96 ± 0.04 for culture harvested at 30 min after glycine treatment (GLY 30'), and 0.81 ± 0.14 for culture harvested at 10 min after treatment with glycine and APV (GLY/A 10'). **p < 0.001.

the wild-type level in the cortex and ~62% of the wild-type level in the hippocampus) in the 8-week-old transgenic mice compared to wild-type mice (p < 0.03; Figure 2G). Because the p-PAK1 level in the hippocampus of wild-type mice was about 2-fold higher than that in the cortex, the residual level of hippocampal p-PAK1 in the transgenic mice was similar to the level of cortical p-PAK1 in the wild-type mice (p > 0.05; Figure 2G). At postnatal 3 weeks, the transgene had no effect on the level of p-PAK1 (Figure 2H), presumably reflecting the developmentally delayed activity of the α-CamKII promoter (Figure 2C).

Altered Spine Morphology in the Cortex
The dnPAK transgenic mice exhibited normal gross morphology in the forebrain when examined by hematoxylin and eosin staining (data not shown), suggesting that dnPAK does not affect the global anatomy of the forebrain. Since PAK is known to regulate actin remodeling (Bokoch, 2003) and active PAK is localized in dendritic spines (Figure 1), we carried out morphological analysis on the number and structure of spines. In the normal adult brain, the structure of spines is very heterogeneous, and newly formed immature spine-like protrusions are usually smaller and longer than their mature counterparts (Huber et al., 1998; Toni et al., 2001; Trachtenberg et al., 2002). For the spine analysis, we focused on pyramidal neurons in cortical layer II/III and hippocampal area CA1, two types of cells whose synaptic plasticity has been studied extensively (Diamond et al., 1994; Tsien et al., 1996; Trachtenberg et al., 2000). Golgi staining showed that, in layer II/III of the temporal cortex, the mean density of dendritic spines in the transgenic neurons was lower by ~22% compared to the wild-type neurons (p < 0.0001; Figures 3A and 3B). The decreased spine density was observed throughout the proximal to distal segments of the dendrites (Figure 3B) and was not due to nonspecific defects in dendritic arborization, since dendritic length and number of branch points remained unaffected in the transgenic mice (Figure 3D). The transgenic mice also did not differ
Suppression of PAK Catalytic Activity in the PSD of Forebrain Neurons by Expression of dnPAK

A schematic representation of the dnPAK transgene construct.

Northern blot analysis of total RNA from the forebrain of wild-type (WT) and transgenic (TG) mice. The blot was probed with an SV40 pA probe to detect dnPAK.

Western blot analysis of forebrain extracts. dnPAK was detected by a myc antibody, and tubulin served as an internal control for protein loading.

In situ hybridization of brain sagittal sections with a myc probe shows that dnPAK is highly expressed in the cortex and hippocampus (images of higher magnification shown at bottom).

Immunostaining with a myc antibody detects expression of dnPAK in the cortex and hippocampus.

Catalytic activity of PAK1 in total forebrain homogenates from 8-week-old wild-type and transgenic mice. The activity of immunoprecipitated PAK1 was measured by the amount of phosphorylated myelin basic protein (MBP) in an in vitro kinase assay. The gel shows a representative example. The graph depicts the averaged result from three experiments.

* \( p < 0.002 \)

Western blot analysis with the p-PAK T423 antibody in extracts obtained from 8-week-old (G) and 3-week-old (H) mice. The gel shows a representative example. The graph depicts the averaged result from three experiments.

In the cortex and hippocampus, phosphorylation of PAK1 was reduced in the PSD fraction of the 8-week-old transgenic mice. The relative levels of p-PAK1 were 0.28 ± 0.05 in the cortex of transgenic mice, 0.47 ± 0.05 in the cortex of wild-type mice, 0.62 ± 0.04 in the hippocampus of transgenic mice, and 1 in the hippocampus of wild-type mice. Tubulin and actin served as internal controls for protein loading. CX, PSD fraction of cortical extracts; HP, PSD fraction of hippocampal extracts. * \( p < 0.03 \)

In the cortex and hippocampus, phosphorylation of PAK1 was not reduced in PSD fraction of the 3-week-old transgenic mice. The relative levels of p-PAK1 were 0.67 ± 0.13 in the cortex of transgenic mice; 0.70 ± 0.12 in the cortex of wild-type mice; 0.99 ± 0.19 in the hippocampus of transgenic mice; and 1 in the hippocampus of wild-type mice.

From wild-type mice in immunostaining intensity and pattern with an axon terminal marker, growth-associated protein 43 (GAP-43) (Goslin et al., 1990) (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/42/5/773/DC1). This was consistent with the near absence of active PAK in the axons and boutons of mature neurons (Figure 1A). In hippocampal CA1 pyramidal neurons, there was no significant difference in dendritic spine density (Figure 3C) and dendritic length or number of branch points (Figure 3D) between the transgenic and wild-type mice.

