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Genetic enhancement of trace fear memory and cingulate potentiation in mice overexpressing Ca^{2+}/calmodulin-dependent protein kinase IV

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Abstract

Long-term potentiation (LTP) is a key cellular model for studying mechanisms for learning and memory. Previous studies reported that the Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) is critical for gene regulation, and behavioral learning and memory. Less is known about the roles of CaMKIV in cortical plasticity and trace fear memory. Here we have found that LTP was significantly enhanced in the anterior cingulate cortex (ACC) of the mice overexpressing CaMKIV. By contrast, neither α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated basal excitatory synaptic transmission nor *N*-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents were affected. Furthermore, paired-pulse ratio in the transgenic mice is normal. In behavioral tests, we found that the CaMKIV transgenic mice exhibited significant enhancement in trace fear memory, while the acute sensory thresholds were not affected. Our results provide strong evidence that forebrain CaMKIV contributes to trace fear memory by enhancing synaptic potentiation in the ACC.

Introduction

The Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) is a serine-threonine kinase that is activated by elevated intracellular Ca^{2+} and the following activation of CaMK kinase during neuronal activity (Anderson & Kane, 1998; Soderling, 1999). Through phosphorylating the regulatory serine 133 residue, CaMKIV activates the cAMP response element-binding protein (CREB), a well-known transcription factor involved in a number of gene expressions (Bito *et al*., 1996; Shaywitz & Greenberg, 1999). Therefore, CaMKIV is regarded as a potent mediator of Ca^{2+}-induced gene expression associated with neuronal activity (Anderson & Kane, 1998; Soderling, 1999). Studies using genetic manipulation of CaMKIV have revealed its critical roles in synaptic plasticity in the hippocampus, cerebellum, cortex and amygdala (Ho *et al*., 2000; Ribar *et al*., 2000; Kang *et al*., 2001; Wei *et al*., 2002; Boyden *et al*., 2006). Because the CaMKIV–CREB pathway plays a central role in gene expression, the protein synthesis-dependent late-phase long-term potentiation (LTP) and long-term depression (LTD) require the activation of CaMKIV (Ahn *et al*., 1999; Ho *et al*., 2000; Kang *et al*., 2001). However, the early phase of LTP was also impaired in CaMKIV knockout mice (Ho *et al*., 2000; Wei *et al*., 2002). Consistently, acute expression of constitutively active forms of CaMKIV enhances LTP but not LTD in hippocampal slices (Marie *et al*., 2005).

Associated with its role in synaptic plasticity, CaMKIV is reported to be involved in different forms of memory, such as spatial memory, fear memory and motor memory (Ho *et al*., 2000; Ribar *et al*., 2000; Kang *et al*., 2001; Wei *et al*., 2002; Boyden *et al*., 2006). Previous reports on CaMKIV functions, however, vary significantly when obtained from CaMKIV knockout mice and transgenic mice with the expression of a dominant-negative form of CaMKIV (Ho *et al*., 2000; Kang *et al*., 2001; Wei *et al*., 2002). Most recently, it has been reported that CaMKIV is important for some specific motor memory storage (Boyden *et al*., 2006). Genetic deletion of CaMK kinase α or β, the important components in the CaM kinase pathway, also affects contextual fear memory or spatial memory (Peters *et al*., 2003; Blaeser *et al*., 2006; Mizuno *et al*., 2006). All these results suggest that CaMKIV finely regulates different forms of memory, depending on its regional distributions, testing paradigms or even genetic manipulations.

CaMKIV is expressed in the cerebellum, hippocampus, amygdala and various cortical areas including the anterior cingulate cortex (ACC; Wei *et al*., 2002). The ACC plays important roles in higher brain functions such as emotion, learning and memory (Frankland *et al*., 2004; Maviel *et al*., 2004; Zhuo, 2006). Recent studies have shown that LTP and LTD can be induced in the ACC both in *in vitro* brain slices and in *in vivo* whole animals (Wei & Zhuo, 2001; Wu *et al*., 2005a; Zhao *et al*., 2005b). Moreover, the ACC exhibits long-term plastic changes in response to various physiological and pathological manipulations (Wei & Zhuo, 2001; Wu *et al*., 2005a), suggesting that cingulate plasticity may contribute to ACC-related

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brain functions. Trace fear memory requires the activity of the ACC and is sensitive to attention-distracting stimuli (Han *et al*., 2003; Zhao *et al*., 2005a). Using transgenic mice with forebrain overexpression of CaMKIV, we studied the role of CaMKIV in cingulate LTP as well as trace fear memory, and we found both of them were enhanced in the transgenic mice.

Materials and methods

Animals

All adult C57BL/6 mice were purchased from Charles River. Transgenic mice overexpressing CaMKIV were generated in Satoshi Kida's laboratory (Tokyo University of Agriculture, Japan). Briefly, we constructed a transgene that contained a αCaMKII promoter, a hybrid intron in the 5′ untranslated leader, the coding region of CaMKIV fused with the Flag tag sequence at the N-terminus and a polyadenylation signal. The αCaMKII promoter has been known to exhibit strong activity in regions of the forebrain including the hippocampus, cortex and striatum (Mayford *et al*., 1996; Kida *et al*., 2002). Among three lines of transgenic mice generated, transgenic line 2 showed the highest levels of transgene expression in the forebrain area (H. Fukushima, S. Kida and others, unpublished results). Thus, transgene line 2 was used for the present experiments. Control wild-type mice were littermates of transgenic mice. All mice were maintained on a 12 h light : dark cycle with food and water provided *ad libitum*. The Animal Studies Committee at the University of Toronto approved all experimental protocols, which were in accordance with the guidelines of the Canadian Council on Animal Care.

Slice preparation

Mice were anesthetized with 1–2% halothane and decapitated. Coronal brain slices (300 μM) containing the ACC from 6–8-week-old C57BL/6 male mice and mice with CaMKIV overexpression were prepared using standard methods (Wu *et al*., 2005a). Slices were transferred to a submerged recovery chamber with oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 2.5; $CaCl_2$, 2; $MgSO_4$, 2; $NaHCO_3$, 25; NaH_2PO_4, 1; glucose, 10; at room temperature for at least 1 h.

