

Role of Hippocampal $\text{Ca}_v1.2$ Ca^{2+} Channels in NMDA Receptor-Independent Synaptic Plasticity and Spatial Memory

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Current knowledge about the molecular mechanisms of NMDA receptor (NMDAR)-independent long-term potentiation (LTP) in the hippocampus and its function for memory formation in the behaving animal is limited. NMDAR-independent LTP in the CA1 region is thought to require activity of postsynaptic L-type voltage-dependent Ca^{2+} channels ($\text{Ca}_v1.x$), but the underlying channel isoform remains unknown. We evaluated the function of the $\text{Ca}_v1.2$ L-type Ca^{2+} channel for spatial learning, synaptic plasticity, and triggering of learning-associated biochemical processes using a mouse line with an inactivation of the *CACNA1C* ($\text{Ca}_v1.2$) gene in the hippocampus and neocortex ($\text{Ca}_v1.2^{\text{HCKO}}$). This model shows (1) a selective loss of protein synthesis-dependent NMDAR-independent Schaffer collateral/CA1 late-phase LTP (L-LTP), (2) a severe impairment of hippocampus-dependent spatial memory, and (3) decreased activation of the mitogen-activated protein kinase (MAPK) pathway and reduced cAMP response element (CRE)-dependent transcription in CA1 pyramidal neurons. Our results provide strong evidence for a role of L-type Ca^{2+} channel-dependent, NMDAR-independent hippocampal L-LTP in the formation of spatial memory in the behaving animal and for a function of the MAPK/CREB (CRE-binding protein) signaling cascade in linking $\text{Ca}_v1.2$ channel-mediated Ca^{2+} influx to either process.

Key words: calcium channels; learning; memory; NMDA receptor independent; $\text{Ca}_v1.2$; synaptic plasticity

Introduction

Glutamatergic synapses in the CNS can undergo activity-dependent long-lasting changes of synaptic efficacy such as long-term potentiation (LTP) (Bliss and Lomo, 1973). The mechanisms involved in these synaptic modifications are thought to give insight into the molecular processes underlying memory in the behaving animal and, ultimately, in humans (Bliss and Collingridge, 1993; Kandel, 2001; Martin and Morris, 2002; Malenka and Bear, 2004). Depending on the pattern of neuronal stimulation, LTP consists of an early phase (E-LTP) that requires covalent protein modifications and a late phase (L-LTP) that is protein synthesis dependent (Frey et al., 1988). Specifically, L-LTP is believed to be a cellular equivalent of long-lasting memory (Kandel, 2001). In line with this view, inhibition of protein synthesis

not only blocks hippocampal L-LTP but also impairs hippocampus-dependent learning. Both hippocampal synaptic plasticity and various forms of learning-related behavior critically depend on the mitogen-activated protein kinase (MAPK)/extracellular signal-related protein kinase (ERK) cascade and the resulting stimulation of gene transcription and *de novo* protein synthesis [e.g., via cAMP response element-binding protein (CREB)] (English and Sweatt, 1997; Atkins et al., 1998; Hardingham et al., 2001; Kandel, 2001; Wu et al., 2001; Pittenger et al., 2002; Thomas and Huganir, 2004). Induction of L-LTP at Schaffer collateral/CA1 synapses, as well as activation of the ERK/CREB pathway in hippocampal CA1 neurons, requires an increase in the postsynaptic intracellular Ca^{2+} concentration (Shaywitz and Greenberg, 1999; Kandel, 2001). Ca^{2+} influx via L-type Ca^{2+} ($\text{Ca}_v1.x$) channels can specifically trigger the transcription of Ca^{2+} -regulated genes (e.g., *Zif/268*) and brain-derived neurotrophic factor (BDNF), which play a major role in learning (Murphy et al., 1991; West et al., 2001). Ca^{2+} influx via postsynaptic $\text{Ca}_v1.x$ channels can also support a form of NMDA receptor (NMDAR)-independent LTP (Grover and Teyler, 1990; Grover, 1998; Morgan and Teyler, 1999) and sustained CREB phosphorylation with subsequent activation of cAMP response element (CRE)-dependent gene expression in hippocampal neurons (Impey et al., 1996; Dolmetsch et al., 2001). However, the functional significance of these findings for memory formation

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remains unclear, because compelling evidence for a role of L-type Ca²⁺ channel-dependent, NMDAR-independent synaptic plasticity in the behaving animal is missing.

Hippocampal pyramidal neurons express predominantly the Ca_v1.2 channel and only rather low levels of the Ca_v1.3 isoform (Hell et al., 1993; Davare et al., 2001; Sinnegger-Brauns et al., 2004). Accordingly, a knock-out mouse model lacking the Ca_v1.3 channel showed neither a defect in hippocampus-dependent learning nor a defect in hippocampal LTP (Clark et al., 2003). To investigate their role in hippocampal LTP and memory formation, we generated a mouse line (Ca_v1.2^{HCKO}) with an inactivation of the *CACNA1C* (Ca_v1.2) gene, mainly in the hippocampus and neocortex.

Here, we report that Ca_v1.2^{HCKO} mice show a defect in protein synthesis-dependent, NMDAR-independent LTP in the CA1 region that is paralleled by a deficit in spatial learning and an impairment of CREB activation. These findings demonstrate that Ca_v1.2 L-type Ca²⁺ channels serve a critical function in hippocampus-dependent spatial memory by coupling NMDAR-independent synaptic activity to transcriptional events, which are thought to be molecular prerequisites for L-LTP and learning.

Materials and Methods

Conditional inactivation of the Ca_v1.2 gene. The generation of mice lacking Ca_v1.2 channels is described in supplemental Methods (available at www.jneurosci.org as supplemental material).

Behavior. Animals were maintained on a 12 h light/dark cycle and had access to water and food *ad libitum*. Litter-matched male mice of each genotype at the age of 8–15 weeks were used in all experiments. Experiments were performed with the investigator unaware of the genotype. Behavioral analysis was done as described previously (Kleppisch et al., 2003) and is delineated in detail in supplemental Methods (available at www.jneurosci.org as supplemental material).

Field EPSP recordings. Field EPSPs (fEPSPs) and whole-cell currents from CA1 pyramidal neurons were recorded in transverse hippocampal slices using the Axoclamp 2B amplifier (Molecular Devices, Union City, CA), and an EPC9 and the Pulse software (HEKA, Lambrecht/Pfalz, Germany), respectively, and a submersion-type recording chamber as described previously (Kleppisch et al., 2003). Experiments are delineated in detail in supplemental Methods (available at www.jneurosci.org as supplemental material).