To quantitatively compare the structure of individual spines between wild-type and transgenic mice, we first measured spine length (the radial distance from tip of spine head to dendritic shaft) of Golgi-stained pyramidal neurons (Meng et al., 2002). Cortical neurons in the transgenic mice exhibited a significant shift in the overall spine distribution toward spines of shorter length, indicating a lower proportion of longer, filopodia-like spines relative to wild-type neurons (Figure 4A). This is opposite to the increased spine length observed in other forms of mental retardation, including fragile X syndrome (O’Donnell and Warren, 2002). Next, we examined the size of the spine head by conducting electron microscopic analyses of the length of the PSD and the cross-sectional area of the spine head (Figure 4B). As previously shown, these two parameters are representative of the volume of spines and hence can be used to detect changes in spine size (Luo et al., 1996; Toni et al., 2001; Meng et al., 2002). In layer II/III of the temporal cortex, frequency distribution plots revealed a significant shift in the overall distribution toward spines with longer PSD (\( p < 0.02 \); Kolmogorov-Smirnov test) and larger spine head area (\( p < 0.004 \); Kolmogorov-Smirnov test) in transgenic neurons relative to wild-type neurons (Figure 4C). Consistently, both mean PSD length (\( p < 0.04 \)) and mean spine head area (\( p < 0.02 \)) of the entire spine population were significantly greater in transgenic than in wild-type neurons in the cortex (Figure 4D). By contrast, the wild-type and transgenic mice did not differ in mean PSD length or in mean spine head area (\( p > 0.05 \); \( n = 3 \) mice each) in the hippocampal area CA1.

As visualized in single-section electron micrographs, larger spines often have a discontinuous PSD and are classified as complex or perforated spines, whereas smaller spines always have a continuous PSD and are...
Figure 3. Decreased Spine Density in Cortical Layer II/III Pyramidal Neurons of dnPAK Transgenic Mice

(A) Photomicrographs of representative dendritic segments of cortical layer II/III pyramidal neurons from adult wild-type (left) and transgenic (right) mice. (B) Spine density in primary apical dendrites of layer II/III pyramidal neurons in temporal cortex from wild-type (white) and transgenic (black) mice. (Left) The mean spine density, i.e., the number of spines per 8 μm dendritic segment, was lower in the transgenic (8.45 ± 0.31) than in the wild-type neurons (10.88 ± 0.38; n = 3 mice, 30 neurons each). ***p < 0.0001. (Right) Spine density in each 8 μm long dendritic segment is plotted against the consecutive segments, starting with the most proximal (i.e., segment number 1) to the most distal (segment number 10) from the cell soma. Spine density in all segments was significantly lower in transgenic than in wild-type neurons. *p < 0.05; **p < 0.01.

(C) Spine density in primary apical dendrites of hippocampal CA1 pyramidal neurons. (Left) The mean spine density was indistinguishable between the transgenic (9.16 ± 0.26) and wild-type neurons (9.13 ± 0.25; n = 3 mice, 30 neurons each). (Right) Spine density in most dendritic segments was comparable between transgenic and wild-type neurons. *p < 0.05.

(D) Quantitation of the dendritic length and the number of dendritic branch points. There were no significant differences in all of these measures between adult wild-type and transgenic mice in pyramidal neurons in temporal cortical layer II/III and hippocampal area CA1.

<table>
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<th>Type of neuron</th>
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<th>WT</th>
<th>TG</th>
<th>p values</th>
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<td></td>
<td>Number of branch points</td>
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<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>10.3±0.36</td>
<td>10.2±0.36</td>
<td>p&lt;0.05</td>
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Collectively, cortical pyramidal neurons in the dnPAK transgenic mice had fewer spines and exhibited a shift in the overall spine population toward shorter, larger spines with a perforated PSD compared to wild-type neurons. These results demonstrate a critical role for PAK in dendritic spine morphogenesis and suggest that the level of active PAK influences the extent of spineogenesis.

Altered Presynaptic Structure in the Cortex

In wild-type animals, the PSD matches the presynaptic active zone, and the size of dendritic spines covaries with the number of docked vesicles in the bouton (Harris and Stevens, 1989; Schikorski and Stevens, 1997). To examine whether the structural abnormalities that were observed in the cortical spines of dnPAK transgenic mice were accompanied by any presynaptic structural changes, we counted the number of docked vesicles per bouton in wild-type and transgenic mice. Docked vesicles are identified in electron micrographs as the vesicles that abut the plasma membrane of the active zone (Schikorski and Stevens, 1997). In layer II/III of
temporal cortex, the transgenic neurons exhibited a significant shift in the overall distribution of boutons toward those with a larger pool of docked vesicles compared to wild-type neurons (p < 0.001, Kolmogorov-Smirnov test). Consistently, on average, there was a greater number of docked vesicles per bouton in transgenic than in wild-type mice (p < 0.02; Figure 4F). However, the density of docked vesicles (calculated as the number of docked vesicles per unit length of PSD) did not differ between the transgenic (6.82 ± 0.21 per 10 μm) and wild-type mice (6.28 ± 0.28 per 10 μm, p > 0.05). Thus, the positive correlation between spine size and docked vesicle number was maintained in the transgenic mice: the increased proportion of larger spines (postsynaptic) in transgenic neurons was accompanied by an increased proportion of boutons with a larger pool of docked vesicles (presynaptic). These results indicate that the proportion of larger synapses was greater in the cortex of transgenic mice relative to wild-type mice.