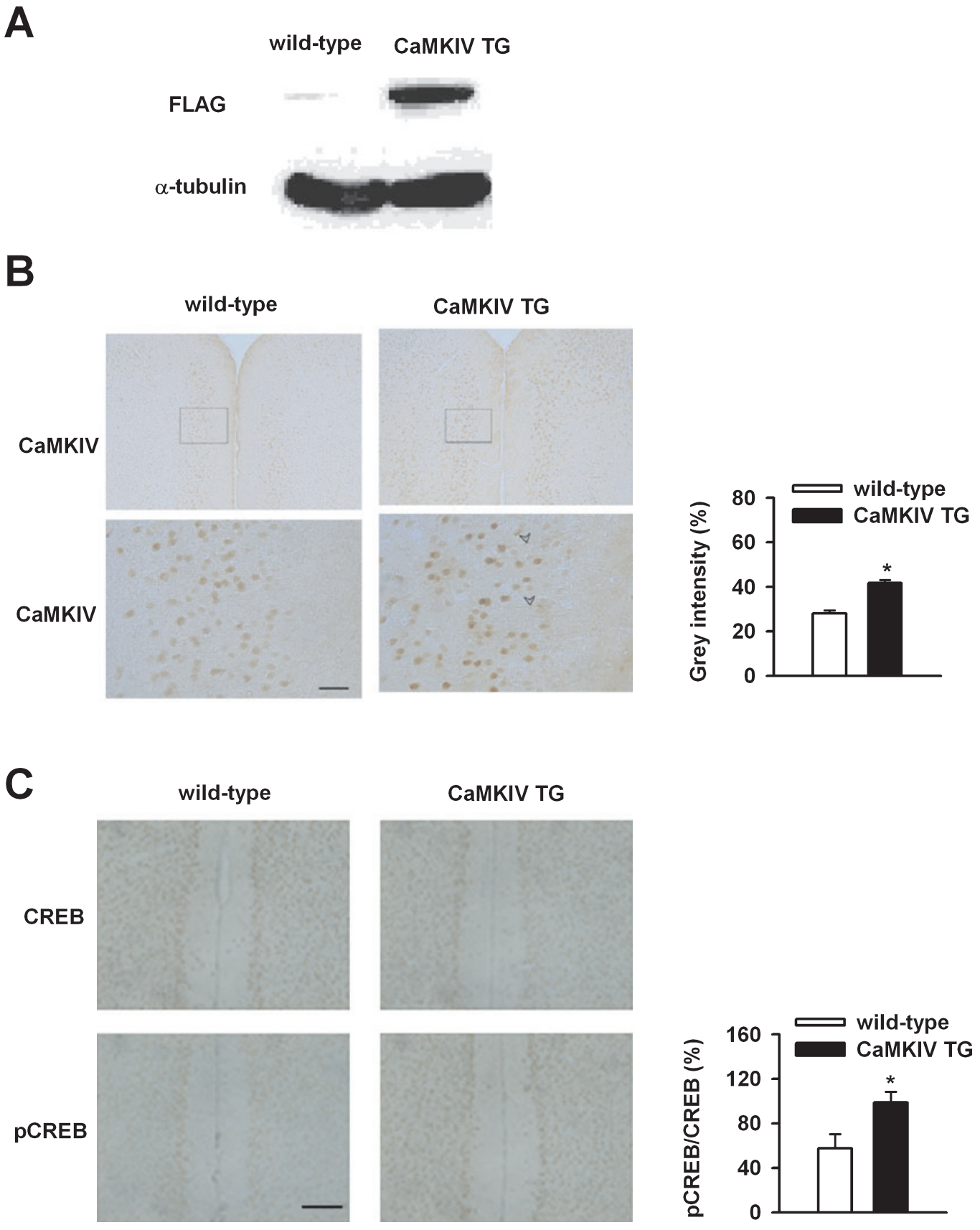
Whole-cell patch-clamp recordings

All electrophysiological experiments were performed at room temperature. An Axioskop 2FS microscope (Zeiss, Germany) with infrared DIC optics was used for visualization of whole-cell patch-clamp recording. Excitatory postsynaptic currents (EPSCs) were recorded from layer II/III neurons with an Axon 200B amplifier (Molecular Devices, CA, USA), and the stimulations were delivered by a bipolar tungsten stimulating electrode placed in layer V of the prefrontal slices. EPSCs were induced by repetitive stimulations (duration is 200 μs, intensity is adjusted to induce EPSCs with an amplitude of 50–100 pA) at 0.02 Hz and neurons were voltage-clamped at −70 mV. The recording pipettes (3–5 MΩ) were filled with solution containing (in mM): K-gluconate, 145; NaCl, 5; $MgCl_2$, 1; EGTA, 0.2; HEPES, 10; Mg-ATP, 2; Na_3-GTP, 0.1 (adjusted to pH 7.2 with KOH). When current–voltage relationships were measured, K-gluconate was replaced by equimolar $CsMeSO_3$ and 5 QX-314 chloride was added in the internal solution. LTP was induced with paired training within 12 min after establishing the whole-cell configuration to avoid washout of intracellular contents that are critical for the establishment of synaptic plasticity (Zhao *et al*., 2005b). The induction protocol (referred to as spike-timing protocol) for LTP involved three paired presynaptic stimuli, which caused three EPSPs (10 ms ahead) with three postsynaptic action potentials (APs) at 30 Hz, paired 15 times every 5 s. The *N*-methyl-D-aspartate (NMDA) receptor-mediated component of EPSCs was pharmacologically isolated in ACSF containing 6-cyano-2,3-dihydroxy-7-nitro-quinoxailne acid (CNQX; 20 μM) and picrotoxin (100 μM). The patch electrodes for NMDA receptor-mediated EPSCs contained (in mM): $CsMeSO_3$, 102; TEA chloride, 5; NaCl, 3.7; BAPTA, 11; EGTA, 0.2; HEPES, 20; MgATP, 2; NaGTP, 0.3; QX-314 chloride, 5 (adjusted to pH 7.2 with CsOH). Neurons were voltage-clamped at −30 mV and NMDA receptor-mediated EPSCs were evoked at 0.05 Hz. Picrotoxin (100 μM) was always present to block g-aminobutyric acid $(GABA)_A$ receptor-mediated inhibitory currents and monitored throughout the synaptic currents. Access resistance was 15–30 MΩ and was monitored throughout the experiment. Data were discarded if access resistance changed more than 15% during an experiment. Statistical comparisons were performed using the Student's *t*-test or two-way ANOVA.

Immunohistochemistry

Mice were anesthetized with isoflurane and perfused with 0.1 mol/L phosphate-buffered saline (PBS; pH 7.2–7.4) via the ascending aorta followed by 4% paraformaldehyde. The brains were then removed and postfixed in the same fixative for 4 h before cryoprotection in PBS containing 30% sucrose overnight at 4 °C. Every fourth section of 25 μm thickness, serially cut through the forebrain containing ACC in cryostat, was collected. One series was used for CaMKIV immunohistochemistry, a second series was for control experiments. Before immunohistochemistry, the sections were incubated in PBS containing 20% methanol and 0.3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. Then sections were processed for immunoreactivity in a routine way. Briefly, sections were sequentially incubated through the following solutions: (i) a solution of 3% bovine serum albumin (BSA; Sigma, St Louis, USA), 0.3% Triton X-100 containing mouse antibody against CaMKIV (1 : 300; BD Biosciences, San Jose, CA, USA), or anti-CREB rabbit antibody (1 : 1000; Cell Signaling Technology) or anti-phosphorylated CREB rabbit antibody (1 : 250; Cell Signaling Technology) for 2 days at −4 °C; and (ii) horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1 : 200; Santa Cruz, CA, USA) in PBS containing 2% BSA and 0.3% Triton X-100 for 24 h at 4 °C. Following each step, the sections were rinsed with PBS (3 × 10 min). Signals were visualized with 3, 3′-diaminobenzidine as chromogen using ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were mounted on gelatine-coated slides, air-dried, cleared and coverslipped for observation. No

FIG. 1. Expression of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) and cAMP response element-binding protein (CREB) in the ACC of CaMKIV transgenic mice. (A) Western blot analysis of transgenic CaMKIV expression in the ACC of wild-type and CaMKIV transgenic mice. The anti-FLAG antibody detected a 60-kDa protein corresponding to CaMKIV. (B) Photomicrographs showing expression of CaMKIV in ACC of wild-type and CaMKIV transgenic mice. The increased expression of CaMKIV in transgenic mice can be observed at a cellular level at a higher magnification (lower panel) of framed areas shown in the upper panel. Note the arrows in the ACC from transgenic mice pointing to the CaMKIV-immunoreactive apical dendrites. Scale bar: 200 μm (upper panel); 35 μm (lower panel). (C) Immunocytochemistry of the expression of phosphorylated CREB and total CREB in the ACC of wild-type and transgenic mice (left panel). The ratios of the number between phosphorylated CREB- and CREB-positive cells are indicated (right panel). *$P < 0.05$ compared with wild-type mice.