Western blot analysis. Western blotting was performed using standard methods. Briefly, brain homogenates were separated on an SDS-PAGE, blotted on a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with Ca_v1.2- and Ca_v1.3-specific antibodies (Moosmang et al., 2003), antibodies against CREB and phospho-CREB (pCREB) Ser¹³³ (Upstate Biotechnology, Lake Placid, NY), and ERK1/2 (Upstate Biotechnology) and phospho-ERK (pERK) Thr²⁰²/Tyr²⁰⁴ (Cell Signaling, Beverly, MA, and Promega, Madison, WI), respectively. Equal loading of slots was ascertained by the use of a polyclonal anti-cGMP-dependent kinase I antibody. Antibodies were visualized by the ECL system (NEN, Boston, MA). For densitometry, blots were digitized and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

Agonist treatment of perfused hippocampal slices. Transverse 400 μm hippocampal slices were continuously perfused with oxygenated artificial CSF (aCSF) at 32°C for 2–3 h before the addition of tetraethylammonium chloride (TEA-Cl) (Sigma, St. Louis, MO). D-APV (50 μM; Sigma) or 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)-butadiene (U0126) (40 μM; Tocris, Ballwin, MO) were added to the perfusate 1 h before and during the stimulation.

Immunocytochemistry. Hippocampal slices were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, and sectioned at 8 μm intervals. Immunocytochemistry for β-galactosidase was performed using standard procedures with a rabbit anti-β-galactosidase antibody at 1:1000 dilution (Cappel, Cochranville, PA) and Cy3 goat-anti-rabbit IgG at 2 μg/ml (Jackson Immunochemicals, West Grove, PA). For immunocytochemistry of phospho-CREB and phospho-ERK1/2, slices were fixed in

ice-cold PBS containing 4% paraformaldehyde for 30 min after the stimulation. A dual-label procedure was used: antibodies were used at 1:1000 dilution and the DNA stain/nuclear marker Hoechst 33342 was used at 200 μg/ml (Sigma). Images were captured on a Zeiss (Oberkochen, Germany) LSM 510 laser scanning confocal microscope.

Quantification of immunocytochemistry. Quantification was performed with Zeiss image analysis software. Briefly, integrated pixel intensity was measured in the desired region. A constant pixel area was used for 12 independent measurements, which were averaged. The averaged integrated pixel intensity for groups of slices was statistically analyzed. Imaging and quantitation were conducted in a blind manner.

Statistics. Data are expressed as mean ± SEM. Statistical evaluations were performed by using ANOVA (repeated measures), and *t* tests were used to assess differences among individual time points.

Results

Regional inactivation of the *CACNA1C* gene in the murine hippocampus

We used the Cre recombinase system, using Nex-Cre transgenic mice (Schwab et al., 2000), to create a mouse line (Ca_v1.2^{HCKO} mice) with an inactivation of the *CACNA1C* gene in the cerebral cortex and hippocampus (see supplemental Results and supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

CA1 pyramidal cells in Ca_v1.2^{HCKO} mice lack Ca_v1.2 L-type Ca²⁺ currents

CA1 pyramidal cells of hippocampal slices from adult control and Ca_v1.2^{HCKO} mice showed robust whole-cell Ca²⁺ inward currents at test potentials positive to −40 mV (Fig. 1*a*). To demonstrate the loss of functional Ca_v1.2 channels, we assessed the dihydropyridine (DHP)-sensitive Ca²⁺ inward currents evoked during voltage-clamp ramps (Fig. 1*d–f*). When the DHP isradipine (10 μM) was added to the bath perfusion after reaching a stable baseline current, we observed a prominent decrease of the Ca²⁺ current amplitude in CA1 neurons from control mice. In sharp contrast, isradipine had virtually no effect on Ca²⁺ currents in CA1 neurons of Ca_v1.2^{HCKO} mice. On average, the portion of the peak Ca²⁺ inward current inhibited by isradipine was 22.3 ± 2.8% (control; *n* = 14) and 4.4 ± 1.8% (Ca_v1.2^{HCKO}; *n* = 9). This is equivalent to >80% reduction of the DHP-sensitive current in CA1 neurons of the mutant mice (*p* < 0.001). The tiny residual DHP-sensitive current is likely caused by the Ca_v1.3 L-type channel. Magee et al. (1996) have suggested that a population of DHP-sensitive Ca²⁺ channels in CA1 pyramidal cells may be active under physiological conditions at potentials as hyperpolarized as −70 mV. Therefore, we tested for a possible impact of the Ca_v1.2 channel knock-out on resting membrane potential (RP) and input resistance (R_N) at RP. Neither parameter was significantly altered in CA1 pyramidal cells of mutant mice (Fig. 1*b,c*). Control and Ca_v1.2^{HCKO} mice exhibited an RP of −70.8 ± 0.9 mV (*n* = 13) and −69.9 ± 1.0 mV (*n* = 10), respectively. Corresponding R_N were 92 ± 8 MΩ (*n* = 8) and 107 ± 12 MΩ (*n* = 6), respectively.

In summary, the data above demonstrate an efficient deletion of the *CACNA1C* gene in hippocampal CA1 neurons of Ca_v1.2^{HCKO} mice without affecting RP and R_N at rest.

NMDAR-independent hippocampal synaptic plasticity critically depends on the function of the Ca_v1.2 Ca²⁺ channel

To evaluate the role of Ca_v1.2 channels in synaptic plasticity, we studied NMDAR-dependent and NMDAR-independent forms of LTP. Initially, we compared basal synaptic transmission in hippocampal slices from control and Ca_v1.2^{HCKO} mice. We ob-