**Enhanced AMPAR- and NMDAR-Mediated Synaptic Transmission in the Cortex**

Accumulating evidence has suggested a positive correlation between size of a synapse and the strength of synaptic transmission (Schikorski and Stevens, 1997; Nusser et al., 1998; Mackenzie et al., 1999; Takumi et al., 1999; Matsuzaki et al., 2001; Murthy et al., 2001). To examine whether the increased proportion of larger synapses in the cortex of dnPAK transgenic mice correlated with an enhancement in synaptic strength, we first measured miniature excitatory postsynaptic currents (mEPSCs) of cortical neurons. mEPSCs are the spontaneous and randomly occurring synaptic currents that are observed after action potentials have been blocked, and one mEPSC constitutes the response to neurotransmitter released from a single vesicle (Bekkers and Stevens, 1994; Regehr and Stevens, 2001). In layer II/III of temporal cortex, the mean amplitude but not the mean frequency of AMPAR-mediated mEPSCs was greater in
transgenic neurons than in wild-type neurons (*p* < 0.02; Figure 5A), suggesting an enhancement in mean AMPAR-mediated synaptic transmission in the transgenic mice. Next, we measured the ratio of NMDAR- vs AMPAR-mediated synaptic currents and found no statistical difference between the wild-type and transgenic neurons (WT, 0.20 ± 0.01, *n* = 18 cells, 5 mice; TG, 0.23 ± 0.01, *n* = 13 cells, 4 mice; *p* > 0.05). Due to the enhanced AMPAR-mediated synaptic transmission, it is likely that mean NMDAR-mediated synaptic transmission was also enhanced in the transgenic mice. Thus, these results suggest that the increased proportion of larger synapses correlated with an enhancement in mean AMPAR- and mean NMDAR-mediated synaptic transmission in the transgenic cortex.

Enhanced LTP and Reduced LTD in the Cortex

To assess whether synaptic plasticity was altered in the transgenic mice, we performed a series of extracellular field recordings from the layer IV to layer II/III synapses in the temporal cortex. The transgenic mice did not differ from wild-type controls in basal synaptic transmission as measured by field potential responses to a range of stimulus intensities (Figure 5B). However, administration of theta-burst stimulation (TBS) at 100 Hz produced LTP with a higher magnitude in transgenic than in wild-type mice (*p* < 0.007, interaction of time × genotype of repeated-measures ANOVA for 1–40 min). Stimulation at 10 Hz produced modestly enhanced potentiation in the transgenic mice relative to wild-type mice (p < 0.007, interaction of time × genotype of repeated-measures ANOVA for 1–40 min).

(G) CA1 LTP induced by TBS was comparable between transgenic (*n* = 10 slices, 5 mice) and wild-type slices (*n* = 8 slices, 5 mice; *p* > 0.05, repeated-measures ANOVA for 31–40 min). An overlay of representative FP traces taken during baseline and at the last few minutes of recording is shown for each genotype.

(F) Synaptic depression was significantly impaired throughout the 15 min LFS in transgenic slices relative to wild-type controls (*p* < 0.002, repeated-measures ANOVA for 0–15 min).

(H) CA1 L-LTP induced by three trains of TBS was comparable between transgenic (*n* = 11 slices, 6 mice) and wild-type slices (*n* = 9 slices, 5 mice; *p* > 0.05, repeated-measures ANOVA for 151–180 min). The initial response at 0–40 min was not significantly different between transgenic and wild-type slices (*p* > 0.05, repeated-measures ANOVA).

Figure 5. Enhanced Mean Synaptic Strength and Impaired Bidirectional Synaptic Plasticity in the Cortex of dnpAK Transgenic Mice

(A) Mean frequency and mean amplitude of AMPAR-mediated mEPSCs in wild-type (white; *n* = 24 cells, 5 mice) and transgenic neurons (black; *n* = 18 cells, 4 mice). While the frequency of mEPSCs was comparable between the two genotypes (WT, 3.11 ± 0.30 Hz; TG, 3.81 ± 0.51 Hz; *p* > 0.05), the mean amplitude of mEPSCs was greater in the transgenic (20.24 ± 2.71 pA) than in the wild-type neurons (12.68 ± 0.57 pA). *p* < 0.02. Example minitraces are shown at the left for each genotype.