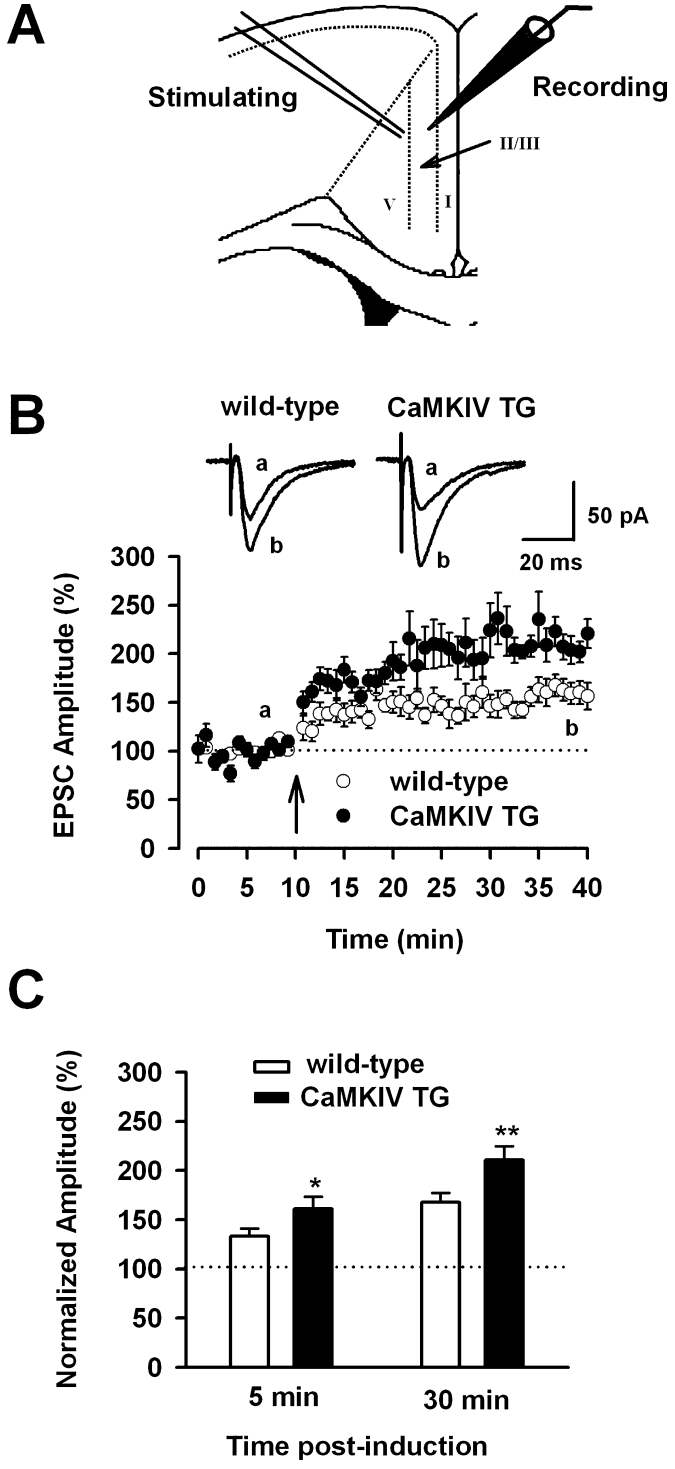


FIG. 2. Enhanced LTP in the ACC in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) Diagram of a slice showing the placement of a whole-cell patch recording and a stimulation electrode in a cingulate slice. (B) LTP is reliably induced in ACC neurons in wild-type mice ($n = 9$). Enhanced LTP is observed in ACC neurons in CaMKIV transgenic (TG) mice ($n = 8$). The insets show overlap of averages of six excitatory postsynaptic currents (EPSCs) at baseline response and 30 min after the LTP training in wild-type and transgenic mice, respectively. (C) Statistical results showed the enhanced LTP at both 5 min and 30 min after LTP induction (post-induction) in the CaMKIV transgenic mice. The dashed line indicates the mean basal synaptic response. $*P < 0.05$ and $**P < 0.01$, compared with wild-type mice.

staining was observed on brain sections when the primary antibody was omitted from the protocol. Images were captured with the assistance of Image-Pro Plus 5.0 software, all the parameters used were kept consistent during capturing.

Western blot

Western blot analysis was carried out as previously described (Hosoda *et al.*, 2004; Wang *et al.*, 2007). The ACC was dissected and homogenized in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1% sodium dodecyl sulfate (SDS), 1 × protease inhibitor cocktail and 1 × phosphatase inhibitor cocktail 1 and 2. Electrophoresis of equal amounts of total protein was performed on 4–12% SDS–polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes at 4 °C for analysis. Membranes were probed with a rabbit polyclonal anti-FLAG Epitope Tag antibody (1 : 1000; Affinity Bio Regents). The membranes were incubated in the appropriate HRP-coupled secondary antibody diluted 1 : 3000 for 1 h followed by enhanced chemiluminescence detection of the proteins. To verify equal loading, membranes were also probed with anti-α-tubulin antibody (1 : 1000; Santa Cruz).

Trace fear memory

Trace fear conditioning was performed in an isolated shock chamber (Medical Associates, St Albans, VT, USA). The conditioned stimulus (CS) used was an 80-db white noise, delivered for 15 s, and the unconditioned stimulus (US) was a 0.5-mA or 0.8-mA electric foot-shock for 0.5 s. Mice were acclimated for 60 s, and were presented with 10 CS–trace–US–intertrial interval (ITI) trials (trace of 30 s, ITI of 210 s). One day after training, mice were acclimated for 60 s and subjected to 10 CS–ITI trials (ITI of 210 s) in a novel chamber to test for trace fear memory (Huerta *et al.*, 2000). All data were recorded using the video-based FreezeFrame fear conditioning system and analysed by Actimetrics Software (Coulbourn Instruments, Wilmette). Average freezing for the baseline and for each trace between CS and US during the training and testing sessions were analysed. Bouts of 1.0 s were used to define freezing (the absence of movement aside from respiration).

Hotplate and tail-flick test

In the hotplate test, mice were placed on a standard thermal hotplate with a 10 × 10-inch black heated surface at 50 or 55 °C (Columbus Instruments, Columbus, OH, USA). The latency in which the mice showed signs of nociception, by either rapid fanning or licking of the hindpaws, was recorded with a cutoff time of 30 s. The spinal nociceptive tail-flick reflex was evoked by radiant heat (Columbus Instruments) applied to the underside of the tail. The latency to reflexive removal of the tail away from the heat was measured by a photocell timer. A cutoff time of 10 s was used to minimize skin damage.