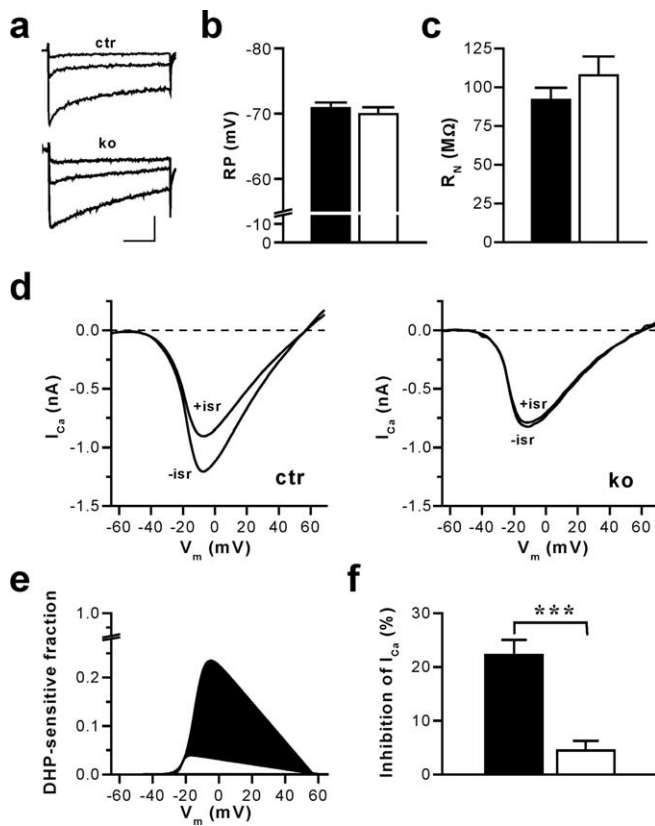


Figure 1. CA1 pyramidal cells of mice with an inactivation of the *CACNA1C* gene (in $Ca_v1.2^{HCKO}$) in the hippocampus display a nearly complete loss of L-type Ca^{2+} currents without changes in RP and R_N . **a**, Ca^{2+} channel currents evoked by voltage-clamp steps in pyramidal neurons of hippocampal slices from control (ctr) and $Ca_v1.2^{HCKO}$ (ko) mice. The traces illustrated correspond to test potentials of -30 , -20 , and -10 mV. The holding potential was -65 mV, and 2 mM Ca^{2+} was used as a charge carrier. Calibration: 50 ms, 100 pA. **b**, Average RP measured in CA1 neurons of hippocampal slices from control (■) ($n = 13$ cells from 6 mice) and $Ca_v1.2^{HCKO}$ (□) ($n = 10$ cells from 6 mice) mice in the current-clamp mode. Means \pm SEM are shown. **c**, Average R_N of hippocampal CA1 neurons from control (■) ($n = 8$ cells from 5 mice) and $Ca_v1.2^{HCKO}$ (□) ($n = 6$ cells, 5 mice) mice determined by injecting hyperpolarizing currents from a membrane potential of -70 mV. Means \pm SEM are shown. **d**, Representative examples of whole-cell Ca^{2+} currents (I_{Ca}) evoked by voltage-clamp ramps from -80 to $+80$ mV (0.5 ms/mV) in hippocampal CA1 neurons from ctr and ko mice before ($-isr$) and after ($+isr$) application of the L-type channel blocker isradipine ($10 \mu M$). The holding potential was -65 mV, and 2 mM Ca^{2+} was used as a charge carrier. **e**, Fraction of the total voltage-dependent Ca^{2+} inward current blocked by isradipine for the representative examples shown in **d**. Fitted $I-V$ curves of the DHP-sensitive current component in control (filled in black) and $Ca_v1.2^{HCKO}$ (filled in white) mice, normalized to the corresponding peak inward current in the absence of isradipine (for details on fit function, see Materials and Methods), are represented. **f**, Average component of the total voltage-dependent Ca^{2+} current block by isradipine. The reduction of the peak inward current in hippocampal CA1 neurons from control (■) ($n = 14$ cells from 6 mice) and $Ca_v1.2^{HCKO}$ (□) ($n = 9$ from 5 mice) mice, shown in percentage, is represented. Data are means \pm SEM ($***p < 0.001$).

served no difference between the two genotypes in the dependency of the fEPSP slope on the stimulus intensity (Fig. 2a) and the fiber volley amplitude (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Paired-pulse facilitation (PPF), a form of short-term plasticity thought to rely on presynaptic mechanisms, was also normal in the $Ca_v1.2^{HCKO}$ mice (Fig. 2b). Moreover, $Ca_v1.2^{HCKO}$ mice showed normal LTP in response to a weak theta burst (Fig. 2c), which is known to induce NMDAR-dependent LTP (Bliss and Collingridge, 1993; Kleppisch et al., 1999). Collectively, these findings show that mutant mice lack a general defect in basal synaptic transmission and have normal NMDAR-dependent LTP.

