Impaired Synaptic Plasticity and cAMP Response Element-Binding Protein Activation in Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase Type IV/Gr-Deficient Mice

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The Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) is a key effector of neuronal Ca\textsuperscript{2+} signaling; its function was analyzed by targeted gene disruption in mice. CaMKIV/Gr-deficient mice exhibited impaired neuronal cAMP-responsive element binding protein (CREB) phosphorylation and Ca\textsuperscript{2+}/CREB-dependent gene expression. They were also deficient in two forms of synaptic plasticity: long-term potentiation (LTP) and long-term depression (LTD), which are proposed to be important for the formation of long-term memory (LTM). In particular, later phases of LTP/LTD require activation of Ca\textsuperscript{2+}-dependent gene transcription, whereas manipulations that interfere with Ca\textsuperscript{2+} entry disrupt both synaptic plasticity and LTM responses (Chen and Tonegawa, 1997).

Several transducers of Ca\textsuperscript{2+} signaling have been implicated in synaptic plasticity and LTM. These include the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type II (CaMKII), the inactivation or antagonization of which impairs LTP in hippocampal CA1 neurons and compromises spatial memory (Silva et al., 1992a,b; Bach et al., 1995; Mayford et al., 1996; Cho et al., 1998; Giese et al., 1998). Ca\textsuperscript{2+}/calmodulin-dependent adenyl cyclases also promote synaptic plasticity, learning, and memory via activation of cAMP-regulated protein kinase A (PKA) and the transcription factor cAMP response element binding protein (CREB), which phosphorylates on the regulatory Ser133 residue (Shaywitz and Mayford, 1999).

Key words: calcium/calmodulin-dependent kinase; synaptic plasticity; LTD; CREB; memory

Ca\textsuperscript{2+} signaling pathways play a pivotal role in synaptic plasticity and memory formation (Dubnau and Tully, 1998; Silva et al., 1998; Mayford and Kandel, 1999). Elevation of intracellular Ca\textsuperscript{2+} is necessary for the induction of different forms of synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD), which are proposed to be important for the formation of long-term memory (LTM). In particular, later phases of LTP/LTD require activation of Ca\textsuperscript{2+}-dependent gene transcription, whereas manipulations that interfere with Ca\textsuperscript{2+} entry disrupt both synaptic plasticity and LTM responses (Chen and Tonegawa, 1997).

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The Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) is of particular interest in neuronal Ca\textsuperscript{2+} signaling because of its expression profile and its function as a transcriptional activator. CaMKIV/Gr is expressed in both nuclei and cytosol of neurons of several brain regions, including the cortex, cerebellum, hippocampus, and amygdala (Ohmstede et al., 1989; Jensen et al., 1991a,b; Means et al., 1991; Ohmstede et al., 1991). It activates several transcription factors, including CREB, which it phosphorylates on the regulatory Ser133 residue (Mathews et al., 1994; Sun et al., 1994), the CREB-related factor ATF-1, the MADS-box family members SRF and MEF2D, and the transcriptional coactivator p300/CREB-binding protein (CBP) (Miranti et al., 1995; Enslen et al., 1996; Sun et al., 1996; Chawla et al., 1998; Hu et al., 1999; Blaeser et al., 2000). CaMKIV/Gr also phosphorylates and regulates the function of synaptic proteins including the actin binding protein synapsin I (Ohmstede et al., 1989) and the microtubule regulator Stathmin/OP18 (Melander Gradin et al., 1997).

CaMKIV/Gr has been implicated in various aspects of neuronal Ca\textsuperscript{2+} signaling, including CREB phosphorylation and gene expression in response to excitatory neurotransmission (Bito et al., 1996), production of and responsiveness to neurotrophic factors (Fink-beiner et al., 1997; Shieh et al., 1998; Tao et al., 1998), and synaptic plasticity (Ahn et al., 1999). Notwithstanding these findings, the role of CaMKIV/Gr in neuronal Ca\textsuperscript{2+} signaling remains unclear and at times in dispute (Impey et al., 1998). In this study, we report on the derivation and use of CaMKIV/Gr-deficient mice to elucidate the contributions of CaMKIV/Gr to Ca\textsuperscript{2+}-regulated adaptive neuronal responses, including synaptic plasticity, gene expression, and learning and memory.

MATERIALS AND METHODS

Generation of CaMKIV/Gr knockout mice

Genomic DNA fragments spanning CaMKIV/Gr exon III were isolated by screening a genomic DNA library derived from the mouse strain L129 with the entire murine CaMKIV/Gr cDNA. A 16.5 kb genomic DNA clone was isolated that comprised the 79-bp-long CaMKIV/Gr exon III and its...
surrounding intrinsic sequences. A 2.9 kb fragment flanking 5' to the targeted exon was derived by cloning 2.17 with NotI (N) and XhoI (X), then ligated into unique NotI-XhoI sites 5' of the neomycin resistance gene of pBluescript KS (a gift of Dr. Al Core and Dr. D. Frame) into embryonic stem (ES) cells (Genome Systems, St. Louis, MO). These were then grown on murine embryonic fibroblasts in the presence of leukemia inhibitory factor and subjected to double selection with G418 at 200 μg/ml and ganciclovir at 0.12 μg/ml. Double drug-resistant colonies were screened for homologous recombination by Southern blotting using as a probe a 400 bp EcoRI fragment immediately distal to the 3' flanking DNA sequence.

Disruption of the targeted sequence resulted in the introduction of a BamHI fragment into the calcium binding domain, or a 67% decrease in the size of the BamHI fragment, which normally hybridizes with this probe from 12.5 to 7.5 kb.

Heterozygous CaMKIV/Gr-deficient ES cells were injected into C57BL/6 blastocysts that were reimplanted in pseudopregnant females. Several male chimeras were identified by agouti coat color, and those showing >80% agouti coats were mated to C57BL/6 females. Agouti offspring were screened for heterozygotes (HET) by Southern blot and PCR analysis, and HET animals were bred to generate F2 hybrid homozygous-deficient (KO) mice and wild-type (WT) littermate controls. F2 hybrid siblings were used for all of the behavioral studies and most of the other experiments except where indicated. The mice used in these studies were on a F1 background with a 1:1 ratio of WT to KO progeny. All mouse protocols were in accordance with National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Washington University School of Medicine.

**RT-PCR analysis**

cDNA was derived from total brain RNA of WT, HET, and KO mice by reverse transcription using an oligo dT primer, then subjected to RT-PCR analysis using the following exon III sequence flanking primers: 5'-TCCCTCTGGCGTATCTTCG-3' (sense primer; bp 153–172 of murine CaMKIV/Gr cDNA) and 5'-CTGGATCTGTGGGTGTCT-3' (antisense primer; bp 377–357 of CaMKIV/Gr cDNA) (Jones et al., 1991).