Open-field activity

To record horizontal locomotor activity we used the Activity Monitor system from Medical Associates (43.2 × 43.2 × 30.5 cm; MED-associates, St Albans, VT, USA). Briefly, this system uses paired sets of photo beams to detect movement in the open-field, and movement is recorded as beam breaks. The open-field is placed inside an isolation

chamber with dim illumination and a fan. Each subject was placed in the center of the open-field and activity was measured for 30 min.

Data analysis and statistics

Results were analysed by *t*-test, paired *t*-test, one-way ANOVA or two-way ANOVA followed by *post hoc* Student's–Newman–Keuls test to identify significant differences. All data are expressed as mean ± SEM. In all cases, $P < 0.05$ was considered statistically significant.

Results

CaMKIV is functionally overexpressed in ACC neurons in transgenic mice

First, to examine the expression of CaMKIV transgene in the ACC under the control of αCaMKII promoter, we performed Western blotting analysis using anti-FLAG antibody. As predicted, we observed the transgenic expression of CaMKIV protein in the ACC of transgenic mice (Fig. 1A) as compared with wild-type mice. Immunohistochemistry experiments were then performed to examine CaMKIV expression and phosphorylated CREB in ACC neurons. There is a significantly increased CaMKIV expression in transgenic mice than that in wild-type mice (Figs 1B; $n = 6$ sections/two mice for each group, $P < 0.05$, unpaired *t*-test). Notably, CaMKIV is expressed in apical dendrites in some ACC neurons from transgenic mice. We next examined the effect of CaMKIV overexpression in the ACC by analysing the level of phosphorylated CREB at Serine 133. The level of phosphorylated CREB in the ACC was higher in CaMKIV mice ($n = 6$ mice) than wild-type mice littermates ($n = 4$ mice), whereas the total amount of CREB was not affected by the transgenic overexpression of CaMKIV (wild-type, $n = 4$; CaMKIV mice, $n = 6$; Fig. 1D). Quantitative analysis showed that the level of phosphorylated CREB in the ACC of CaMKIV transgenic mice was significantly higher than wild-type mice ($F_{1,8} = 6.338$, $P < 0.05$), suggesting that the overexpression of CaMKIV results in increased activity, thereby resulting in increased levels of phosphorylated CREB in the ACC of transgenic mice.

Enhanced LTP in the ACC in transgenic mice overexpressing CaMKIV

We have previously shown the deficit of LTP in the ACC in the CaMKIV knockout mice using field potential recording (Wei *et al*., 2002). We want to know whether overexpression of CaMKIV would enhance synaptic transmission or LTP in the ACC. Conventional whole-cell patch-clamp recordings were performed from visually identified pyramidal neurons in layer II/III of ACC. Evoked EPSCs, which are mainly mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Wu *et al*., 2005b), were obtained by delivering focal electrical stimulation to layer V (Fig. 2A).

Our previous studies have shown that ACC LTP could be induced *in vitro* and required NMDA receptors, adenylyl cyclases 1 and 8, CaMKIV (Wei *et al*., 2002; Liauw *et al*., 2005; Zhao *et al*., 2005b). Here, we first studied LTP in the ACC in transgenic mice overexpressing CaMKIV. LTP is reliably induced by spike-timing protocol in the ACC neurons in wild-type mice (161.0 ± 12.2% of baseline response, $n = 9$ neurons/five mice; $P < 0.05$ compared with baseline response before the stimulation, paired *t*-test; Fig. 2B and C). Using the same protocol, LTP is significantly increased in the CaMKIV overexpression mice (210.8 ± 14.2% of baseline response, $n = 8$ neurons/four mice; $P < 0.01$ compared with wild-type mice, unpaired *t*-test; Fig. 2B and C). We also compared 5 min after LTP induction in wild-type and transgenic mice. A significant increase of early potentiation was observed in transgenic mice compared with wild-type mice (wild-type, 133.3 ± 7.9%, $n = 9$ neurons/five mice; CaMKIV transgenic, 168.0 ± 9.3%, $n = 8$ neurons/four mice; $P < 0.05$, unpaired *t*-test; Fig. 2C). These results indicate that CaMKIV contributes to synaptic potentiation in the ACC and overexpression of CaMKIV increases LTP.

Normal basal synaptic transmission in CaMKIV transgenic mice

Next, we want to know whether the altered LTP is due to the change in basal synaptic transmission in the CaMKIV overexpression mice. To address this question, we first studied AMPA receptor-mediated EPSCs in wild-type and transgenic mice. The input–output relationship of AMPA EPSCs in CaMKIV mice ($n = 7$ neurons) was similar to those in wild-type mice ($n = 6$ neurons) at various stimulus intensities ($P > 0.05$, two-way ANOVA; Fig. 3A). To examine the voltage dependence of AMPA EPSCs, the currents were recorded at different holding potentials from −70 mV to +50 mV with 20-mV steps. The current–voltage (*I–V*) relationships of EPSCs were then plotted. We found that there is no difference in the *I–V* relationship of AMPA receptor-mediated EPSCs between the transgenic mice ($n = 6$ neurons) and wild-type mice ($n = 6$ neurons; $P > 0.05$, two-way ANOVA; Fig. 3B). To test whether presynaptic function was altered in CaMKIV overexpression mice, we measured paired-pulse facilitation (PPF) in slices of wild-type and transgenic mice. We found that there was no significant difference in the PPF between wild-type ($n = 5$ neurons) and CaMKIV transgenic mice ($n = 5$ neurons; $P > 0.05$, two-way ANOVA; Fig. 4).

Because the NMDA receptor is critical in cingulate LTP, we then tested whether NMDA receptor function is altered in the transgenic mice. Therefore, both input–output and *I–V* relationships of NMDA receptor-mediated EPSCs were examined. We found that there is no difference in the input–output relationship of NMDA EPSCs between wild-type ($n = 5$ neurons) and CaMKIV overexpression mice ($n = 6$ neurons; Fig. 5A). Moreover, the *I–V* relationship of the NMDA EPSCs remained unchanged in CaMKIV mice ($n = 8$ neurons) in comparison with wild-type mice ($n = 5$ neurons; Fig. 5B). Taken together, these results indicate that function of postsynaptic AMPA and NMDA receptors and basal synaptic transmission are not altered in mice overexpressing CaMKIV.