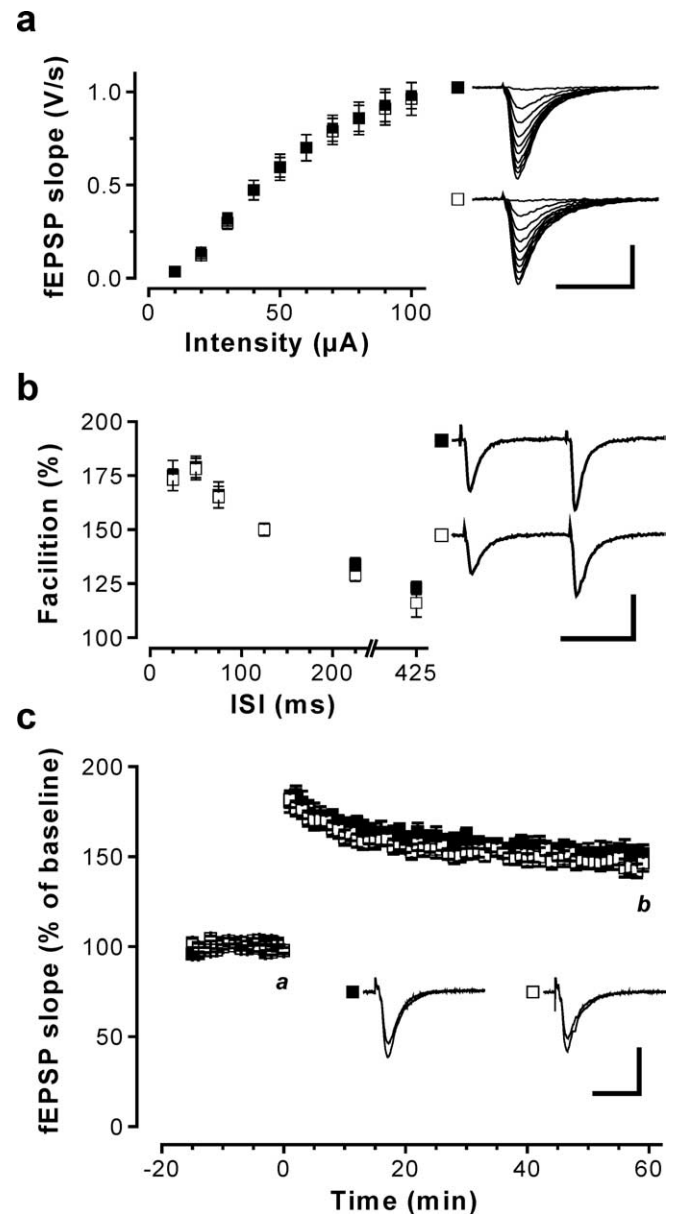


Figure 2. Basal synaptic transmission and NMDAR-dependent synaptic plasticity in the hippocampal CA1 region of $Ca_v1.2^{HCKO}$ mice. fEPSPs in response to stimulation of the Schaffer collaterals were recorded in slices from control (■) and $Ca_v1.2^{HCKO}$ (□) mice. **a**, Input–output relationship curves obtained from control (37 slices from 16 mice) and $Ca_v1.2^{HCKO}$ mice (41 slices from 21 mice) were not different. Representative recordings are shown on the right (calibration: 20 ms, 1 mV). **b**, Short-term plasticity was assessed by measuring PPF at interstimulus intervals (ISIs) of 25 , 50 , 75 , 125 , 225 , and 425 ms. PPF was not altered in control ($n = 27$ slices from 12 mice) compared with $Ca_v1.2^{HCKO}$ mice ($n = 25$ slices from 13 mice). Representative PPF recordings for the 75 ms interval are shown on the right (calibration: 50 ms, 1 mV). **c**, NMDAR-dependent Schaffer collateral/CA1 LTP was induced by theta-burst stimulation (12 times 4 pulses at 100 Hz; 200 ms pause) applied at time 0 . The time course of the fEPSP slope (mean \pm SEM) was obtained from control (8 slices from 3 mice) and $Ca_v1.2^{HCKO}$ (9 slices from 4 mice) slices. Representative fEPSPs recorded at the times indicated (**a**, **b**) are shown in the corresponding inset. Calibration: 20 ms, 1 mV. Error bars indicate SE.

Contrary to the situation in NMDAR-dependent synaptic plasticity, the $Ca_v1.2$ Ca^{2+} channel might be critical for NMDAR-independent synaptic plasticity. Robust NMDAR-independent LTP can be induced using 200 Hz tetanus (Grover and Teyler, 1990). Taking into account their postsynaptic localization, a recent report suggests that activation of $Ca_v1.x$ channels by 200 Hz tetanic stimulation triggers a retrograde signal, leading

to presynaptic BDNF release and, ultimately, the induction of a presynaptic component of LTP (Zakharenko et al., 2003). Indeed, after a 200 Hz tetanus, $Ca_v1.2^{HCKO}$ mice showed significantly reduced LTP compared with control mice (factor genotype, $F = 4.72$; $p = 0.048$; ANOVA); ~ 2 h after the tetanus, fEPSP slope was 136.5 ± 6.5 ($n = 7$) versus 167.6 ± 10.5 ($n = 10$). To explore whether this decrease in LTP was caused by a reduction of the NMDAR-independent component, we further examined 200 Hz LTP in the presence of the NMDAR antagonist APV ($50 \mu M$). Under these conditions, again, $Ca_v1.2^{HCKO}$ showed significantly decreased LTP (factor genotype, $F = 7.55$; $p = 0.018$; ANOVA) (Fig. 3a). Moreover, the extent of the reduction in LTP in the presence of APV was nearly identical to that without APV, arguing for a selective defect in NMDAR-independent LTP in $Ca_v1.2^{HCKO}$ mice. To further substantiate the evidence for a specific role of $Ca_v1.2$ channels in NMDAR-independent LTP, we used multiple 100 Hz tetanic stimulation (Impey et al., 1996) and examined L-LTP in the presence of APV for up to 4 h after the tetanus. Corroborating the results presented above, significant L-LTP was observed exclusively in the control but not in $Ca_v1.2^{HCKO}$ mice (factor genotype, $F = 6.28$; $p = 0.02$; ANOVA) (Fig. 3b).

An alternative test for NMDAR-independent synaptic plasticity is to study LTP_K [i.e., LTP induced by brief superfusion of hippocampal slices with the potassium channel blocker TEA-Cl (25 mM)]. Importantly, LTP_K is thought to depend mostly on L-type channel-mediated Ca^{2+} influx (Aniksztejn and Ben-Ari, 1991; Zakharenko et al., 2001). We compared LTP in control and $Ca_v1.2^{HCKO}$ mice after 15 min of superfusion with a modified TEA-containing aCSF and APV ($50 \mu M$) present throughout the experiment. Under these conditions, control mice exhibited robust LTP, even 2 h after washout of TEA (147.4 ± 11.3 ; $n = 15$) (Fig. 3c). In sharp contrast, mutant mice showed only marginal LTP 2 h after treatment with TEA (113.1 ± 4.47 ; $n = 11$; $p < 0.05$) (Fig. 3c). LTP in control mice 2 h after superfusion with TEA might represent the so-called late-phase LTP, a hallmark of which is the dependence on *de novo* protein synthesis (Frey et al., 1988; Kandel, 2001). To test for this obligatory feature, we performed additional experiments with hippocampal slices from control and mutant mice in which the inhibitor of protein synthesis, anisomycin ($20 \mu M$), was present throughout the experiment. Anisomycin significantly reduced hippocampal LTP observed 2 h after treatment with TEA in slices from control mice (121.8 ± 8.3 ; $n = 8$; $p < 0.05$) compared with LTP without anisomycin, but had no effect in slices from $Ca_v1.2^{HCKO}$ mice (114.4 ± 12.9 ; $n = 8$) (Fig. 3d). Together, these findings clearly demonstrate that NMDAR-independent, protein synthesis-dependent L-LTP in the hippocampal CA1 region depends mostly on $Ca_v1.2$ L-type Ca^{2+} channels.