**Histological and immunohistochemical analysis**

For histological analysis, freshly dissected brains of adult KO mice and WT littermate controls were fixed overnight in phosphate-buffered 4% paraformaldehyde. For immunohistochemistry, paraformaldehyde-fixed brains or hippocampal slices of adult male CaMKIV/Gr KO mice and their WT littermate controls were cryoprotected for 48 hr in 15% sucrose in Dulbecco's PBS (D-PBS). Immunohistochemical analysis was performed on free-floating sections cut at 35 μm on a cryostat. After blocking in 3% normal goat serum in PBS for 30 min, sections were incubated with a 1:1000 dilution of murine monoclonal anti-CaMKIV/Gr (Clontech, Palo Alto, CA), 1:1000 dilution of a glutamate receptor antibody, then either sham-treated or depolarized by treatment with 60 mM glutamate. The slices were then washed and fixed in 4% paraformaldehyde for at least 1 hr before being placed in 10% PBS, then dehydrated through a graded series of ethanol, and subjected to Giemsa staining after standard procedures. For immunohistochemistry, paraffin-embedded sections of adult male CaMKIV/Gr-deficient mice were stained with mouse monoclonal anti-CaMKIV/Gr catalytic domain antibody, anti-CaMKIIα antibody, and anti-PKA catalytic subunit (PKAc) antibody (Transduction Laboratories); goat polyclonal anti-CaMKIV/Gr c-terminal peptide antibody, rabbit polyclonal anti-ERK antibody, and mouse monoclonal anti-CREB antibodies (Santa Cruz Biotechnology); and phospho-specific anti-ERK (pERK) and anti-CREB (pCREB) antibodies (New England Biolabs). The blots were developed using horseradish peroxidase-conjugated secondary antibodies and enzyme-linked chemiluminescence (ECL, Amersham, Arlington Heights, IL)

**Hippocampal electrophysiology**

Male WT and CaMKIV/Gr KO littermate mice at 6–8 weeks of age were anesthetized with inhaled halothane. Transverse slices of hippocampus were rapidly prepared and maintained in an interface chamber at 28°C, where they were superfused with artificial CSF (ACSF) consisting of (in mM): 124 NaCl, 4.4 KCl, 1.2 CaCl2, 1.0 MgSO4, 25 NaHCO3, 1.0 HEPES, 11 glucose, pH 7.35 with NaOH. Electrodes were filled with a 3 M potassium acetate and 0.5 M CaCl2, and testes of adult WT, HET, and KO littermate mice were tested in a blinded fashion, and their CaMKIV/Gr status was later established by genotyping and CaMKIV/Gr immunoblotting. Neurons were cultured in serum-free medium overnight before stimulation, then either sham-treated or depolarized by treatment with 60 mM KCl for 10 min. The cells were then lysed with 1% boiling sample buffer and boiled for 10 min. Protein concentrations of the respective samples were determined, and 12.5 μg protein of each lysate sample were loaded and resolved on 10% SDS-polyacrylamide gels, using a standard SDS-PAGE protocol. The gels were then transferred to nitrocellulose membranes and subjected to immunoblotting.

**Immunoblotting**

Whole brain and testes homogenates were derived from dissected brains and testes of adult WT, HET, and KO littermate mice using a Polytron homogenizer. Homogenates were cleared by high-speed centrifugation, and 50 μg protein in samples were resolved by SDS-PAGE, then transferred to nitrocellulose membranes for immunoblotting. Membranes of whole brain homogenates or neuronal cell culture lysates were blocked in fat-free milk, then probed with one or more of the following antibodies as indicated: mouse monoclonal anti-CaMKIV/Gr catalytic domain antibody, anti-CaMKIIα antibody, and anti-PKA catalytic subunit (PKAc) antibody (Transduction Laboratories); goat polyclonal anti-CaMKIV/Gr c-terminal peptide antibody, rabbit polyclonal anti-ERK antibody, and mouse monoclonal anti-CREB antibodies (Santa Cruz Biotechnology); and phospho-specific anti-ERK (pERK) and anti-CREB (pCREB) antibodies (New England Biolabs). The blots were developed using horseradish peroxidase-conjugated secondary antibodies and enzyme-linked chemiluminescence (ECL, Amersham, Arlington Heights, IL)

**pCREB and c-Fos induction by glutamate-perfusion and restraint-stress paradigms**

For pCREB and c-Fos staining of glutamate-perfused hippocampal slices, the latter were prepared and maintained for 2 hr in an interface chamber at 28°C superfused with ACSF. They were then perfused with sodium glutamate (100 mM) for the indicated periods after which they were fixed in paraformaldehyde and analyzed as indicated above. For pCREB staining in the context of restraint-stress, adult male CaMKIV/Gr KO mice and their WT littermate controls were either left undehandled or subjected to 30 min of restraint stress (2 min of immobilization followed by 28 min of both). Mice were anesthetized with 1 ml of 2.5% Avertin and transcardially perfused with D-PBS followed by 4% paraformaldehyde in D-PBS and processed for pCREB staining as described above. Analysis of c-Fos induction on restraint-stress was performed as above except that the mice were subjected to statistical analysis, a p < 0.05 was considered significant.

**Cerebellar LTD studies**

Mouse embryonic cerebellar cultures were prepared and maintained as described previously (Linden, 1996). After 10–16 d in vitro, perforated-plate slices (Garcia-Bustos et al., 1992) were attached to Purkinje neuron somata were fixed with a solution containing (in mM): 95 CsSO4, 15 CsCl, 8 MgCl2, 10 HEPES, pH 7.35 with CsOH. Electrode tips were filled with a small amount of this solution, and the shanks were backfilled with this solution supplemented with 0.3 M CsCl and a concentration of 300 μg/ml. Stable access resistance of <12 MΩ could be obtained within 10 min of gigaseal formation. A holding potential of -70 mV was imposed. Iontophoresis electrodes (1 μm tip diameter) were filled with 10 mM glutamate (in 10 mM HEPES, pH 7.1 with NaOH) and positioned ~20 μm away from the large-caliber dendrites. Test pulses of glutamate were delivered using negative current pulses (600–900 nA, 40–110 msec duration, 0.05 Hz). After acquisition of baseline responses, the stimuli were added and each consecutive test pulse combined with a 3-sec-long depolarization step to 0 mV. Cells were maintained in solution (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 0.8 MgCl2, 10 HEPES, 10 glucose, 0.0005% tetrodotoxin, and 0.02 picric acid. Additional LTP was induced with 7.35 with NaOH at a flow rate of 0.5 ml/min. Experiments were conducted at room temperature. Bis-fura-2 ratio imaging of intracellular free Ca was accomplished by measuring the background-corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system as described previously (Linden, 1996).

**Derivation and stimulation of primary cortical neuronal cell cultures**

Primary mouse neuronal cultures were prepared as described (Dugan et al., 1999) from neocortices of embryonic day 14 (E14) fetuses conceived by mating C3H/HeJx/C57Bl/6J matings (F0 generation). Two cultures were derived from each fetal brain, one to serve as a control and the other as the test sample. Neuronal cultures derived from fetuses of the same litter were tested in a blinded fashion, and their CaMKIV/Gr status was later established by genotyping and CaMKIV/Gr immunoblotting. Neurons were cultured in serum-free medium overnight before stimulation, then either sham-treated or depolarized by treatment with 60 mm KCl for 10 min. The cells were then lysed with 1% boiling sample buffer and boiled for 10 min. Protein concentrations of the respective samples were determined, and 12.5 μg protein of each lysate sample were loaded and resolved on 10% SDS-polyacrylamide gels, using a standard SDS-PAGE protocol. The gels were then transferred to nitrocellulose membranes and subjected to immunoblotting.