Enhanced trace fear memory in CaMKIV transgenic mice

Synaptic activities in the ACC are important for trace fear memory (Zhao *et al*., 2005a; 2006). Therefore, the enhanced LTP in the ACC may lead to improved trace fear memory. To test this idea, wild-type and CaMKIV overexpression mice were examined in trace fear conditioning paradigm with a weak electric shock (0.5 mA). Trace fear memory conditioning differs from the classic delay paradigm in that the animal must sustain attention during the trace interval to learn the CS–US association (Huerta *et al*., 2000; Han *et al*., 2003). Before conditioning, CaMKIV transgenic mice ($n = 8$ mice) displayed similar movement in the conditioning chamber compared with wild-type mice ($n = 7$ mice; $F_{1,13} = 0.43$, $P = 0.52$, one-way ANOVA, data not shown). During 10 CS–US pairing training, both mice showed increasing freezing throughout the training session. However, CaMKIV overexpression mice displayed dramatically increased freezing response starting from

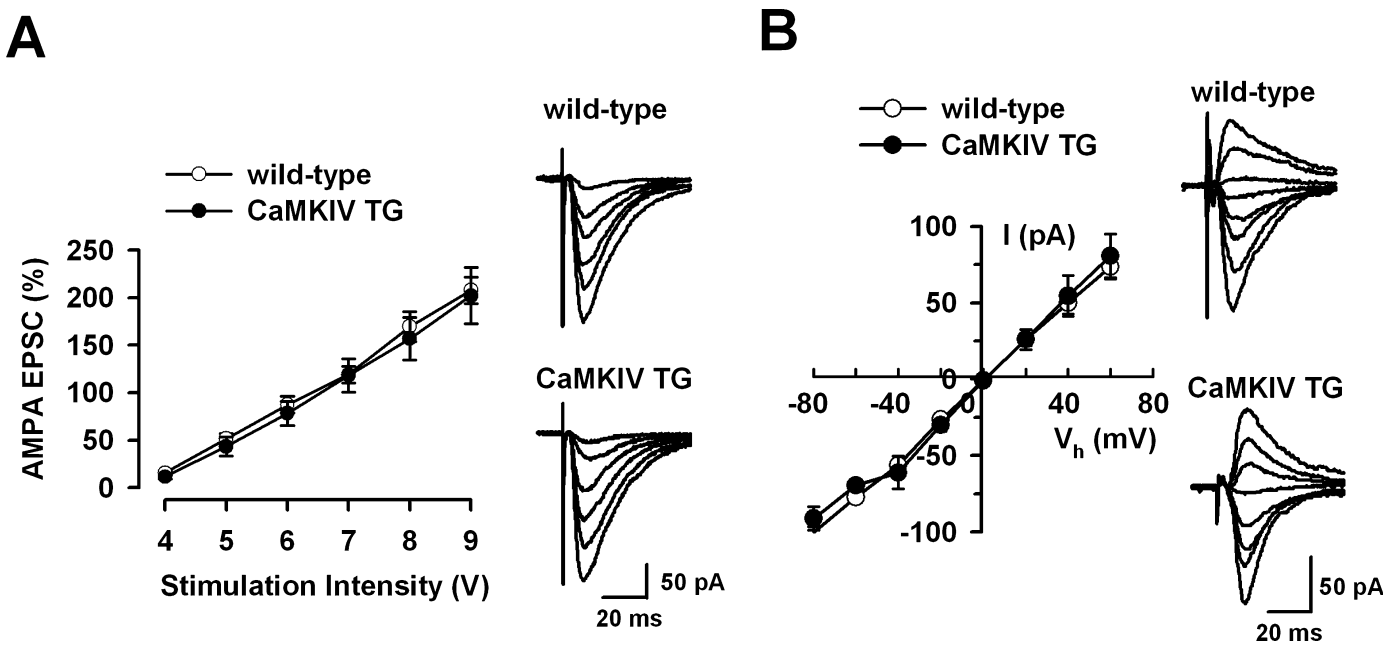


FIG. 3. Normal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated synaptic transmission in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) Input–output relationship for AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) in CaMKIV transgenic ($n = 7$) and wild-type mice ($n = 6$). The input–output relationship for AMPA receptor-mediated EPSCs in the transgenic mice is unaltered as compared with wild-type mice. (B) No difference in current–voltage plots for AMPA receptor-mediated EPSCs between CaMKIV transgenic ($n = 6$) and wild-type mice ($n = 6$).

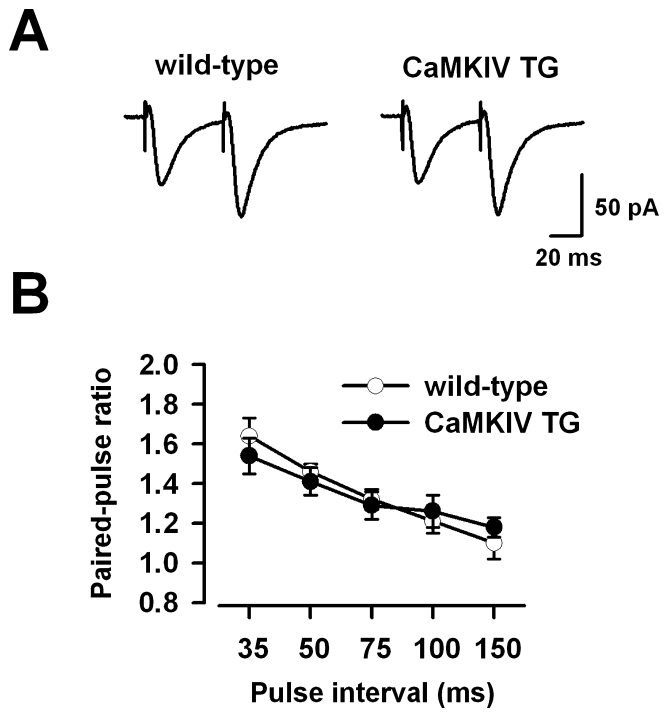


FIG. 4. Normal presynaptic release probability in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) Sample traces of PPF at the 50-ms interval recorded from CaMKIV transgenic and wild-type mice. (B) Pooled results showing that PPF does not differ in CaMKIV transgenic ($n = 5$) and wild-type ($n = 5$) mice.

the third trial compared with wild-type mice ($P < 0.01$, unpaired *t*-test; Fig. 6A and C). Moreover, CaMKIV overexpression mice reached the highest level freezing at the fourth trace, while wild-type mice did at the fifth trace (Fig. 6A). These data show that CaMKIV overexpression mice can learn faster in trace fear conditioning. In the test session (24 h post-training), however, the transgenic mice showed no difference in freezing as compared with wild-type mice ($F_{1,13} = 2.30$, $P > 0.05$, two-way ANOVA; Fig. 6B and C).

Considering unchanged 24 h trace fear memory may be due to the electric shock (0.5 mA) being too weak to be consolidated, we then tested the trace fear memory with a strong electric shock (0.8 mA). Similar to the 0.5-mA electrical shock, both mice showed increasing freezing throughout the training session during 10 CS–US pairing training (Fig. 7A). Interestingly, CaMKIV transgenic mice ($n = 7$ mice) displayed similar freezing response compared with wild-type mice ($n = 10$ mice) during 10 CS–US pairing training ($P > 0.05$; Fig. 7A and C). However, on the test session 24 h post-training, the transgenic mice showed significantly increased freezing response compared with wild-type mice ($P < 0.05$; Fig. 7B and C). Taken together, these results suggest that mice overexpressing CaMKIV are enhanced in fear memory. However, the enhancement in acquisition or 24 h post-training retention of trace fear memory is dependent on the shock intensity.