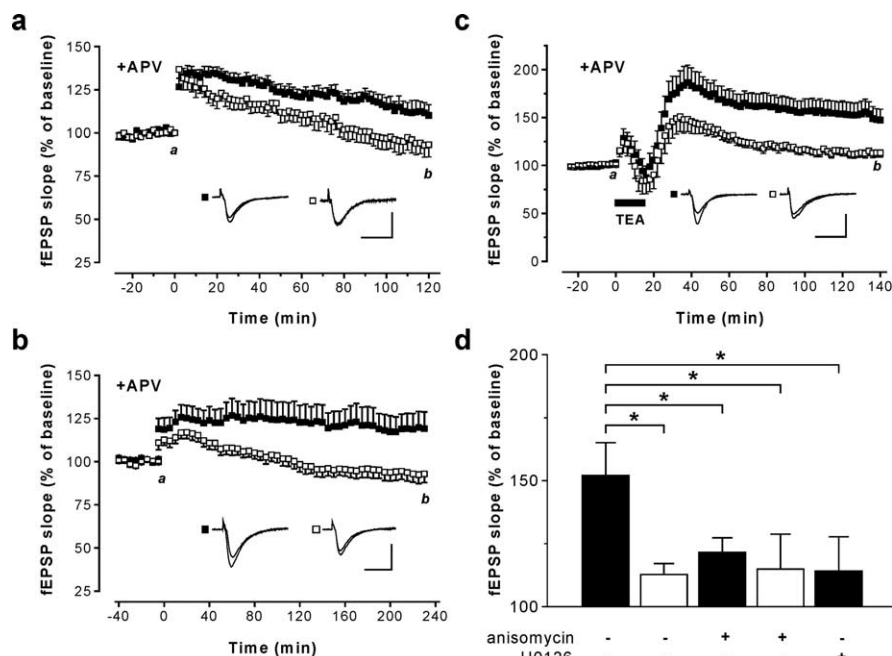


Figure 3. NMDAR-independent LTP in the hippocampal CA1 region of $Ca_v1.2^{HCKO}$ mice. fEPSPs in response to stimulation of the Schaffer collaterals were recorded in slices from control (■) and $Ca_v1.2^{HCKO}$ (□) mice. *a*, LTP was induced at time 0 by a 200 Hz tetanic stimulation with the NMDAR antagonist APV ($50 \mu M$) present throughout the experiment. The time course of the fEPSP slope represents mean \pm SEM of experiments in eight slices from five mice for each genotype. Representative fEPSPs recorded at the times indicated (*a*, *b*) are shown in the corresponding inset. *b*, LTP was induced at time 0 by multiple strong 100 Hz tetani with the NMDAR antagonist APV ($50 \mu M$) present throughout the experiment. The time course of the fEPSP slope represents the mean \pm SEM in control (10 slices from 6 mice) and $Ca_v1.2^{HCKO}$ (14 slices from 6 mice) slices. Representative fEPSPs recorded at the times indicated (*a*, *b*) are shown in the corresponding inset. *c*, LTP_K was induced by superfusing hippocampal slices with aCSF containing 25 mM potassium channel blocker TEA for 15 min (indicated by the bar). The NMDAR antagonist, APV ($50 \mu M$), was present throughout the experiment. The time course of the fEPSP slope represents the mean \pm SEM in control (15 slices from 12 mice) and $Ca_v1.2^{HCKO}$ mice (11 slices from 7 mice). Representative fEPSPs recorded at the times indicated (*a*, *b*) are shown in the corresponding inset. *d*, Effect of the protein synthesis inhibitor anisomycin ($20 \mu M$) and the MEK1/2 inhibitor U0126 ($40 \mu M$) on L-LTP_K in control and $Ca_v1.2^{HCKO}$ mice. ■, The magnitude of LTP_K 2 h after treatment with TEA in control mice in normal aCSF, in the presence of anisomycin ($n = 8$ slices from 5 mice) and U0126 ($n = 7$ slices from 5 mice) is illustrated. □, The magnitude of LTP_K 2 h after treatment with TEA in control mice in normal aCSF and in the presence of anisomycin ($n = 11$ slices from 7 mice), respectively, is illustrated. *Significant difference ($p < 0.05$). Calibrations: 20 ms, 1 mV. Error bars indicate SE.

$Ca_v1.2^{HCKO}$ mice have normal basic motor functions, exploratory behavior, anxiety, and visual acuity

Does the lack of NMDAR-independent L-LTP in $Ca_v1.2^{HCKO}$ mice correlate with altered spatial memory? The performance of $Ca_v1.2^{HCKO}$ mice in learning tasks might, in principle, be affected by defects in other behavioral traits. Therefore, we initially performed a series of control tests. Arguing against nonspecific effects of the *CACNA1C* gene inactivation on exploratory behavior and anxiety, control and $Ca_v1.2^{HCKO}$ mice ($n = 10$ for both groups) showed no significant differences in the open field test. The total number of fields entered by control mice (119 ± 20 on day 1 and 89 ± 9 on day 4) matched that of $Ca_v1.2^{HCKO}$ mice (112 ± 19 on day 1 and 70 ± 18 on day 4). For both genotypes, the vast majority were entries into outer fields, amounting to 114 ± 18 (day 1) and 85 ± 7 (day 4) in control mice, and 105 ± 19 (day 1) and 70 ± 15 (day 4) in the $Ca_v1.2^{HCKO}$ mice. The following findings rule out a deficit in general motor ability in the $Ca_v1.2$ knock-out. First, control and $Ca_v1.2^{HCKO}$ mice ($n = 10$ for both groups) performed equally in a rotarod test. The mean duration that mice of both genotypes managed to keep balance on the accelerating rotarod increased similarly to a maximal level at day 3 and remained constant throughout the following 2 d of examination. Second, swim speed in a water maze pool was the same in