**Hippocampal EPSPs were measured after the blockade of AMPA/kainate receptors using 20 μM CNOX (for at least 30 min treatment). The Mg2+ concentration used in these studies is 1 mM, and the residual EPSPs were completely blocked by 100 μM 2-APB. A comparison of AMPA receptor-mediated responses was observed. Stimulations at different intensities were used to generate the fiber volley and NMDA receptor-mediated EPSP slope plot. Data are presented as mean ± SEM. One-way ANOVA (with Dunnett’s multiple range test for post hoc comparison) and Student’s t test were used for statistical analysis, a p < 0.05 was considered significant.**
were restrained for 1 hr and then returned to their cages for 2 hr before they were killed and analyzed. Staining intensity was quantitated using NIH Image software and analyzed for statistical significance between genotypes by ANOVA.

Behavioral analyses

F2 hybrid CaMKIV/Gr KO mice \((n = 14)\) and WT littermate controls \((n = 12)\) that were 3–4 months of age when behavioral testing began were evaluated for the following tests. All tests were performed in a blinded fashion.

One hour locomotor activity test. Testing was performed in transparent \((47.6 \times 25.4 \times 20.6 \text{ cm})\) polystyrene cages. Movement was monitored by photocells, and their output was fed to an on-line computer (Hamilton-Kendall, Chichester, UK). A transparent Plexiglas frame \((15 \times 52 \text{ cm})\) was used to define a 33 \(\times 33 \text{ cm}\) central zone and a peripheral or surrounding zone that was 5.5 cm wide with the sides of the cage being the outermost boundary. Variables that were analyzed included the total distance traveled in the center area and the distance traveled in the periphery. Testing took place between 7:30 A.M. and 3 P.M. in a quiet room continuously illuminated by fluorescent lights.

Sensomotor battery. The general sensomotor capabilities of the mice were evaluated using the four tests described below according to previously published methods (Wozniak et al., 1990; Brosnan-Watters et al., 1996, 1999). The inclined screen test involved placing mice on an elevated wire mesh grid (10 \(\times 10\) cm) that was placed across a square that was 15 × 52 cm and inclined to 60°. Each mouse was placed in the middle of the screen with its head oriented downward and scored for either how long it remained on the screen or how long it took to turn 180° and climb to the top. The maximum time allowed was 60 sec.

A maximum score of 60 sec was given if an animal did not fall. In the platform task, the mouse was timed for how long it remained on an elevated \((47 \text{ cm above the floor})\) circular platform \((1.0 \text{ cm thick}, 3.0 \text{ cm diameter})\). A maximum score of 60 sec on the platform task was assigned to the mouse if it remained on the platform for the entire duration of that time or if it could climb down, without falling, on a very thin pole that supported the platform. For the ledge test, each mouse was timed for how long it could maintain its balance on a narrow \((0.75 \text{ cm thick})\) Plexiglas ledge without falling \((60 \text{ sec maximum})\). A score of 60 sec was also assigned if the mouse traversed the entire length \((51 \text{ cm})\) of the Plexiglas ledge and returned to the starting place under 60 sec without falling. For the walking initiation test, mice were placed in the middle of a square outlined by white cloth tape \((21 \times 21 \text{ cm})\) on a smooth black surface of a large table top, and the time it took each mouse to leave the square \(\text{(place all four paws outside of the tape)}\) was recorded, with a maximum time of 60 sec allowed.

Accelerated rotorod test. Mice were tested for their ability to maintain balance on a rotating rod \((\text{rotorod})\) that gradually increased its rotational speed over time. The protocol consisted of two phases: habituation/adaptation \((3 \text{ d})\) and accelerated rotorod testing \((5 \text{ d})\). During habituation/adaptation, the mice were trained to remain on a stationary rod \((3 \text{ cm diameter})\) set on a rotating apparatus when it was rotating at a constant speed \((2.5 \text{ rpm})\) on days 2 and 3. Training on the rod that rotated at a constant speed involved two daily sessions, each containing three consecutive 1 min trials with a 10 min interval separating the two sessions. Two days after completing habituation, a 2-day period was designated on the accelerating rotorod test, which involved quantifying how long mice could remain on the rod when its rotational speed was accelerated from 2.5 to 20 rpm in 3 min. Each of the five daily test sessions consisted of three 3 min trials, and each trial was separated by 10 min. The time the mice spent on the rod without falling was recorded for each trial.

Morris water navigation task. Mice were trained in a round pool of opaque water to learn the location of a platform that could be used to escape out of the water. The pool was 100 cm in its inner diameter, 20.5 cm deep, was filled to a depth of 17.5 cm with water maintained at a temperature of 24 \(\pm 1^{\circ}\text{C}\), and was located in the center of a room containing many distinct spatial cues. All trials were videotaped with an overhead camera. Mice were first trained on the Fruity Pebbles maze to evaluate working (trial-dependent) memory according to previously published procedures (Wozniak et al., 1990). The food reward that was used during testing \((\text{Fruity Pebbles cereal})\) was introduced into the cage before testing. Each mouse was placed on the platform for 30 sec and then placed into a holding cage for 30 sec. After a pretest acclimation period, during which a mouse was placed on the platform for 60 sec and then placed into a holding cage for 30 sec, the same 60 sec “probe” trial was given to each mouse to determine if it could find the platform. The mice received two consecutive trials followed by a 1 hr rest period and then two more consecutive trials. Probe trials were given at the end of day 4 and 3 d after day 5 of reversal training. Other aspects of reversal training were similar to those used during the probe phase. A total of 20 trials \((5 \text{ blocks of four trials} \text{ of escape latency and path length data as well as data from two probe trials were collected for both the place and the reversal conditions. In addition, mean swimming speed scores were computed for each day within the three test conditions (cued, place, and reversal) by dividing the mean escape latency for a given day by the mean path length for that day. Swimming speed scores were calculated to provide an additional measure for comparing the swimming capabilities of the two groups."

Radial arm maze. Mice were food-restricted to \(\sim 85\%\) of their \text{ad libitum} weight and trained on the win-shift spatial discrimination in the radial arm maze to evaluate working (trial-dependent) memory according to previously published procedures (Wozniak et al., 1990). The food reward that was used during testing \((\text{Fruity Pebbles cereal})\) was introduced into the cage before testing. Each mouse was placed on the platform for 30 sec and then placed into a holding cage for 30 sec. After a pretest acclimation period, during which a mouse was placed on the platform for 60 sec and then placed into a holding cage for 30 sec, the same 60 sec “probe” trial was given to each mouse to determine if it could find the platform. The mice received two consecutive trials followed by a 1 hr rest period and then two more consecutive trials. Probe trials were given at the end of day 4 and 3 d after day 5 of reversal training. Other aspects of reversal training were similar to those used during the probe phase. A total of 20 trials \((5 \text{ blocks of four trials} \text{ of escape latency and path length data as well as data from two probe trials were collected for both the place and the reversal conditions. In addition, mean swimming speed scores were computed for each day within the three test conditions (cued, place, and reversal) by dividing the mean escape latency for a given day by the mean path length for that day. Swimming speed scores were calculated to provide an additional measure for comparing the swimming capabilities of the two groups."