Normal acute sensory thresholds in CaMKIV transgenic mice

To determine whether the increase in trace fear memory of CaMKIV transgenic mice is attributable to changes in pain sensitivity to the foot-shock, we measured acute sensory thresholds in the hotplate and tail-flick tests. We found there is no difference between nociceptive responses of CaMKIV overexpression ($n = 6$ mice) and wild-type mice ($n = 7$ mice) for the hotplate set at 50 or 55 °C ($F_{1,13} = 0.01$, $P = 0.62$ for 50 °C; $F_{1,13} = 0.51$, $P = 0.81$ for 55 °C, one-way ANOVA; Fig. 8A). Similarly, no difference was found in the tail-flick latency of CaMKIV overexpression mice compared with wild-type mice ($F_{1,13} = 0.36$, $P = 0.53$, one-way ANOVA; Fig. 8B). We also

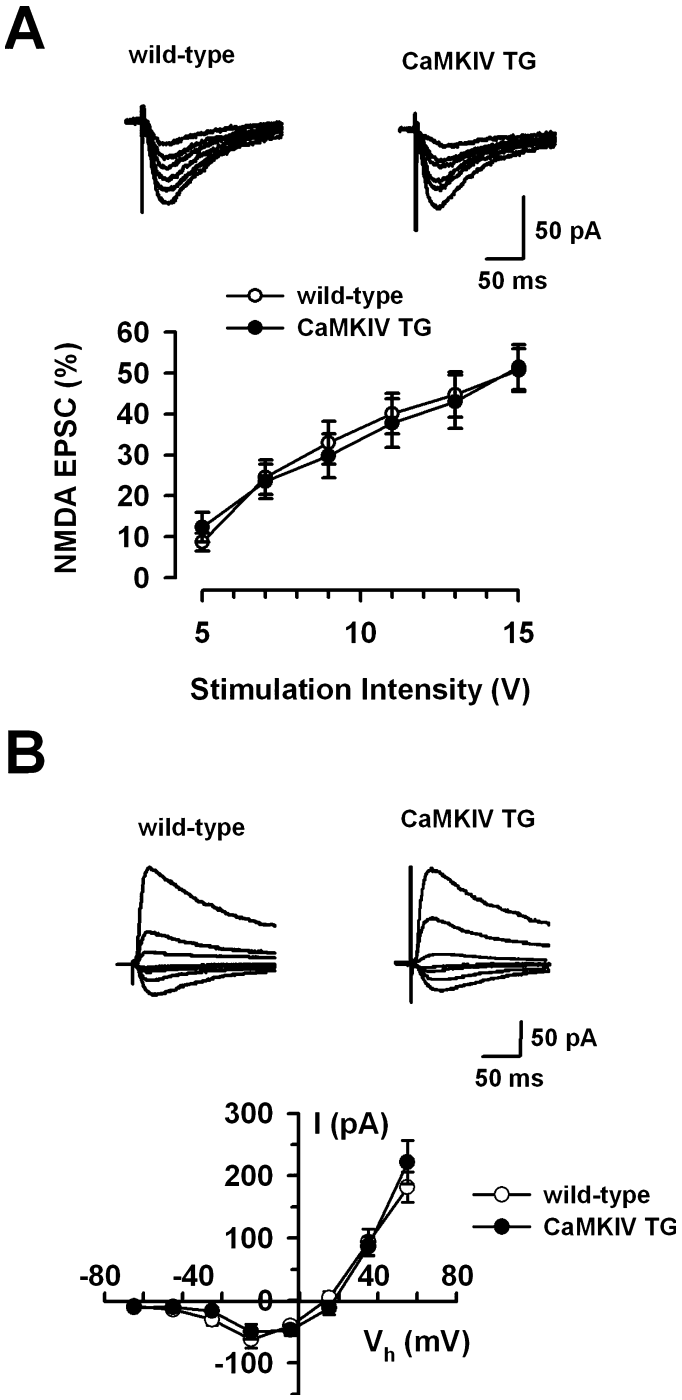


FIG. 5. Normal *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) No difference in the input–output relationship for NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) between CaMKIV transgenic ($n = 6$) and wild-type mice ($n = 5$). (B) No difference in current–voltage plots for NMDA receptor-mediated EPSCs between CaMKIV transgenic ($n = 8$) and wild-type mice ($n = 5$).

tested the freezing responses to tone and we found that there is no difference between wild-type ($n = 17$ mice) and CaMKIV transgenic mice ($n = 15$ mice; $F_{1,30} = 0.22$, $P = 0.64$, one-way ANOVA; Fig. 8C). To test for changes in mobility, locomotor activity was recorded in an open-field. We found no significant differences between wild-type and transgenic mice in the total distance traveled within the open-field for 30 min (wild-type, $n = 6$ mice; CaMKIV transgenic mice, $n = 7$ mice, $F_{1,11} = 0.02$, $P = 0.89$, one-way ANOVA; Fig. 8D). These results indicate that mice overexpressing CaMKIV are normal in locomotor activity and in sensitivity to acute painful stimuli or tone.

Discussion

Despite its high expression in the ACC, less is known about the function of CaMKIV in the ACC (Wei *et al*., 2002). Using transgenic mice with forebrain overexpression of CaMKIV, we investigated the role of CaMKIV in cingulate plasticity as well as ACC-related brain function. We found that overexpression of CaMKIV enhanced trace fear memory and LTP in the ACC, while acute sensory responses and basal synaptic transmission are normal. These results suggest that activity-dependent activation of CaMKIV is important for LTP induction in the ACC and ACC-related function such as trace fear memory. Our results confirm and extend the previous work in the hippocampus and cerebellum that CaMKIV plays critical roles in synaptic plasticity and memory.

Although our current study showed the correlation of enhanced LTP in the ACC and trace fear memory, we have to keep in mind that there is no direct evidence to link between the electrophysiological and the behavioral results in the present study, given that the overexpression of CaMKIV applies throughout the forebrain. The other brain regions such as hippocampus and amygdala may also be involved in enhanced trace fear memory observed in CaMKIV overexpression mice. In particular, the hippocampus has been implicated in trace fear conditioning (Huerta *et al*., 2000). We have been focusing on synaptic response in layer II/II neurons evoked by stimulation of layer V, which might represent more integrative synapses in the ACC (Wang & Shyu, 2004; Zhuo, 2007). We cannot rule out the possible involvement of neurons in other layers of the ACC.

CaMKIV in the cingulate LTP

Our previous studies demonstrated that glutamatergic synapses in the ACC can undergo long-term plasticity, such as LTP and LTD (Wei *et al*., 2002; Liauw *et al*., 2005; Wu *et al*., 2005a; Zhao *et al*., 2005b). NMDA receptor including both NR2A and NR2B subunit is involved in LTP induction in the ACC (Zhao *et al*., 2005b). Genetic deletion of downstream molecules as adenylyl cyclases 1 and 8, CaMKIV abolished cingulate LTP (Wei *et al*., 2002; Liauw *et al*., 2005). Our recent results further indicated that LTP in the ACC requires the recruitment of GluR1 AMPA receptors; and such events are rapid and completed within 5–10 min after LTP induction (Toyoda *et al*., 2007). These findings suggest that cingulate LTP share certain common Ca^{2+}-related signaling pathways with hippocampal LTP.