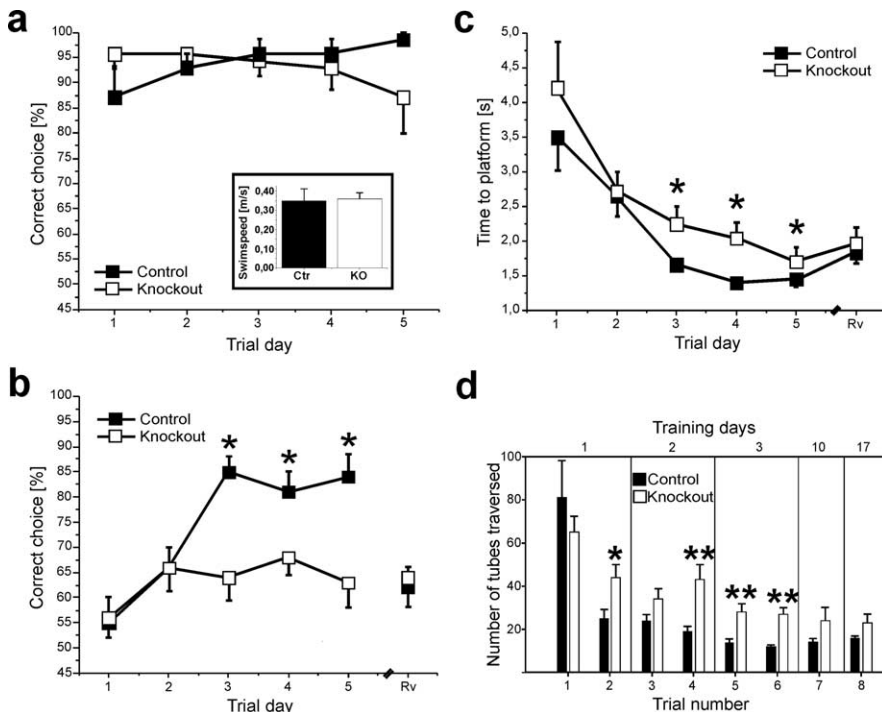


Figure 4. Spatial memory defects in $Ca_v1.2^{HCKO}$ mice. **a**, Correct-choice rate of mice trained to find the correct platform in a visual acuity task by using the only a visible landmark in a dimly lit room. There were no differences between the $Ca_v1.2^{HCKO}$ (\square) animals ($n = 7$) and the control (\blacksquare) mice ($n = 7$) in the percentage of correct choices during the trial days (10 trials per day). Inset, Swim speed. $Ca_v1.2^{HCKO}$ (\square) animals ($n = 10$) present the same swim-speed rate (meters/second) compared with control (\blacksquare) mice ($n = 10$). Data are expressed as mean + SEM. **b**, **c**, The performance of $Ca_v1.2^{HCKO}$ mice in the discriminatory water-maze task. **b**, The graph represents the correct-choice rate of mice trained to find the correct platform in a discriminatory water maze by using the distal cues surrounding it. The $Ca_v1.2^{HCKO}$ (\square) animals ($n = 10$) display a significantly ($p < 0.05$) lower correct-choice rate from day 3 (10 trials per day) than the control (\blacksquare) mice ($n = 10$). During a control reversal trial (Rv), the percentage of correct choices made by control mice dropped by a similar extent to values near chance level, indicating that spatial searching strategies were used. **c**, The graph represents the escape latencies of mice that found the correct platform. The $Ca_v1.2^{HCKO}$ (\square) animals ($n = 10$) displayed a longer latency, starting from day 3 (10 trials per day) than the control (\blacksquare) mice ($n = 10$). **d**, Spatial learning in a labyrinth maze made of transparent, brightly lit glass tubes, with a single correct way leading to a dark box. Performance was assessed by the number of tubes traversed by control (\blacksquare) and $Ca_v1.2^{HCKO}$ (\square) mice until they reached the dark box. Two trials were performed on the first 3 d ($n = 11$ animals for both groups) and one trial on days 10 and 17 ($n = 5$ animals of both groups). Data are expressed as mean + SEM. * $p < 0.05$; ** $p < 0.01$.

knock-out and control groups (control mice, 0.35 ± 0.06 m/s; $Ca_v1.2^{HCKO}$ mice, 0.36 ± 0.03 m/s) (Fig. 4a).

It is a general problem when studying mutants in a task using distal cues (like the two learning tasks used in this study) to rule out a deficit based on an inability to properly see them. Therefore, we trained mice in a visual acuity, discriminatory water-maze task, in which they were required to identify the correct platform, kept in a permanent position and marked by a single large, distal, well lit landmark in a dimly lit room with no additional distal cues (for details, see Materials and Methods). In this test, again, there was no difference in the performance of control and $Ca_v1.2^{HCKO}$ mice (Fig. 4a). Collectively, these findings allow us to separate a learning defect from other kinds of behavioral changes (e.g., perceptual, motivational, or attentional) in $Ca_v1.2^{HCKO}$ mice.

$Ca_v1.2^{HCKO}$ mice display severely impaired hippocampus-dependent spatial memory

Next, we chose two mouse-friendly learning tasks that tax spatial reference memory but require different sets of sensory and motor abilities. Initially, we examined hippocampus-dependent spatial learning in a well established discriminatory water-maze task (Arns et al., 1999; Steckler et al., 1999; Kleppisch et al., 2003), adapted from the paradigm originally described by Morris et al.

(1986b) for rats. This task has been proven to be hippocampus dependent using excitotoxic lesions (Arns et al., 1999). The water-maze spatial-discrimination task differs from the more frequently used place-navigation paradigm in several aspects. First, we used visible instead of hidden platforms with the consequence that animals are not required to first learn that there is a platform at all. Second, escape latencies are relatively short, providing the advantage of greatly reduced swim stress. Third, the setup with two platforms gives the opportunity to assess spatial learning using a probability parameter, the frequency of correct choices (Fig. 4b), that is less likely to be affected by changes in other behavioral, perceptual, or motor functions than a time (e.g., the water escape latency) or distance (e.g., the path length in the pool) parameter (Fig. 4c).

$Ca_v1.2^{HCKO}$ and control mice were trained to identify a fixed platform visually identical to a second sinkable platform. Both groups showed the same initial rate of correct choices and an equivalent increase of this parameter from day 1 to day 2. However, we observed a dramatic difference in the performance of control and mutant mice beyond trial day 2 (Fig. 4b). Whereas $Ca_v1.2^{HCKO}$ mice did not further improve their correct choice score from the value at day 2 (~65%), the performance of their control siblings significantly improved to >80%. In addition, when knock-out animals found the correct platform, their water escape latency was significantly increased (Fig. 4c). These findings suggest a severe impairment of spatial learning in the $Ca_v1.2^{HCKO}$ mice.

To test for the possibility that mice used salient cues provided by the platforms for discrimination, the correct platform was moved to the opposite quadrant (reversal) and animals were tested again. During this reversal trial, the percentage of correct choices made by control mice dropped to values near chance level (Fig. 4b). This rules out that the two platforms were discriminated merely based on their appearance but confirms that control animals used spatial cues in the environment for successful navigation.

To further substantiate that $Ca_v1.2$ -mediated, NMDAR-independent synaptic plasticity is crucial for spatial memory formation, we sought after an alternative learning task preferably differing from the water maze in various aspects, such as task complexity, response requirement, and the nature of the reinforcer. Therefore, we further examined mice in a validated spatial-learning labyrinth paradigm that has been shown to be well suited to resolve alterations in spatial memory [e.g., using NR2B (NMDAR 2B)-overexpressing transgenic mice] (Tang et al., 1999; Adelsberger et al., 2005). The task consists of the repeated walk through a labyrinth made of clear, transparent acrylic glass tubes with a length of 50 cm each to a familiar dark box in a laboratory environment presenting distal cues (for details, see Materials and Methods). The test is primarily based on

the positive motivation of the animals and avoids the escape from forced swimming associated with water-maze tests (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

During the initial trial, the number of tubes traversed (which represents the path length) before reaching the dark box was not significantly different for control and $Ca_v1.2^{HCKO}$ mice (Fig. 4*d*). This indicates equally effective searching patterns in naive animals of either genotype. The number of tubes traversed is expected to decline during training to an extent depending on the efficiency of spatial learning strategies and the formation of spatial memories. In nice agreement with the differences observed between the two genotypes in the discriminatory water maze, $Ca_v1.2^{HCKO}$ mice showed significantly longer path lengths than the control mice during all subsequent trial days (Fig. 4*d*). The coincidence of a defect in NMDAR-independent protein synthesis-dependent L-LTP with a defect in two spatial learning tasks strongly suggests a functional link between these processes.