Statistical analyses for the behavioral tests. The data from the behavioral tests were analyzed using ANOVA models. In some instances, one-way ANOVAs were conducted for a given variable or test, e.g., number of correct responses in a given day of a given maze or field of view or number of head dips. In other instances such as for the variables generated in the Morris water navigation or radial arm maze tasks, two-way ANOVAs were used for a given variable that contained one between-subjects variable, group (F2 hybrid CaMKIV/Gr KO mice and WT littermates), and one within-subjects variable, blocks of trials or test days. Simple main effects of group or other contrasts were conducted after a significant main effect of group or a significant group by blocks of trials or test days interactions. \(\alpha\) levels were adjusted for within-subjects variables containing more than two levels using the Huynh-Feldt correction to control for violations of sphericity/compound symmetry.
RESULTS

Generation of CaMKIV/Gr-deficient mice

The CaMKIV/Gr gene includes 13 exons that extend over at least 42 kb of DNA (Sun et al., 1995). It encodes three proteins, the α and β isoforms of CaMKIV/Gr and calspерmin, that are derived by alternative transcriptional initiation and alternative splicing (Ohmstedt et al., 1991; Sun et al., 1995). Our targeting strategy aimed to disrupt CaMKIV/Gr-α and -β expression while sparring that of calspерmin by avoiding targeting of any of the promoter sequences of the gene or calspерmin coding sequences. To this end, a targeting vector was constructed that was designed to replace the 79 bp exon 3 of CaMKIV/Gr (encoding the ATP binding site in the catalytic domain of the kinase) with a phosphoglycerate kinase-neomycin resistance cassette (Fig. 1A). Replacement of CaMKIV/Gr exon III with a neomycin resistance gene was predicted to result in the generation of out of frame, alternatively spliced kinase transcripts that skip the inserted neomycin cassette and encode a catalytically inactive 55 amino acid peptide before terminating at a premature stop codon. On the basis of our previous studies on N-terminal, catalytically inactive CaMKIV/Gr fragments, such a peptide would fail to be expressed in targeted cells because of instability/degradation (Chatila et al., 1996).

Three of 83 G418 and ganciclovir-resistant ES clones underwent homologous recombination as determined by Southern blot analysis using a hybridization probe external to the region of homology contained within the targeting vector. Several male chimeras were generated from two ES clones that transmitted the mutant allele through the germline. Matings of CaMKIV/Gr HET mice resulted in generation of CaMKIV/Gr KO mice (Fig. 1B) in numbers consistent with autosomal recessive Mendelian inheritance (data not shown). KO mice of both sexes were grossly indistinguishable from their WT and HET littermates and were fertile.

Northern blot analysis revealed equivalent levels of CaMKIV/Gr transcripts in brains, testes, and thymi of WT and mutant animals (data not shown). However, RT-PCR analysis using flanking primers confirmed the absence from transcripts of
KO mice of targeted exon III sequence, whereas HET mice manifested both WT and exon III-deficient transcripts (Fig. 1C). Expression of CaMKIV/Gr protein in targeted animals was evaluated by immunoblotting using two different anti-CaMKIV/Gr antibodies: one directed at the C-terminal peptide and the other at the catalytic domain (Fig. 1D). In both cases, CaMKIV/Gr protein expression was found to be totally absent in KO mice and reduced by 50% in HET mice as compared with WT littermates. In contrast, expression of the testis-specific calbindin protein, as detected by the anti-CaMKIV/Gr C-terminal peptide antibody, was normal (Fig. 1D). Similarly, there was normal expression in CaMKIV/Gr KO mice of other protein kinases such as CaMKIIa, ERK1 and 2, RSK-2, and PKA, and of the CaMKIV/Gr substrate CREB (Fig. 1E).

To determine whether CaMKIV/Gr deficiency causes gross neuroanatomical abnormalities, we compared coronal sections from brains of WT and KO mice using different stains (Fig. 2). Histological analysis revealed the gross hippocampal morphology of WT and KO mice to be indistinguishable. Giemsa (Fig. 2A, B) and Nissl (data not shown) staining revealed the cellular composition and orientation of different hippocampal substructures to be comparable in WT and KO mice (Fig. 2A, B). The neocortex showed the normal layered structure (data not shown). Calbindin immunostaining revealed apparently normal mossy fiber projections to the CA3 (Fig. 2C–F). Cerebellar histology appeared grossly normal (Fig. 2G,H), and calbindin immunostaining visualized Purkinje cell dendritic arborization in both WT and KO animals (Fig. 2I,J).

**Impaired LTP in CA1 neurons of CaMKIV/Gr KO mice**

To address the role of CaMKIV/Gr in synaptic plasticity at the Schaffer collateral/CA1 synapse in the hippocampus, we examined two major forms of synaptic plasticity: LTP and LTD. First, we examined the competency of basal synaptic transmission in hippocampal slices of WT and KO mice. Paired-pulse facilitation, a simple form of plasticity, in KO slices was similar to that of WT controls at different intervals measured (WT: n = 15 slices/8 mice; KO: n = 13 slices/9 mice) (Fig. 3A). No obvious difference in baseline EPSPs was observed in slices of WT and KO mice (data not shown). To test whether NMDA receptor-mediated responses may be affected in KO mice, we measured NMDA receptor-mediated field EPSPs in the presence of AMPA/kainate receptor blockade (CNQX, 20 μM). We found that NMDA receptor-mediated responses in hippocampal slices of KO mice were not significantly different from those of WT mice (WT: n = 11 slices/7 mice; KO: n = 5 slices/5 mice) (Fig. 3B).

Next we examined LTP induction in hippocampal slices of WT and KO mice after tetanic stimulation. Strong tetanic stimulation (two trains, 100 Hz for 1 sec/train, delivered at a 20 sec interval) (Zhuo et al., 1999) induced robust and sustained potentiation of synaptic responses (n = 7 slices/7 mice, 241.7 ± 18.5% of control,
comparing 45 min after strong tetanus to before stimulation,

$p$ comparing 45 min after strong tetanus to before stimulation,

$A$ KO mice.

Figure 3.