(B–H) Extracellular recordings in the temporal cortex layer II/III (B–F) and in the hippocampal area CA1 (G and H) from wild-type (open circle) and transgenic (filled circle) mice. (B) Input-output curves plotting the changes in fEPSP amplitude and their corresponding presynaptic stimulus intensity. *n* = 20 slices, 5 mice each. (C) Cortical LTP induced by TBS was enhanced in transgenic slices (*n* = 18 slices, 6 mice) relative to wild-type controls (*n* = 15 slices, 5 mice; *p* < 0.04, repeated-measures ANOVA for 31–40 min). An overlay of representative FP traces taken during baseline and at the last few minutes of recording is shown for each genotype.

(D) Stimulation at 10 Hz produced modestly enhanced potentiation in the transgenic mice relative to wild-type mice (*n* = 18 slices, 5 mice; *p* > 0.007, interaction of time × genotype of repeated-measures ANOVA for 1–40 min).

(E) Cortical LTD induced by 1 Hz stimulation was significantly impaired in transgenic slices (*n* = 15 slices, 7 mice) relative to wild-type controls (*n* = 15 slices, 8 mice; *p* < 0.03, repeated-measures ANOVA for 66–75 min). An overlay of representative FP traces taken during baseline and at the last few minutes of recording is shown for each genotype.

(F) Synaptic depression was significantly impaired throughout the 15 min LFS in transgenic slices relative to wild-type controls (*p* < 0.002, repeated-measures ANOVA for 0–15 min).

(G) CA1 LTP induced by TBS was comparable between transgenic (*n* = 10 slices, 5 mice) and wild-type slices (*n* = 8 slices, 5 mice; *p* > 0.05, repeated-measures ANOVA for 51–60 min).

(H) CA1 L-LTP induced by three trains of TBS was comparable between transgenic (*n* = 11 slices, 6 mice) and wild-type slices (*n* = 9 slices, 5 mice; *p* > 0.05, repeated-measures ANOVA for 151–180 min). The initial response at 0–40 min was not significantly different between transgenic and wild-type slices (*p* > 0.05, repeated-measures ANOVA).
for responses at 1–40 min; Figure 5D). LTD induced by low-frequency stimulation (LFS, 800 pulses at 1 Hz) was significantly reduced in transgenic slices compared to wild-type controls (p < 0.03, repeated-measures ANOVA for responses at 66–75 min; Figure 5E). Interestingly, the decrease in synaptic depression in transgenic slices occurred immediately following the onset of LFS (p < 0.006 at 8.5 s) and lasted throughout the 15 min LFS delivery period (p < 0.002, repeated-measures ANOVA for responses at 0–15 min; Figure 5F). This suggests that the induction phase of LTD was impaired in transgenic mice compared to wild-type mice. In contrast to the abnormal plasticity observed in the cortex, the transgenic mice did not differ from wild-type mice in various stimulation paradigms (basal transmission [data not shown], paired-pulse facilitation [data not shown], TBS-induced LTP [p > 0.05, repeated-measures ANOVA for responses at 51–60 min; Figure 5G] and late LTP [L-LTP] induced by three trains of TBS [p > 0.05, repeated-measures ANOVA for responses at 151–180 min; Figure 5H]) in the hippocampus. The absence of detectable alteration in synaptic plasticity and spine morphology in the hippocampus of transgenic mice could be explained by the fact that the level of active PAK in the hippocampus of transgenic mice remained similar to that in the cortex of wild-type mice (Figure 2G). Thus, it is possible that PAK inhibition did not reach the critical threshold that is necessary to result in morphological and physiological alterations in the hippocampus. Overall, our results demonstrate a correlation between abnormal synaptic morphology, synaptic strength, and bidirectional modifiability (enhanced LTP and reduced LTD) in the cortical neurons of dnPAK transgenic mice.

Normal Acquisition and Impaired Consolidation/Retention of Spatial Memory
To examine whether the structural and electrophysiological defects of the cortical synapses in dnPAK transgenic mice lead to deficits in learning and memory, we carried out behavioral analyses. In a battery of tests for general behaviors, including open field, accelerating rotarod, light/dark transition, hot plate, social interaction, and Porsolt’s forced swim test (Crowley, 2000), transgenic mice performed similarly to wild-type mice (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/42/5/773/DC1). This indicates that locomotor activity, pain perception, sensorimotor response, motor coordination, and anxiety level were normal in the transgenic mice.

We then subjected these mice to the hidden-platform version of the Morris water maze, in which the acquisition of spatial memory is known to depend on the hippocampus (Martin and Morris, 2002). The transgenic mice learned as quickly as wild-type mice: the decreases in escape latency (time to reach the platform) and path length (distance traveled to reach the platform) were equivalent between wild-type and transgenic mice (Figure 6A). In the probe trial conducted immediately after the completion of the training session, transgenic mice performed as well as wild-type controls, as indicated by the similar search time in the target quadrant (wt, 43.2% ± 3.0%, n = 15; TG, 44.1% ± 4.5%, n = 13; p > 0.05) and the similar number of target platform location crossings (wt, 8.07 ± 1.19; TG, 8.69 ± 1.41; p > 0.05; Figure 6B). These results indicate normal learning in dnPAK transgenic mice.