CaMKIV is required for synaptic plasticity, particularly for late-phase LTP and LTD, in the hippocampus and cerebellum (Ahn *et al*., 1999; Ho *et al*., 2000; Kang *et al*., 2001). Considering that the late phase of plasticity is dependent on protein synthesis, it is believed that the CaMKIV–CREB pathway is involved (Shaywitz & Greenberg, 1999). Consistently, it has been shown that LTP is associated with the increased activity of CaMKIV and phospho-CREB (Kasahara *et al*., 2001). Using transgenic mice expressing a dominant-negative form of CaMKIV, Kang *et al*. (2001) found that late-phase but not early-phase LTP is disrupted in the hippocampus. Using CaMKIV knockout mice, Ho *et al*. (2000) reported the impaired late-phase LTP in the hippocampus. However, the onset of defect in LTP is early, which is within the first 5 min after tetanization. Impaired early-phase LTP was

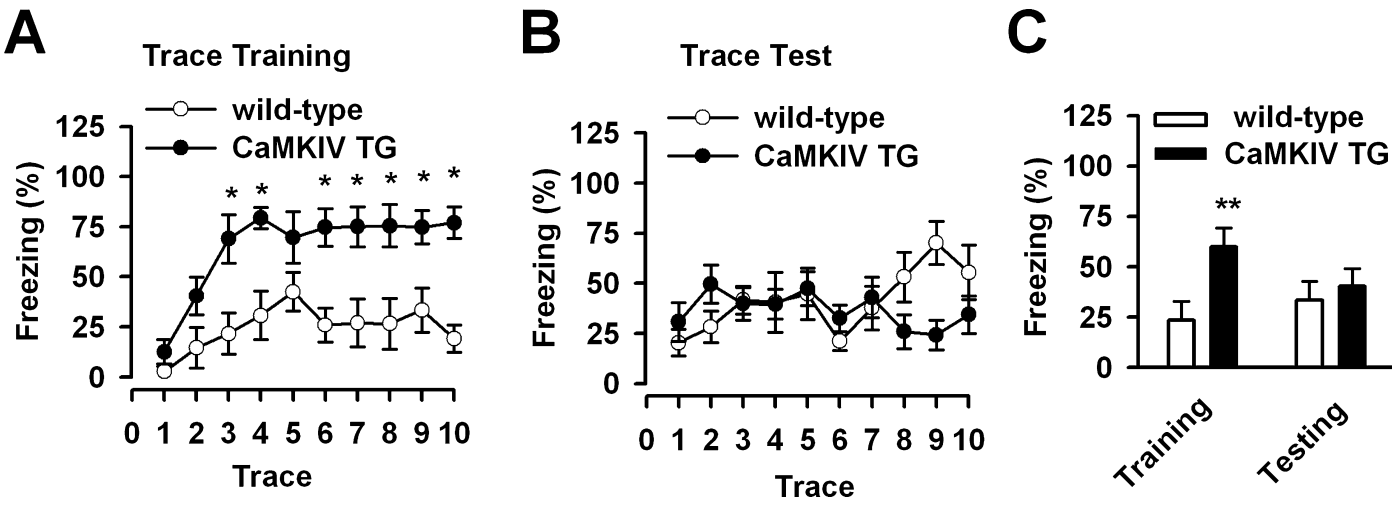


FIG. 6. Enhanced learning of trace fear conditioning at weak shock intensity in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) CaMKIV transgenic mice ($n = 8$) showed significantly increased freezing compared with wild-type mice ($n = 7$) during trace training at 0.5-mA intensity electric shock. (B) At 0.5-mA intensity electric shock, CaMKIV transgenic mice showed comparable freezing within the traces of the 24-h retention test compared with wild-type mice. (C) Statistical results showed that CaMKIV transgenic mice showed increased average freezing during trace training at 0.5-mA intensity electric shock compared with wild-type mice. $*P < 0.05$ and $**P < 0.01$.

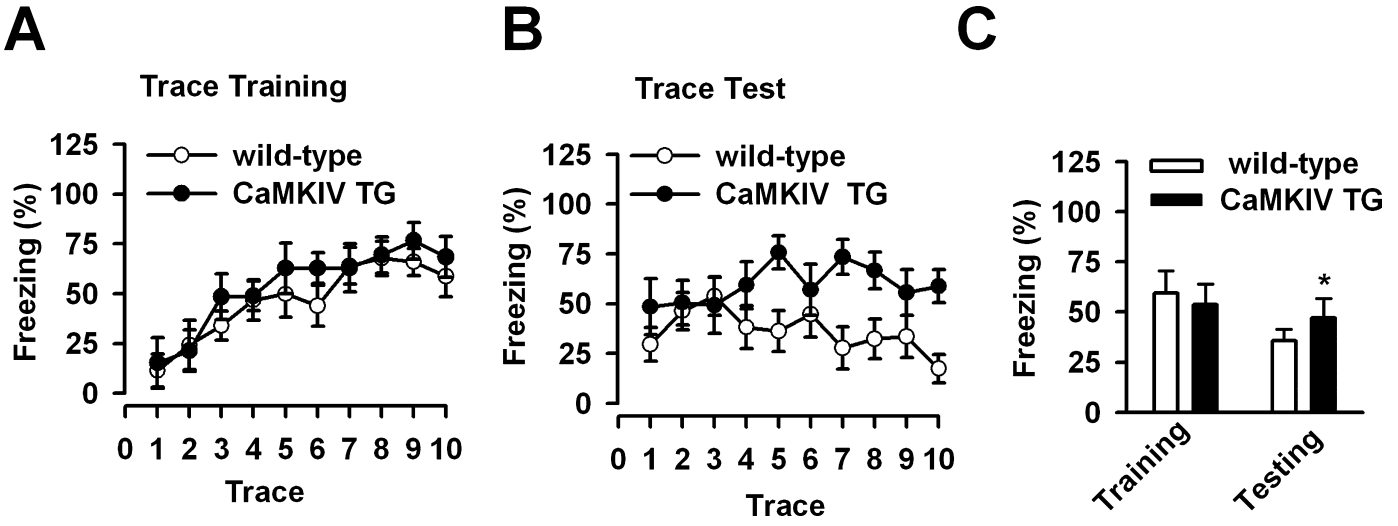


FIG. 7. Enhanced memory of trace fear conditioning at strong shock intensity in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) At 0.8-mA intensity electric shock, CaMKIV transgenic mice ($n = 7$) showed similar freezing compared with wild-type mice ($n = 10$) during trace training. (B) At 0.8-mA intensity electric shock, CaMKIV transgenic mice showed increased freezing within the traces of the 24-h retention test compared with wild-type mice. (C) Statistical results showed that CaMKIV transgenic mice showed increased average freezing during the 24-h retention test compared with wild-type mice. $*P < 0.05$.

also found in the amygdala, ACC, insular cortex and somatosensory cortex in CaMKIV knockout mice (Wei *et al*., 2002). During the early phase of LTP, gene expression in the nucleus is unlikely to affect LTP formation (Shaywitz & Greenberg, 1999). Therefore, the contribution of CaMKIV in synaptic plasticity is not only dependent on the CaMKIV–CREB pathway, but also resides in pathways independent of Ca^{2+} influx-triggered gene expression. This might explain why we have observed the hyperphosphorylation of CREB in the ACC in transgenic mice under basal conditions while the LTP is enhanced but not occluded.