CaMKIV/Gr KO mice (WT: LTP induced by two-train tetanic stimulation was blocked in slices of traces before and 45 min after stimulation are shown in the insets (min) induced LTD in the CA1 region of the hippocampus from WT mice 5

vals (WT: mediated EPSPs (WT:

$T$ slices/8 mice, 60.2

0.05) (Fig. 3

$C$ was still observed in these slices (Fig. 3

1 Hz stimulation with before the stimulation, $C$

was studied in both WT and knockout mice (Fig. 3

protein synthesis-independent phase (E-LTP) lasting for up to 1 hr and lasts for up to several days (Frey et al., 1988, 1993; Huang and Kandel, 1994; Nguyen et al., 1994). The former can be recapitulated by a single high-frequency tetanic stimulation, whereas the latter requires multiple-train tetanization (Huang and Kandel, 1994). To test whether the defect in synaptic potentiation

in CA1 neurons of KO mice involves both forms of LTP, we examined LTP induction in slices of WT and KO mice during single-train tetanic stimulation (100 Hz for 1 sec) as compared with multiple-train stimulation (four trains, 100 Hz for 1 sec/train, delivered at 5 min intervals) (Huang and Kandel, 1994). Although the amount of potentiation 30 min after a one-train tetanic stimulation in WT and KO mice was not significantly different (WT: $n = 10$ slices/9 mice, 144.1 ± 16.4%; KO: $n = 8$ slices/8 mice, 142.9 ± 13.6%) (Fig. 4A), synaptic potentiation at 60 min induced by four-train stimulations in slices of KO mice was significantly smaller than that of WT mice (WT: $n = 7$ slices/7 mice, 205.0 ± 18.2%; KO: $n = 6$ slices/6 mice, 125.0 ± 7.2%, $p < 0.05$).

As in the case of two-train stimulation, the defect in L-LTP induction in the four-train stimulation paradigm was notable at very early time points, even as repeat tetanization was in progress (Fig. 4B). These results consistently suggest that CaMKIV/Gr is critical for long-term persistence of synaptic potentiation induced by two- or four-train tetanic stimulation of hippocampal slices.

Absence late-phase LTD in cerebellar Purkinje cells of CaMKIV/Gr KO mice

Cerebellar LTD is a cellular model system of information storage that has been suggested to underlie certain forms of motor learning (for review, see Daniel et al., 1998). In cerebellar LTD, a persistent, input-specific attenuation of the parallel fiber–Purkinje neuron synapse is produced when parallel fiber and climbing fiber inputs to a Purkinje neuron are stimulated together at low frequency. Similar to hippocampal LTP, cerebellar LTD has also been demonstrated to be composed of an early, protein synthesis-independent phase lasting for 1–2 hr and a late phase that is protein synthesis dependent and lasts for several hours (Linden, 1996; Murashima and Hirano, 1999). Both CREB and CaMKIV/Gr have been implicated in the establishment of a late phase of cerebellar LTD. Particle-mediated transfection of cultured Purkinje neurons
with an expression vector encoding a dominant inhibitory form of CREB resulted in a nearly complete blockade of the late phase of LTD (Ahn et al., 1999). Also, although inhibitors of PKA or the mitogen-activated protein kinase/ribosomal 56 kinase (MAPK/RSK) cascade were without effect on the late phase, transfection with expression vectors encoding a CaMK inhibitor peptide, dominant negative forms of CaMKIV/Gr, or a calmodulin trap localized to the nucleus produced attenuation of the late phase of LTD.

As a further test of this hypothesis, LTD was assessed in cerebellar cultures prepared from CaMKIV/Gr KO mice. Because Ca$^{2+}$ influx via voltage-gated Ca channels and activation of the glutamate receptor mGluR1 are required for LTD induction, alterations in these signals in the KO could block cerebellar LTD. However, when these functions were measured using bis-fura-2 microfluorimetry, no difference was seen between KO and WT Purkinje neurons (Table 1). Likewise, the amplitude of mEPSCs was unaltered in KO neurons. There were, however, two significant alterations in the basal physiology of KO Purkinje neurons: the frequency of mEPSCs was reduced by ~70%, and $R_{\text{in}}$ was increased by ~250% (Table 1). Although we did not quantitate the morphology of the Purkinje neurons in culture, it appeared as if the dendritic Ca concentration for Purkinje neurons and peak somatic Ca for granule neurons. Depolarization-evoked Ca$_{\text{d}}$ was measured in cells that were incubated in normal (2 mM Ca-containing) external saline and stimulated with a 3 sec depolarizing pulse from -80 to 0 mV. Quisqualate-evoked Ca$_{\text{d}}$, a measure of group I mGluR function, was measured in cells that were incubated in 0 Ca/0.2 EGTA external saline and stimulated with a pressure pulse of 100 $\mu$m quisqualate (dissolved in 0 Ca/0.2 EGTA saline, 4 psi, 2 sec). This pressure pulse delivered agonist to the entire cell. Resting values were measured immediately before stimulation. Depolarization-evoked values were measured as the peak during a 30 sec measuring period after the onset of depolarization, and quisqualate-evoked values were measured as the peak during a 120 sec measuring period after the onset of the pressure pulse. *p < 0.01 compared with WT, Student’s t test.

### Table 1. Effects of CaMKIV/Gr deletion on some basal properties of Purkinje neurons in culture

<table>
<thead>
<tr>
<th>Measure</th>
<th>C57BL6 WT</th>
<th>CaMKIV/Gr KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{in}}$ (MΩ)</td>
<td>175 ± 30</td>
<td>458 ± 55*</td>
</tr>
<tr>
<td>mEPSC frequency (sec)</td>
<td>8.3 ± 2.7</td>
<td>2.7 ± 0.8*</td>
</tr>
<tr>
<td>mEPSC amplitude (pA)</td>
<td>25 ± 6</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Resting Ca$_{\text{d}}$, 2 mM external (nM)</td>
<td>109 ± 17</td>
<td>114 ± 120</td>
</tr>
<tr>
<td>Depolarization-evoked Ca$_{\text{d}}$, (nM)</td>
<td>506 ± 54</td>
<td>483 ± 49</td>
</tr>
<tr>
<td>Resting Ca$_{\text{d}}$, 0 mM external (nM)</td>
<td>32 ± 8</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>Quisqualate-evoked Ca$_{\text{d}}$, (nM)</td>
<td>166 ± 31</td>
<td>175 ± 30</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 10 cells. $R_{\text{in}}$ was determined by measuring the sustained current deflection during a voltage step from -80 to -90 mV. mEPSCs were measured over a 200 sec continuous interval before any additional manipulation (depolarization or quisqualate application). Bis-fura-2 microfluorimetric measurements of depolarization-evoked and quisqualate-evoked Ca$_{\text{d}}$ were conducted in cells separate from those of the LTD experiments. Values are peak proximal dendritic Ca$_{\text{d}}$ concentration in C57BL6 wt. Values were measured immediately before stimulation. Depolarization-evoked Ca$_{\text{d}}$ values were measured as the peak during a 30 sec measuring period after the onset of depolarization, and quisqualate-evoked Ca$_{\text{d}}$ values were measured as the peak during a 120 sec measuring period after the onset of the pressure pulse. *p < 0.01 compared with WT, Student’s t test.