Interestingly, the transgenic mice failed to retain their memory compared to wild-type mice, as demonstrated by the comparison of two additional probe trials conducted at 1 and 21 days after the completion of training. At 1 day after training, transgenic mice remembered the platform location equally well as wild-type mice (search time in the target quadrant: wt, 47.4% ± 2.3%, n = 34; TG, 44.5% ± 2.2%, n = 34, p > 0.05; number of platform crossings: wt, 8.56 ± 0.77; TG, 7.44 ± 0.66, p > 0.05; Figure 6B). By contrast, at 21 days after training, transgenic mice were significantly impaired in the probe trial relative to wild-type mice (search time in the target quadrant: wt, 44.4% ± 2.1%; TG, 35.5% ± 2.5%, p < 0.01; number of platform crossings: wt, 8.44 ± 0.73; TG, 5.62 ± 0.80, p < 0.02; Figure 6B). These results suggest that the acquisition of spatial memory was unaffected while the consolidation/retention of this memory was impaired in the transgenic mice.

Normal Short-Term Contextual Fear Conditioning and Attenuated Consolidation/Retention
To fully study the consequence of cortical structural and physiological alterations on memory consolidation, we subjected the wild-type and transgenic mice to a contextual fear conditioning task, in which the acquisition of contextual fear memory is known to depend on the hippocampus (Tonegawa et al., 2003). The freezing responses were comparable between wild-type and transgenic mice at 40 min (wt, 32.3% ± 3.8%, n = 20; TG, 25.9% ± 4.1%, n = 18, p > 0.05; Figure 6C) and 7 hr (wt, 27.1% ± 4.0%, n = 20; TG, 33.9% ± 3.5%, n = 17, p > 0.05) after training. However, at 24 hr after training, transgenic mice exhibited a significant reduction in context-dependent freezing relative to wild-type controls (wt, 33.3% ± 4.1%, n = 18; TG, 20.6% ± 3.5%, n = 18, p < 0.03; Figure 6C). A similar deficit in long-term contextual fear memory was observed for line #110 (data not shown). In addition, the transgenic mice displayed normal tone-associated memory at 48 hr after training (wt, 32.3% ± 4.6%, n = 18; TG, 31.1% ± 3.9%, n = 18, p > 0.05; Figure 6C). These results suggest an impairment in consolidation/retention of contextual fear memory in the transgenic mice, which is consistent with the water maze results.

Discussion
The aim of this study was to investigate the relationship between spine structure and synaptic function in the context of memory capability. To this end, we generated and characterized transgenic mice in which the catalytic activity of PAK, a critical regulator of actin remodeling, was inhibited in postnatal forebrain neurons. In dnPAK transgenic mice, cortical neurons had fewer spines and an increased proportion of shorter, larger spines relative to wild-type mice. Cortical synapses of these transgenic mice also displayed a corresponding alteration in the size of boutons. These abnormalities in basal cortical synaptic morphology correlated with enhanced mean synaptic strength and impaired bidirectional modificabil-
Cortical Synaptic Morphology and Long-Term Memory

Figure 6. Normal Acquisition and Impaired Consolidation/Retention of Spatial and Contextual Fear Memories in dnPAK Transgenic Mice

(A) Normal acquisition of spatial memory in the hidden-platform Morris water maze. The decreases in escape latency (left panel) and path length (right panel) were equivalent between the wild-type (open circle) and transgenic mice (filled circle; n = 34 each, p > 0.05, repeated-measures ANOVA).

(B) Results of probe trials given on day 14 (end of training, left), day 15 (1 day after the completion of training, middle), and day 35 (21 days after the completion of training, right). (Upper panel) Mean percent of time spent searching in each quadrant. Dotted lines depict chance level (25%) for random searching. (Lower panel) Mean number of platform crossings. There were significant group differences in both measures on day 35 but not on days 14 and 15. *p < 0.05; **p < 0.01. T, target quadrant; L, adjacent left quadrant; R, adjacent right quadrant; O, opposite quadrant.

(C) Freezing responses for the wild-type and transgenic mice in fear conditioning. The transgenic mice showed a significantly impaired context-associated fear memory at 24 hr but not at 40 min or 7 hr after conditioning. At 48 hr after conditioning, the transgenic mice exhibited a comparable freezing response to a novel (unconditioned) context ("pre-tone" period) as well as an intact tone-associated fear memory relative to wild-type mice. *p < 0.03.

ity (enhanced LTP and reduced LTD). In contrast, spine morphology and synaptic plasticity in the hippocampus were unaltered in dnPAK transgenic mice. Notably, dnPAK transgenic mice also displayed specific impairments in the consolidation/retention phase of hippocampus-dependent memories. Thus, these results demonstrated a critical role of PAK in cortical spine morphogenesis and suggested critical relationships among basal cortical synaptic structure, bidirectional modifiability of synaptic strength, and long-term memory capability.