Activation of the MAPK pathway is impaired in $Ca_v1.2^{HCKO}$ mice

There is comprehensive evidence supporting the view that the MAPK signaling cascade is involved in synaptic plasticity in the CA1 area of the mammalian hippocampus and in various forms of long-term memory (e.g., spatial learning and fear conditioning) (Thomas and Huganir, 2004). Experiments with neuronal cell cultures have shown that Ca^{2+} influx can efficiently activate ERK/MAPK (Rosen et al., 1994; Dolmetsch et al., 2001; Hardingham et al., 2001). To assess the function of $Ca_v1.2$ L-type Ca^{2+} channels for the activation of ERK1/2, we asked whether phosphorylation and nuclear translocation of ERK occurs in postsynaptic CA1 pyramidal neurons after treatment with TEA (i.e., under experimental conditions resulting in robust L-LTP_K in Schaffer collateral/CA1 synapses) (compare Fig. 3*c*). As visualized by confocal immunofluorescence, superfusion with TEA caused a strong increase in phosphorylated, activated ERK in the postsynaptic dendrites (Fig. 5*a*). In addition, we were able to detect phospho-ERK-positive nuclei exclusively in TEA-treated slices, but not in untreated control slices. Remarkably, neither nuclear translocation nor phosphorylation of ERK1/2 in CA1 pyramidal cells of control mice was affected by addition of the NMDAR antagonist APV. In contrast to the findings in control mice, TEA-treated hippocampal CA1 pyramidal cells of $Ca_v1.2^{HCKO}$ mice showed only a delicate dendritic phospho-ERK signal and only a negligible number

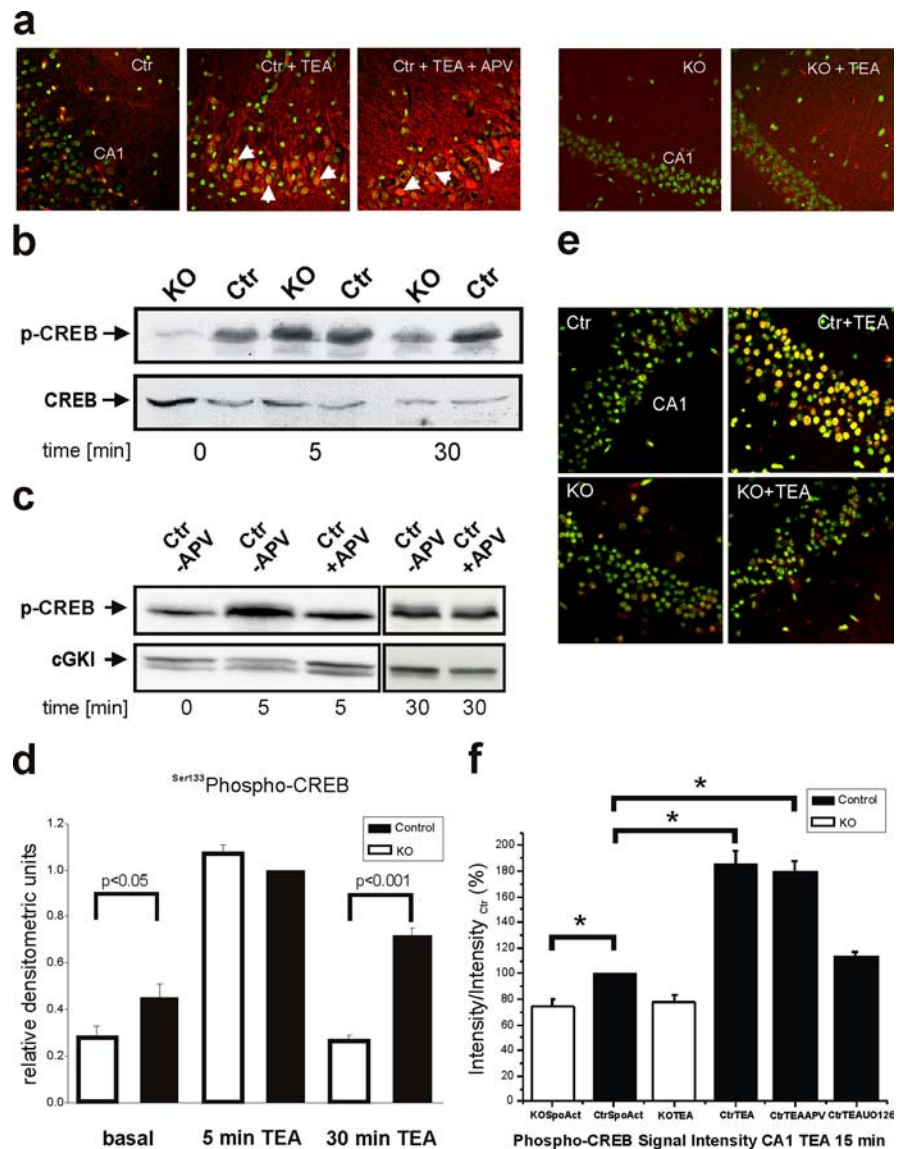


Figure 5. Disturbed MAPK activation and impaired phosphorylation of CREB by stimulus paradigms that generate L-LTP in area CA1 of $Ca_v1.2^{HCKO}$ mice. *a*, Representative examples of phospho-ERK1/2 immunocytochemistry in a untreated control (Ctr), in slices that were treated with a stimulus paradigm (TEA) that generates L-LTP in the presence or absence of the NMDAR antagonist APV, and in untreated and TEA treated knock-out (KO) slices. Green, Hoechst 33258 nuclear marker; red, phospho-ERK1/2; yellow, overlay. Arrowheads, Nuclear localization of ERK1/2. Images are at 400 \times (original) magnification. *b*, Western blot of the time course of CREB Ser¹³³ phosphorylation in hippocampal neurons depolarized with TEA in KO or Ctr mice. Ca^{2+} influx through $Ca_v1.2$ L-type Ca^{2+} channels results in sustained CREB phosphorylation (30 min). Blots ($n = 6$ independent experiments) were stripped and reprobed with anti-CREB. *c*, Western blot of the time course of CREB Ser¹³³ phosphorylation in hippocampal neurons depolarized with TEA in (Ctr) mice in the presence (+APV) or absence (-APV) of the NMDAR antagonist APV. NMDAR activity contributes to the early phase (5 min) but not to the late phase of CREB phosphorylation (30 min). Blots ($n = 6$ independent experiments) were stripped and reprobed with anti-cGMP-dependent protein kinase I (anti-cGKI). *d*, Densitometric analysis of Western blots for CREB Ser¹³³ phosphorylation. Data are normalized phospho-CREB Ser¹³³ values in control mice after 5 min of TEA treatment. *e*, Representative examples of phospho-CREB immunofluorescence in untreated control and knock-out slices, and in slices that were treated with a stimulus paradigm (TEA) that generates L-LTP. Images are at 400 \times (original) magnification. Green, Hoechst 33258 nuclear marker; red, phospho-CREB Ser¹³³; yellow, overlay. *f*, Fold increase in phospho-CREB immunocytochemistry compared with untreated slices [spontaneous activity (SpoAct)]. Slices were stimulated with a paradigm (TEA) that generates L-LTP in the presence and absence of APV. Slices ($n = 12$ per condition) were fixed 30 min after tetanus. *Significant differences ($p < 0.05$). Error bars indicate SE.

of phospho-ERK-positive nuclei. To test whether the defect of TEA-induced NMDAR-independent L-LTP observed in $Ca_v1.2^{HCKO}$ mice results from impaired MAPK cascade signaling, we examined the effect of an inhibitor of the MAPK pathway, U0126, on this form of synaptic plasticity in hippocampal slices

of control mice. Supporting our hypothesis, U0126 (40 μ M) mimicked the effect of the protein synthesis inhibitor anisomycin in these mice (Fig. 3*d*). We therefore concluded that ERK activity is required for NMDAR-independent, protein synthesis-dependent L-LTP in hippocampal CA1 neurons.