**Figure 5.** A late phase of cerebellar LTD is attenuated in cultured Purkinje neurons derived from CaMKIV/Gr KO mice. After acquisition of baseline responses to glutamate test pulses, glutamate/depolarization conjunction (6 pairings) is applied at $t = 0$ min. A late phase of LTD is induced in C57BL6 wt ($n = 7$) but not CaMKIV/Gr KO ($n = 9$) Purkinje neurons. Doubling the number of conjunctive pairings ($2 \times 12$ pairings) failed to rescue the late phase in CaMKIV/Gr KO Purkinje neurons ($n = 5$). Error bars show the SEM. Representative current traces are from the time points indicated on the graph. Calibration: 30 pA, 2 sec.

**Figure 6.** Decreased basal and depolarization-induced pCREB levels in primary cortical neurons of KO E14 mice relative to WT and HET littersmates. Lysate samples of untreated or KCl-treated (60 mM, 10 min) neurons were immunoblotted for pCREB, total CREB, pERK, total ERK, CaMKIV/Gr, and CaMKIIa, respectively, using specific antibodies. Results are derived from a study performed in a blinded fashion on one litter. Similar results were found in an independent study on another litter.

**Impaired CREB activation in CaMKIV/Gr KO mice**

Because CaMKIV/Gr has been implicated as a major CREB kinase, and because CREB-dependent gene transcription has been implicated in the maintenance of synaptic plasticity, we examined CREB activation in CaMKIV/Gr KO neurons under different stimulation paradigms. First, we examined depolarization-induced pCREB formation in primary cortical neuronal cell cultures derived from E14 WT, HET, and KO littermate fetuses. Figure 6 demonstrates that KO neurons exhibited low basal pCREB levels, which increased modestly during depolarization induced by KCl.
treatment. In contrast, both WT and HET neurons exhibited substantially higher basal pCREB levels, which in turn were markedly upregulated after KCl addition. The deficit in pCREB formation in KO neurons did not reflect lack or dysfunction of other CREB activation pathways, in that ERK kinase activation, monitored by anti-pERK antibody, proceeded equally well in neurons of all three genotypes (Fig. 6).

Next, we examined pCREB formation in hippocampal slices of KO mice and WT littermate controls during treatment with glutamate. Immunohistochemical analysis revealed minimal pCREB staining in untreated WT and KO hippocampal slices. In contrast, glutamate treatment readily induced nuclear pCREB staining in a large number of CA1 neurons of WT slices, whereas only faint staining was observed in a limited number of CA1 neurons of KO slices (Fig. 7A). Similarly defective pCREB formation was also observed at an early time point (15 min) after glutamate treatment (data not shown). c-Fos expression was also examined at 45 min after glutamate treatment and was found to be greatly decreased in CA1 neurons of KO mice compared with controls (Fig. 7B). Image densitometric analysis confirmed the defective formation in glutamate-perfused KO CA1 neurons of both pCREB and c-Fos as compared with WT controls ($p = 0.007$ and $<0.0001$, respectively; $n = 4$ slices/4 animals for each group) (Fig. 7C). These results suggested impaired CREB activation and CREB- and Ca$^{2+}$-dependent gene expression in CA1 neurons of KO mice under conditions simulating excitatory neurotransmission.

To evaluate CREB activation in KO mice in the context of an adaptive behavioral response, we analyzed pCREB staining in KO and WT mice after restraint-stress. This stimulus is associated with immediate-early activation gene expression in several brain regions, including the cerebral cortex and the hippocampus (Mella et al., 1994; Cullinan et al., 1995) and proceeds by an NMDA-dependent mechanism consistent with activation of Ca$^{2+}$-dependent gene expression (Titze-de-Almeida et al., 1994; Bozas et al., 1997). WT animals subjected to 30 min of restraint-stress exhibited heightened pCREB staining in several brain regions, including the hippocampus (dentate, CA1, and CA3 areas) and cerebral cortex, relative to unrestrained animals (Fig. 8A). pCREB staining was most prominent immediately after the restraint-stress period and declined by 2 hr thereafter (Fig. 8A). In contrast, pCREB immunoreactivity after restraint-stress was consistently and significantly decreased in cortical neurons ($p < 0.0001$; $n = 3$ mice each for WT and KO groups) and in hippocampal CA1 neurons ($p = 0.0038$) of KO mice as compared with WT littermate controls (Fig. 8A,C). pCREB immunoreactivity was also decreased in CA3 neurons, but this decrease was marginally nonsignificant ($p = 0.083$). Expression of c-Fos was also examined as a marker of Ca$^{2+}$- and CREB-dependent gene expression. c-Fos immunoreactivity was observed in several brain regions of restraint-stressed WT mice, including cerebral cortex and hippocampus, but was profoundly deficient in KO mice (Fig. 8B). Neurons of the somatosensory cortex of KO mice appeared to be the most severely affected, with virtually no c-Fos immunoreactivity observed in these cells as compared with their counterparts in WT littermates (Fig. 8B). The impact of CaMKIV/Gr deficiency was region-specific in that no significant differences in pCREB expression were observed in other brain regions, such as the amygdala (Fig. 8C). These results identified CaMKIV/Gr as a physiologically relevant CREB kinase that may operate in a brain region-specific manner.

**CaMKIV/Gr KO mice exhibit normal locomotion and sensorimotor functions but decreased walking initiation**

To characterize the behavioral phenotype of CaMKIV/Gr KO mice, they and control WT littermates were first evaluated on a 1 hr locomotor activity test and a battery of sensorimotor tests, including ledge, platform, inclined screen, and walking initiation (Brosnan-Watters et al., 1996; Chiesa et al., 1998). Results of ANOVAs conducted on test variables indicated that the groups performed similarly on the 1 hr locomotor activity, ledge, platform, and inclined screen tests (data not shown). However, they differed significantly in their performance on the walking initiation test ($F(1,24) = 8.51, p = 0.008$) (Fig. 9A). Subsequent contrasts indicated that the KO mice took significantly longer to leave the square compared with the WT mice on the first test session, $F(1,24) = 4.49, p = 0.045$. The KO mice also took longer to leave the square on test session 2, but these differences only approached significance $F(1,24) = 3.50, p = 0.074$.

The general coordination and motor skills of KO mice were further evaluated by testing them on a rotating rod (rotorod) that gradually increased its rotational speed over time. During the first (habituation/adaptation) phase of training, the KO and WT groups performed equally well in learning how to maintain their balance,
first on a stationary rod and then on the same rod that was rotated at a slow constant speed (data not shown). The data from the accelerating rod condition are shown in Figure 9B and reveal that the two groups performed similarly during the accelerating rod condition. The performance of the two groups gradually improved over the 5 d test period, indicative of enhancement of motor skills with practice. This was confirmed by an ANOVA of these data that yielded a nonsignificant main effect of group and a significant effect of test days ($F_{(4,76)} = 4.61, p = 0.008$).