Role of PAK in Spinogenesis

Spinogenesis occurs not only during development of the brain but also in adulthood in response to plasticity-inducing stimuli (Moser et al., 1994; Kleim et al., 2002; Knott et al., 2002). A role for PAK in developmental spinogenesis was suggested by a recent in vitro study in which transfection of a dnPAK construct into cultured 10-day-old hippocampal neurons was shown to block ephrin B-induced spine development (Penzes et al., 2003). The lower spine density that was observed in the cortex of dnPAK transgenic mice could in part reflect PAK’s role in developmental spinogenesis. However, we believe that the contribution of developmental effects should be minimal, since endogenous PAK activity is not detectably inhibited in the transgenic mice through the end of the third postnatal week, by which time developmental spinogenesis is nearly complete in the cortex (Blue and Pamavelas, 1983; Markus and Petit, 1987; Micheva and Beaulieu, 1996). Therefore, it is likely that the lower spine density in transgenic mice indicates a critical role of PAK in activity-induced spinogenesis in the adult brain. Consistent with this role, we found that, in mature neurons, active PAK is associated with the PSD of spines and is elevated upon NMDAR activation.

Through its function in actin remodeling, PAK might mediate de novo spine formation and/or spine duplication: the two proposed modes of activity-dependent spinogenesis (Yuste and Bonhoeffer, 2001). During de novo spine formation (Engert and Bonhoeffer, 1999; Fiala et al., 2002), active PAK could be required for the formation of new protrusions from dendritic shafts (Figure 7A), perhaps by promoting actin polymerization via phosphorylation of LIM kinase and inactivation of cofilin/
actin-depolymerizing factor (Edwards et al., 1999). In this case, the increased proportion of larger perforated spines (which usually represent stronger synapses, see below) that is observed in dnPAK transgenic mice could be explained as a consequence of homeostatic compensation (Turrigiano and Nelson, 2000) for reduced spine number, in order to maintain the overall output of individual neurons (Figure 7A). Alternatively, during spine duplication in which preexisting spines enlarge, undergo PSD perforation, and split to form new simple spines (Toni et al., 1999, 2001), active PAK could be required for the splitting of the enlarged perforated spines (Figure 7B), possibly by disassembling actin-myosin filaments that associate with the spine plasma membrane (Morales and Fifkova, 1989) via phosphorylation and inactivation of myosin light chain kinase (Sanders et al., 1999). In this case, PAK inhibition would directly lead to a greater proportion of larger perforated spines in the transgenic mice (Figure 7B).

Association between Synapse Size and Strength of Synaptic Transmission

In a synapse, spine size has been shown to correlate with bouton size and the number of docked vesicles in the bouton (Harris and Stevens, 1989; Schikorski and Stevens, 1997). Consistent with these observations, we found that the increased proportion of larger spines in transgenic cortical neurons correlated with an increased proportion of boutons with a larger pool of docked vesicles, while the density of docked vesicles did not differ between transgenic and wild-type neurons. Since the docked vesicle pool coincides with the readily-releasable pool (RRP) that determines the probability of neurotransmitter release (Murthy et al., 2001), our results suggested a larger RRP and possibly a greater release probability per synapse in the transgenic neurons (Figure 7C). A greater release probability per synapse together with a lower synapse number (due to the lower spine density) could explain the unaltered frequency of AMPAR-mediated mEPSC that was observed in the transgenic neurons. Since endogenous active PAK is nearly absent in the axons and boutons of mature neurons, the observed presynaptic alteration is likely a secondary effect of the postsynaptic alteration, perhaps occurring through communication between pre- and postsynaptic terminals via various molecules, including cell adhesion molecules and secreted proteins (Scheiffele, 2003).

Spine size has also been shown to correlate with the strength of synaptic transmission, as larger spines have more AMPARs and NMDARs than smaller spines (Nusser et al., 1998; Mackenzie et al., 1999; Takumi et al., 1999; Matsuzaki et al., 2001). We did not directly examine the number of AMPARs and NMDARs; however, we observed a greater mean amplitude of AMPAR-mediated mEPSCs as well as an unaltered ratio of NMDAR-to AMPAR-mediated synaptic currents in transgenic cortical neurons relative to wild-type controls. These observations suggested enhanced mean AMPAR- and NMDAR-mediated synaptic transmission in the transgenic neurons, thus providing evidence for an association between spine size and strength of synaptic transmission (Figure 7C). Taken together, our findings from
pre- and postsynaptic terminals in cortical synapses of *dnPAK* transgenic mice indicated that the shift in spine distribution toward spines of larger size was associated with a shift in the corresponding synapse distribution toward synapses of larger size and stronger transmission.