In the present study, we found that early-phase LTP at both 5 min and 30 min after induction is significantly enhanced in the transgenic mice with forebrain overexpression of CaMKIV. However, the input–output relationship or *I–V* curve of AMPA and NMDA receptor-mediated EPSCs in CaMKIV overexpression mice were unaltered compared with those in wild-type mice, suggesting that the increased LTP is not due to the functional modification of postsynaptic NMDA and AMPA receptors. Moreover, there is no change in presynaptic release probability because PPF is also normal in the transgenic mice. Several implications could explain the role of CaMKIV in early-phase LTP. Firstly, synaptic proteins required for LTP may have been primed by overexpression of CaMKIV. A similar mechanism was interpreted for the facilitation of late-phase LTP in transgenic mice expressing constitutively active CREB (Barco *et al*., 2002). Secondly, the change of synaptic structure in CaMKIV transgenic mice may underlie the enhanced LTP. CaMKIV has been demonstrated to promote microtubule assembly by phosphorylating the stathmin, a microtubule regulator important in spine morphology and synaptic plasticity (Melander Gradin *et al*., 1997; Shumyatsky *et al*., 2005). Consistently, expression of the active form of CaMKIV in cortical culture induced

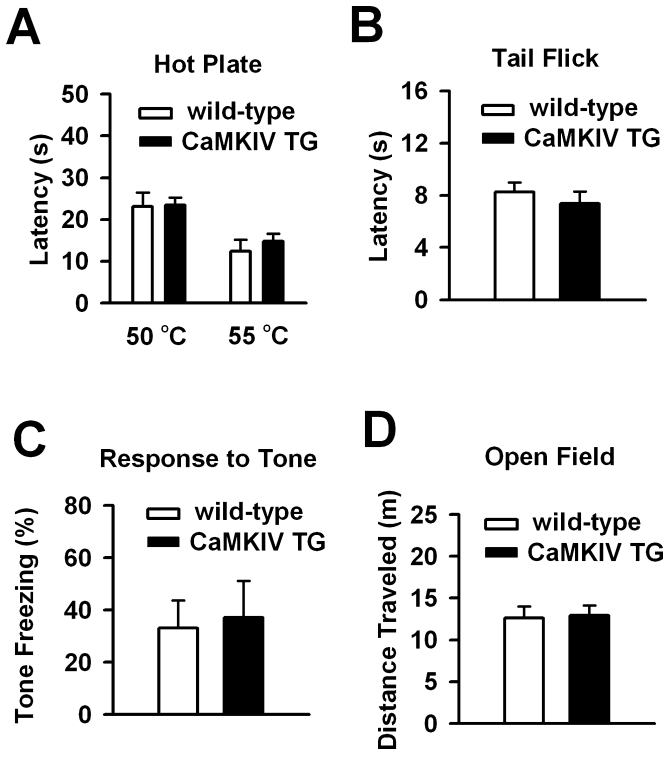


FIG. 8. Normal acute sensory responses and locomotor activities in Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) transgenic mice. (A) No difference in hotplate response latencies between CaMKIV transgenic ($n = 7$) and wild-type mice ($n = 6$) on the 50 °C or 55 °C hotplate. (B) Similar tail-flick responses were detected in CaMKIV transgenic mice ($n = 7$) compared with that in wild-type mice ($n = 6$). (C) No difference in response to tone was observed between CaMKIV overexpressing ($n = 15$) and wild-type mice ($n = 17$). (D) CaMKIV transgenic mice showed normal locomotor activities in the open-field test.

dendritic growth (Redmond *et al*., 2002). Thirdly, recruitment of silent synapses in CaMKIV overexpression mice may explain the enhanced LTP. It has been reported that acute expression of the active form of CREB via *in vivo* viral-mediated gene transfer in the hippocampus could generate silent synapses and enhance LTP (Marie *et al*., 2005).

Due to the limitation of using whole-cell patch-clamp recordings, we did not examine the late-phase LTP in CaMKIV overexpression mice in the present study. Future study is needed to address this question, although we believe that CaMKIV overexpression would affect the late phase of LTP.

CaMKIV in the trace fear memory

CaMKIV is originally revealed in cerebellar granule cells, and was shown to be involved in motor functions and motor memory storage (Ribar *et al*., 2000; Boyden *et al*., 2006). Studies were also focused on the hippocampus, where CaMKIV is shown to be involved in certain hippocampus-dependent memory, although results vary from different groups (Ho *et al*., 2000; Kang *et al*., 2001). The broad distribution of CaMKIV in forebrain areas suggest its important role in higher brain functions, such as emotion, attention and memory (Wei *et al*., 2002). Indeed, CaMKIV knockout mice exhibit abnormal injury and fear-induced ultrasonic vocalizations as well as altered anxiety (Ko *et al*., 2005) and stress-like behaviours (Shum *et al*., 2005).

Genetic deletion of CaMKIV abolished LTP in the ACC (Wei *et al*., 2002). However, the possible role of CaMKIV in the ACC-related function is still unknown. ACC contributes to attention-related memory, such as trace fear memory (Han *et al*., 2003; Zhao *et al*., 2005b). Trace fear conditioning requires animals to sustain attention during the trace interval to learn the conditioning and unconditioning stimuli association (Huerta *et al*., 2000; Han *et al*., 2003). Our previous studies have shown that the abolishment of LTP in the ACC is correlated with loss in trace fear memory. For instance, genetic deletion of fragile X mental retardation 1 (FMR1) caused the deficit in ACC LTP and trace fear memory (Zhao *et al*., 2005b). Mice with peripheral inflammation showed a deficit in expression but not acquisition of trace fear memory. In the same mice, there is a significant increase in the presynaptic release of glutamate in the ACC (Zhao *et al*., 2006). Because forebrain overexpression of CaMKIV enhanced the ACC LTP, we decided to examine trace fear memory in the transgenic mice.

Our results showed that trace fear memory was significantly enhanced in the mice with forebrain overexpression of CaMKIV. The results suggest that CaMKIV affects the ability to sustain attention in a manner that is needed for retaining of the memory. We have shown that LTP in the ACC was significantly enhanced in the transgenic mice, and such enhancement of LTP may, at least in part, contribute to the improved trace fear memory. However, considering the well-known role of CaMKIV as mediator for activity-dependent gene expression (Anderson & Kane, 1998; Soderling, 1999), the enhanced trace fear memory may also involve the altered CaMKIV–CREB pathway in the transgenic mice. We have an interesting finding that different intensities of shock induced distinct memory phenotypes in CaMKIV overexpression mice, that is, the enhanced learning during trace training at weak shock intensity while the enhanced memory during tract test at strong shock intensity. This result suggests that CaMKIV is involved in intensity-dependent trace fear memory. However, there is no clear explanation for the result right now and future study is needed for exploring its potential mechanism.

In summary, we showed that CaMKIV contributes to cingulate LTP and trace fear memory. This is the first evidence to demonstrate that genetic overexpression of CaMKIV enhances ACC-related synaptic function and behavior. Together with our previous reports using CaMKIV knockout mice (Wei *et al*., 2002), our findings consistently demonstrate that CaMKIV is critical for synaptic potentiation in the ACC. Dissecting the molecular mechanisms for LTP in the ACC will be helpful to understand the synaptic basis for functional excitatory transmission and plasticity in ACC-related functions such as trace fear memory.

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Abbreviations

ACC, anterior cingulate cortex; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BSA, bovine serum albumin; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; CREB, cAMP response element-binding protein; CS, conditioned stimulus; EPSC, excitatory postsynaptic current; HRP, horseradish peroxidase; ITI, intertrial interval; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PPF, paired-pulse facilitation; SDS, sodium dodecyl sulfate; US, unconditioned stimulus.

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