CaMKIV/Gr KO mice are not impaired in tests of spatial memory

Because impaired LTP in CA1 neurons has frequently been associated with poor spatial memory, we examined the performance of CaMKIV/Gr KO mice in two tests of spatial learning and memory: the Morris water maze and the eight-arm radial maze tests (Olton et al., 1978; Morris et al., 1982). The Morris water navigation task was used to evaluate spatial reference memory during cued (visible platform), place (submerged platform), and reversal trials (submerged platform in a different location). Cued training was also included to determine whether nonassociative factors were likely to confound interpretation of spatial learning/memory performance. To enhance the sensitivity of the test, we increased the degree of difficulty of the reversal trials relative to that of the place trials by substantially increasing the interval between the second and third trials from ~35 sec to 1 hr.

Figure 10 depicts the escape latency ($A$) and path length data ($B$) as a function of blocks of trials for the cued, place, and reversal trials. Results of ANOVAs conducted on these data revealed that the KO mice and WT littermate controls performed similarly on all three types of trials. Both groups showing steep learning curves on the cued and reversal trials, indicating substantial improvement over trial blocks for both latency and path length. In addition, the results of analyses on mean daily swimming speed scores showed that the KO and WT mice did not differ on this variable during cued, place, or reversal trials. These findings further substantiate the escape latency and path length data from the cued trials...
crossings) than the WT mice ($F_{1,24} = 5.20, p = 0.032$) (Fig. 10C). The KO mice also tended to perform better than the WT mice with respect to the time spent in the target quadrant with the differences being marginally nonsignificant ($F_{1,24} = 3.90, p = 0.060$) (Fig. 10D).

The second probe trial associated with reversal training was conducted 3 d after the last reversal trial to make the probe test more difficult by increasing the retention interval between the last training trial and the subsequent (second) probe trial. When the mice were tested on the second probe trial associated with reversal training, the KO and WT mice performed very similarly with regard to both platform crossings and time spent in the target quadrant (Fig. 10C,D).

We next tested CaMKIV/Gr KO mice and WT littermate controls on a win-shift spatial discrimination in the radial arm maze, which provided a measure of working (trial-dependent) memory capacities (Olton et al., 1978). We also evaluated neophobia (avoidance of a familiar food in a novel environment) and the time required to reach a criterion signifying “habituation” to assess the possibility that differences in emotionality between the groups might affect acquisition performance. Figure 11A shows that the KO mice took longer to begin eating in the radial maze compared with the WT mice on both test days. Results of the two-way ANOVA on the neophobia data indicated that there was a marginally nonsignificant main effect of group ($F_{1,22} = 4.18, p = 0.053$), a significant main effect of test day ($F_{1,22} = 7.26, p = 0.013$), and a nonsignificant group by test day interaction. KO mice took significantly longer to begin eating on test day 2 ($F_{1,22} = 5.49, p = 0.029$), whereas differences observed on test day 1 were nonsignificant. Data pertaining to habituation and acquisition are depicted in Figure 11B–D, which shows that the habituation and acquisition performance of the two groups was very similar. The WT and KO mice did not differ in terms of the number of days required to reach the habituation or acquisition criteria or on the number of errors (retracing) to criterion during acquisition.

**DISCUSSION**

**CaMKIV/Gr and synaptic plasticity**

By using mutant mice derived by targeted gene disruption, an important role for CaMKIV/Gr was demonstrated in hippocampal and cerebellar synaptic plasticity and in neuronal CREB activation. In CA1 neurons, LTP formation depends on Ca$^{2+}$ influx via NMDA receptor channels. Although the function of these channels in KO mice appeared intact, nevertheless at least some of the downstream Ca$^{2+}$-dependent cellular signaling cascades were affected by CaMKIV/Gr deficiency, as evidenced by failure to induce sustained potentiation (L-LTP) after multiple-train high-frequency tetanization and by impaired CREB activation and Ca$^{2+}$-dependent gene transcription. In contrast, a transient form of synaptic potentiation (E-LTP) induced by a single tetanization was unimpaired. On the basis of previous studies documenting the dependence of L-LTP but not E-LTP on CREB activation and on de novo protein synthesis, defective CREB activation and Ca$^{2+}$- and CREB-dependent gene transcription may contribute to the failure of LTP to persist in repeatedly tetanized CA1 neurons of KO mice (Frey et al., 1988, 1993; Bourc'his et al., 1994; Huang and Kandel, 1994). However, it should be noted that the defect in L-LTP induction is notable very early (within the first 5 min) after both two- and four-train tetanization, at which time gene expression is unlikely to be involved in LTP formation. This would suggest a contribution by CaMKIV/Gr to early events in L-LTP formation independent of Ca$^{2+}$ influx-triggered gene expression.

One nontranscriptional mechanism by which CaMKIV/Gr may contribute to L-LTP could involve phosphorylation by resident kinase molecules at postsynaptic densities of ion channels, cytoskeletal proteins, and their regulators. A precedent for ion channel phosphorylation as a contributing mechanism to LTP formation is provided by the case of CaMKII, which phosphorylates Ser-831 of GluRI subunits of AMPA channels to promote channel conductance (Barria et al., 1997a,b; Derkach et al., 1999). Modulation of...
cytoskeletal protein function provides another mechanism by which CaMK may stabilize dendritic spines at synapses. The previous demonstration that CaMKIV/Gr promotes microtubule assembly by phosphorylating the microtubule regulator stathmin/OP18 illustrates one potential mechanism by which CaMKIV/Gr may regulate synaptic architecture and promote synaptic plasticity (Melander Gradin et al., 1997).

The finding that the late phase of cerebellar LTD was suppressed in cultured Purkinje neurons derived from CaMKIV/Gr KO mice suggests that CaMKIV/Gr is necessary for the expression of the late phase. This is consistent with previous findings that the late phase of cerebellar LTD could be blocked by nonspecific CaMK inhibitors (which do not distinguish between CaMKII and CaMKIV), two different dominant-negative CaMKIV constructs, or expression of a nuclear calmodulin trap (Ahn et al., 1999). It is also consistent with the previous demonstration that the late phase of cerebellar LTD is CREB dependent (Ahn et al., 1999). However, there are some limitations to the interpretation of these results that should be noted. First, some aspects of the basal physiology of the *in vitro* cultured CaMKIV/Gr KO Purkinje neurons are clearly abnormal. The frequency of mEPSCs is reduced and *R*_{input} is increased, both consistent with a reduction in the extent of the dendritic arbor of *in vitro* cultured Purkinje neurons. Although there is no particular reason to believe that the *in vitro* alterations suppress a late phase of LTD through nonspecific mechanisms, this possibility cannot be ignored entirely. Second, although CaMKIV/Gr is strongly expressed in the nucleus of neonatal Purkinje neurons, adult Purkinje neurons express CaMKIV/Gr only weakly (Sakagami et al., 1992; Sakagami and Kondo, 1993a), suggesting that this pathway may not necessarily underlie a late phase of cerebellar LTD throughout the life span.