### Cortical Bidirectional Modifiability of Synaptic Strength and Long-Term Memory Storage

Accumulating evidence has suggested that bidirectional modifiability of synaptic strength is critical for a neural network to function as an effective memory system (Willshaw and Dayan, 1990; Migaud et al., 1998; Huh et al., 2000; Paulsen and Sejnowski, 2000; Zeng et al., 2001). Consistent with this notion, *dnPAK* transgenic mice, which exhibited behavioral deficits in the consolidation/re-entainment of spatial and contextual fear memories, displayed impaired bidirectional modifiability, namely, enhanced LTP and reduced LTD, in the temporal cortex (Figure 7D). It is possible and even likely that the alterations in synaptic morphology and plasticity are not restricted to the temporal cortex and occur in other cortical areas. However, the behavioral deficits were highly specific to the consolidation/re-entainment and not observed in the acquisition of these hippocampus-dependent memories, strongly suggesting that sufficiently normal wiring and computational functions are maintained in the forebrain of the transgenic mice. Indeed, associated with their normal acquisition of these hippocampus-dependent memories (Figure 6), we found normal hippocampal synaptic plasticity (LTP and L-LTP) in *dnPAK* transgenic mice (Figures 5G and 5H). A similar dissociation between hippocampal and cortical plasticity was previously observed in mice heterozygous for a null mutation of α-CamKII (α-CamKII 

### Underlying Mechanism of Impaired Bidirectional Modifiability

How does the inhibition of PAK activity lead to the impaired bidirectional modifiability in *dnPAK* transgenic mice? In principle, the impaired bidirectional modifiability could reflect either a direct effect of PAK inhibition or an indirect outcome of PAK inhibition, perhaps through alteration in basal synaptic structure. Interestingly, the effects of PAK inhibition on bidirectional plasticity are already apparent in the induction phase while, as previously shown, it is not the induction phase but the maintenance phase of plasticity that requires actin remodeling (Kruecker et al., 2000; Fukazawa et al., 2003), which likely involves PAK activity. Thus, it is likely that inhibition of PAK activity in the transgenic mice does not directly account for the altered induction of plasticity. Rather, we believe that the altered induction of bidirectional plasticity reflects the altered basal distribution of synapse size.

Previous studies have identified a few key variables that determine the sign of bidirectional modifiability, i.e., whether a synapse undergoes LTP or LTD following stimulation. It is thought that a synapse is more likely to undergo potentiation and less likely to undergo depression if it exhibits slower vesicle depletion presynaptically and/or a higher level of NMDAR-mediated Ca\(^{2+}\) influx postsynaptically (Malenka and Nicoll, 1999; Gottschalk et al., 1998; Huber et al., 1998; Lisman, 2001). The larger cortical synapses in *dnPAK* transgenic mice could fulfill both of these criteria. Presynaptically, these larger boutons would exhibit slower vesicle depletion because they have a larger pool of docked vesicles than smaller boutons (Zucker, 1989; Dobrunz and Stevens, 1997; Murthy et al., 2001). Postsynaptically, these larger spines would allow more NMDAR-mediated Ca\(^{2+}\) influx because they have more NMDARs as well as more AMPARs that can produce greater depolarization, leading to enhanced NMDAR activation relative to smaller spines (Mackenzie et al., 1999; Matsuzaki et al., 2001). Thus, the increased proportion of larger synapses in the transgenic mice would result in an increased proportion of potentiated synapses following TBS, leading to enhanced LTP, but a decreased proportion of depressed synapses following LTD, leading to reduced LTD. Hence, by modulating the induction of plasticity, the shift in basal synapse distribution toward larger synapses would impair bidirectional modifiability of the network favoring LTD over LTP. Consistent with this possibility, an increased proportion of larger spines has recently been observed in the visual cortex of dark-reared rats, which exhibits enhanced LTP and reduced LTD (Kirkwood et al., 1996) (W. Wallace and M.F. Bear, personal communication). Strikingly, in these rats, exposure to light reverses the altered basal distribution of spine size as well as the impaired bidirectional modifiability.

Taken together, the correlatory observations of altered distribution of synapse size and impaired bidirectional modifiability of synaptic strength in the cortex and deficient memory consolidation in *dnPAK* transgenic mice lead us to propose that the basal distribution of synapse size in the cortex must be within a range that is appropriate for bidirectional modifiability in order for a population of neurons to function as an effective memory network. Thus, in addition to structural plasticity of
individual synapses, memory consolidation would depend on the basal synaptic structure in a population of synapses in the cortex. Future studies employing genetic or pharmacological perturbations will help to further understand how basal synaptic structure affects bidirectional modifiability and memory capability. Hopefully, such studies will provide mechanistic insight into the causes of cognitive dysfunction in mental retardation, since many of the genes that are implicated in mental retardation, including PAK3, are involved in the regulation of synaptic structure (Chelly and Mandel, 2001).

Experimental Procedures

**Generation of Transgenic Mice**

The dnPAK transgene construct contained the 8.5 kb α-CamKII promoter, myc-tagged AID-PAK, and SV40 intron/polyA from pMSG [Pharmacia]. The cDNA for AID-PAK, encoding amino acids (aa) 78–136 of PAK3 (corresponding to aa 83–149 of PAK1) (Zhao et al., 1998), was amplified by polymerase chain reaction from mouse cDNA and cloned into the pCMV-6myc vector to generate an in-frame fusion between the N terminus of AID-PAK and the C terminus of the myc tag. The myc-tagged AID-PAK fragment was then cloned between the α-CamKII promoter and SV40 intron/polyA to generate pMLH101. The 10 kb SalI fragment of pMLH101 containing the dnPAK transgene was purified and microinjected into C57BL6/Ey-gotes to generate transgenic mice. All wild-type and transgenic mice used in this study are of the C57BL6 genetic background.

**Northern Blot Analysis and In Situ Hybridization**

Total RNA was isolated from mouse forebrains, and RNA blots were hybridized to 32P-labeled DNA probes containing SV40 intron/polyA to detect expression of dnPAK. For in situ hybridization, frozen mouse brains were sectioned (14 μm) with a cryostat (Leica), fixed, and hybridized with 35S-labeled RNA probes.

**Western Blot Analysis, PSD Fractionation, and Kinase Assay**

Mouse forebrains were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), placed on ice for 5 min, and then centrifuged (10,000 g, 30 min). Both the soluble and -insoluble fractions were isolated and analyzed by Western blot analysis. The level of p-PAK1 in the hippocampus of wild-type mice was always determined as 100%. The relative level of p-PAK was quantified with the aid of densitometry software from the scanned films using the OpenLab Software Program (Improvement). The level of p-PAK was determined using a computerized image analysis system (Collins et al., 2002).

**Electrophysiology**

From 2- to 3-month-old male littermates, transverse hippocampal slices were prepared and left to recover for at least 1 hr before recording in artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM dextrose. CA1 field potentials (FP) evoked by Schaffer collateral stimulation and FP in layer II/III of the hippocampus were measured using multielectrode arrays. The measurements were performed by an experimenter blind to the genotype.
of NMDAR-EPSC/AMPAR-EPSC ratio was done in ACSF containing 2 mM Mg²⁺, 10 μM bicuculline, and 50 μM glycine as described (Myme et al., 2003). NMDAR-dependent and AMPAR-dependent responses were discriminated based on their distinct kinetics and voltage dependence. Thus, as an index of the NMDA-mediated response, we used the value of the currents recorded at -40 mV and measured 100 ms after the response onset. The AMPAR-mediated response was taken from the peak amplitude response recorded at -80 mV.

Behavioral Tests

Animals used were all male littermates at 2–4 months of age. Open field, accelerating rotarod, light/dark transition, hot plate, social interaction, and Porsolt’s forced swim tests were all carried out as previously described (Crowley, 2000). The hidden platform Morris water maze was carried out as previously described (Zeng et al., 2001). Mice were subjected to water maze training at four trials per day with intertrial intervals of 3 min. Each training trial ended either when the mice reached the platform or after 60 s elapsed. Probe trials, i.e., 60 s swimming sessions in the absence of platform, were conducted at 1 hr, 1 day, or 21 days after the last training session. Mice that were tested for several probe trials were always trained for an extra four-trial session after the previous probe trial to prevent memory extinction. The swimming traces were digitized and analyzed by Image WM program (O’Hara & Co). Fear conditioning was carried out as previously described (Zeng et al., 2001). On day 1, mice were placed in the training chamber (Coulbourn Instruments) (chamber A) for 2 min before the onset of a 30 s white noise tone that coterminated with a 2 s shock (0.7 mA intensity), after which mice remained for another 30 s. To test contextual memory, at 40 min, 7 hr, or 24 hr after training, mice were placed back into chamber A for 3 min. Video images were digitized, and the percentage of freezing time was analyzed by Image FZ program (O’Hara & Co). Freezing was defined as the absence of all but respiratory movement for a consecutive 2 s period. To test tone-associated memory, on day 3, mice were placed into a new chamber (chamber B) with a different shape and smell from chamber A for 2 min before the onset of a 3 min tone. To avoid the confounding effects of extinction, separate groups of mice were tested at each retention delay.

Animal Handling, Experimental Design, and Data Analysis

The generation and maintenance of mice and all experimental procedures were performed in compliance with National Institute of Health guidelines. All experiments were conducted in a blind fashion. Statistical analyses were carried out using Statview software (SAS). Unless specified otherwise, data were analyzed with Student’s t test; p values greater than 0.05 were regarded as not significant. Values are presented as mean ± SEM.

Acknowledgments

We are grateful of Xiaoning Zhou, Wenjiang Yu, Frank Bushard, and Jayson Derwin for excellent technical assistance. We thank Drs. David Gerber, Thomas McHugh, Matthew Anderson, Audrey Chang, Yasunori Hayashi, Guo-song Liu, Morgan Sheng, and Mark Bear for critical discussions; many members of the Tonegawa lab for helpful advice; Dr. Tsuyoshi Miyakawa for advice on behavioral experiments; Marcia Feinberg, Maria Ericsson, Elizabeth Benechci, and Dr. Kristen Harris for help on electron microscopy. This study was supported by NIH, HHMI, and RIKEN (S.T.) and NIH (A.K.).

Received: February 11, 2004
Revised: April 23, 2004
Accepted: May 6, 2004
Published: June 9, 2004

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