CaMKIV/Gr, pCREB, and Ca^{2+}-dependent gene transcription

The demonstration of profoundly impaired CREB activation and Ca^{2+}-dependent gene transcription in some brain subregions of CaMKIV/Gr KO mice, including the hippocampus and cerebral cortex, is consistent with a prominent role played by CaMKIV/Gr in mediating these activation events. Nevertheless, CaMKIV/Gr is but one of several activators of CREB implicated in neuronal plasticity and adaptive behavioral responses, raising the issue of unique and redundant functions of the respective kinase. The differential impact of CaMKIV/Gr deficiency on CREB phosphorylation in brain regions of restraint-stressed animals suggests that under at least some conditions CREB activators such as CaMKIV/Gr may be used selectively in a tissue-specific manner. It is also possible that different CREB activators may function over distinct time periods. For example, it has been suggested previously that CaMKIV/Gr, by virtue of its constitutive nuclear expression and its amenability to activation by fast-moving Ca^{2+} waves generated at distal synapses, may drive fast-onset CREB activation, whereas other activators such as cAMP/PKA or cascading

Figure 10. CaMKIV/Gr KO mice performed similarly to WT littermates on several aspects of the Morris water navigation task. A, B, Acquisition performance in terms of escape latency and path length is depicted for the cued, place, and reversal conditions as a function of blocks of trials. No differences were found between the groups during any of these conditions with regard to either escape latency (A) or path length (B). C, D, Retention performance on probe trials is shown. During the probe trials the mice were placed in the pool with the platform removed, and aspects of searching behavior were quantified over a 60 sec period. During place training, no differences were found between KO and WT mice in terms of the number of times the mice swam over the former platform location (C, PLATFORM CROSSINGS) or in the amount of time the mice spent searching in the quadrant where the platform used to be located (D, TARGET QUADRANT TIME). However, the CaMKIV/Gr KO mice exhibited a significantly (*) greater number of platform crossings on the first probe trial during reversal training (p = 0.03) and also tended to spend more time in the target quadrant (D), although the difference was marginally nonsignificant (p = 0.06). In D the dotted line represents the amount of time spent in the target quadrant that would be expected on the basis of chance alone. In addition, † signifies that the second probe trial associated with the reversal condition actually occurred 3 d after the completion of reversal training.
Figure 11. Performance of CaMKIV/Gr and WT mice on the radial arm maze. A. Neophobia. CaMKIV/Gr KO mice tended to take longer to begin eating the food reward in the radial arm maze during the first 2 d of habituation compared with the WT littermate controls ($F_{1,22} = 4.18$, $p = 0.053$). Pairwise comparisons conducted within each test day indicated that the CaMKIV/Gr mice took significantly (*) longer to begin eating in the maze on day 2 relative to the WT mice ($F_{1,22} = 5.49$, $p = 0.029$). B. Habituation. Although the CaMKIV/Gr-deficient mice were more reluctant to eat the food reward during the early portions of the habituation compared with the WT mice, the two groups required similar amounts of time to reach criterion for terminating habituation. C, D. Acquisition results. The acquisition performance was not significantly different between the groups in terms of either days or errors to criterion (C). The acquisition performance of the two groups of mice over time is depicted in D, which shows that groups exhibited similar rates of learning in terms of the cumulative percentage of mice reaching criterion as a function of blocks of 6 d.

mitogen-activated protein kinases may activate CREB more slowly (Finkbeiner et al., 1997). Comparative studies on spatial and temporal aspects of CREB activation in animals lacking different CREB activators (and combinations thereof) may provide further insight into unique, redundant, and integrated functions of these pathways.

CaMKIV/Gr in learning and memory

Although LTP formation, CREB activation, and Ca$^{2+}$-dependent gene transcription were all impaired in CA1 neurons of CaMKIV/Gr KO mice, there was no evidence of disturbed spatial learning in the CaMKIV/Gr KO mice compared with WT controls. A relationship between CA1 LTP and spatial memory was surmised from studies on NMDA receptor- and CaMKII-deficient mice, and more recently from studies on mice deficient in other molecules involved in LTP formation such as TrkB (Silva et al., 1992a,b; Tsien et al., 1996; Minichiello et al., 1999). However, deficiency of other molecules, such as AMPA channels, that results in LTP deficiency is not associated with impaired learning in the Morris water maze (Zamanillo et al., 1999). It is possible that the short- and long-term forms of LTP spared by the mutation are sufficient to support spatial learning by CaMKIV/Gr KO mice. An alternative explanation, suggested by the findings of Zamanillo et al. (1999), may be that LTP is not necessary for spatial learning and memory in the context of the Morris water maze and the radial arm maze tasks. In the case of CREB, it is possible that CREB phosphorylation may still proceed during spatial memory induction by pathways other than those invoked in our pCREB studies (KCl depolarization, glutamate stimulation, restraint-stress). An alternative explanation that we favor is that residual CREB activation in CA1 neurons of CaMKIV/Gr KO mice may be sufficient to rescue spatial memory. This would be consistent with the finding that the impact of CREB deficiency on LTM is gene dosage dependent (Gass et al., 1998). If so, then it could be predicted that combined deficiency of CaMKIV/Gr and other Ca$^{2+}$-regulated CREB activating pathways such as ACI or ACVIII, the deficiency of which does not perturb spatial memory (Wong et al., 1999), may sufficiently compromise CREB activation to precipitate spatial memory defects.

Although testing on the Morris water navigation task revealed no evidence of impaired spatial learning or memory in CaMKIV/Gr KO mice, it should be noted that other investigators have reported that certain training conditions may reveal impairments in spatial learning in mice that have CREB deficits (Bourtchuladze et al., 1994; Kogan et al., 1997; Gass et al., 1998). Kogan et al. (1997) reported that the inter-trial interval was an important parameter for demonstrating place learning deficits in CREB mutant mice because impairment was demonstrable when the inter-trial interval was 1 min but not when it was 10 min in duration. However, it is unlikely that this factor can account for the lack of impairment observed in CaMKIV/Gr KO mice during place training on the water navigation task in the present study because a very short inter-trial interval (35 sec) was used.

In addition to their apparently intact spatial memory, CaMKIV/Gr KO mice were also found to be no different from WT controls in terms of general coordination and motor skills. This was attested to by the performance of KO mice in the ledge, plank, platform, and rotord tests as well as the lack of differences in swimming speeds and cued learning performance during the Morris water navigation task. KO mice also demonstrated improved performance on the rotord test as a function of practice, suggesting that at least some forms of motor learning are intact. Nevertheless, the absence in KO mice of a late form of cerebellar LTD
suggests that CaMKIV/Gr may contribute to aspects of motor learning that are dependent on cerebellar LTD formation, such as associative eye blink conditioning and adaptation of the vestibulo-ocular reflex (Daniel et al., 1998). Further studies will be required to determine the role of CaMKIV/Gr in cerebellar LTD-dependent motor learning paradigms.

Finally, impaired performance of CaMKIV/Gr KO mice on the walking initiation test and their failure to rapidly habituate to the fear of eating in a novel environment (neophobia) in the radial arm maze test suggest altered emotionality of the mutant mice compared with their littermate controls (Wozniak et al., 1990; Brosnan-Watters et al., 1996). In light of this premise, it will be interesting to explore the possibility that these mice are impaired on learning tasks that contain a component relating to changes in emotionality.

REFERENCES