Impaired sustained CREB-Ser¹³³ phosphorylation and CRE-dependent transcription in Ca_v1.2^{HCKO} mice

Based on the above conclusion, we next asked whether effectors of ERK1/2 associated with synaptic plasticity and memory are affected in Ca_v1.2^{HCKO} mice. Activation of ERK/MAPK may contribute to memory formation by stimulating many different events, including transcription of memory-related genes (Sweatt, 2004). A major role in these processes has been assigned to the downstream effector of ERK1/2, CREB, a transcription factor related to synaptic plasticity and essential for various forms of memory (Silva et al., 1998; Lonze and Ginty, 2002). CRE-dependent gene expression critically depends on CREB-Ser¹³³ phosphorylation (Brindle and Montminy, 1992), which provides a duration-sensitive switch (i.e., only a sustained pCREB-Ser¹³³ signal is sufficient to activate CRE-mediated transcription) (Bito et al., 1996). Therefore, we next asked whether decreased ERK activity in hippocampal neurons of Ca_v1.2^{HCKO} mice was accompanied by a suppression of the prolonged activation of CREB. Hippocampal extracts obtained from tissue before and at various times after treatment with TEA (25 mM) were blotted with an antibody specifically binding pCREB-Ser¹³³. Hippocampal tissue from Ca_v1.2^{HCKO} mice showed a reduced basal pCREB-Ser¹³³ level [0.28 ± 0.05 relative densitometric units (rdu)] compared with that in tissue from control mice (0.45 ± 0.06 rdu; $p < 0.05$). This difference is likely caused by spontaneous activity of Ca_v1.2 channels. In hippocampi from control mice, TEA treatment induced a strong CREB phosphorylation, detectable at 5 min and lasting at least 30 min (Fig. 5*b,d*). Interestingly, initial TEA-induced CREB phosphorylation (5 min) was also detected in hippocampi from Ca_v1.2^{HCKO} mice. But, in striking contrast with the findings in control animals, CREB phosphorylation was completely reversed within 30 min (control mice, 0.72 ± 0.03 rdu; Ca_v1.2^{HCKO} mice, 0.27 ± 0.02 rdu; $p < 0.001$). Thus, hippocampal neurons of Ca_v1.2^{HCKO} mice specifically lack sustained CREB phosphorylation under these conditions. Additional analysis of TEA-induced CREB activation in hippocampal tissue from control mice in the presence of the NMDAR antagonist APV (Fig. 5*c*) revealed that the early phase (5 min) was caused by an NMDAR-mediated Ca²⁺ influx. Importantly, APV did not affect CREB phosphorylation at 30 min, confirming the role of an NMDAR-independent mechanism for sustained CREB activation.

In the next series of experiments, we used confocal immunofluorescence to visualize nuclear phospho-CREB localization and CRE-dependent transcription in CA1 neurons under conditions leading to L-LTP_K. CRE-dependent transcriptional activity was assessed in control and Ca_v1.2^{HCKO} mice additionally carrying a transgene for a CRE-regulated reporter construct (Impey et al., 1996). TEA treatment caused a strong increase in nuclear pCREB (Fig. 5*e,f*) and CRE-dependent gene transcription (Fig. 6*a,b*) exclusively in hippocampi from control mice. Hippocampal pyramidal cells in Ca_v1.2^{HCKO} mice lacked such effects (Fig. 6*a,b*). Again, neither nuclear translocation nor phosphorylation of CREB (Fig. 5*e,f*) was affected by adding APV. Importantly, the inhibitor of the ERK pathway, U0126 (40 μ M), abolished nuclear pCREB-Ser¹³³ signals (Fig. 5*f*) and blocked activation of CRE-mediated transcription after treatment with TEA (Fig. 6*a,b*). These data indicate that both L-LTP_K and the increases in CRE-

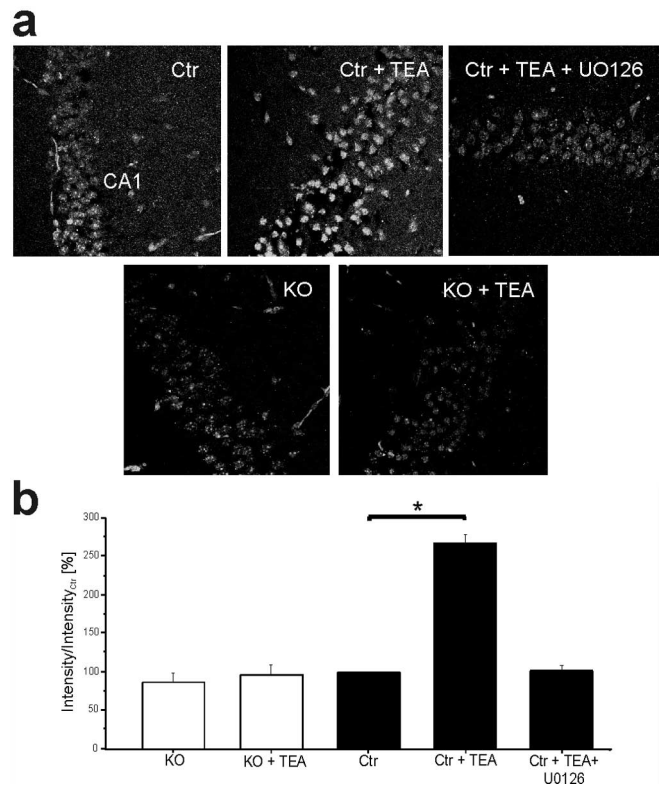


Figure 6. Increases in Cre-LacZ expression by stimulus paradigms that generate L-LTP at Schaffer collateral/CA1 synapses requires Ca_v1.2 L-type Ca²⁺ channel activity. *a*, Representative examples of CRE-LacZ expression in control (Ctr) and knock-out slices (KO) that were untreated, treated with TEA, or treated with TEA and U0126. All experiments were performed in the presence of APV. Green, β -Galactosidase reporter protein. *b*, Percentage of increase in β -galactosidase immunocytochemistry compared with untreated slices. Slices ($n = 12$ per condition) were fixed 240 min after the LTP induction. *Significant difference ($p < 0.05$). Error bars indicate SE.

mediated gene expression are associated with increased ERK1/2 activity.

Discussion

Numerous studies have provided pharmacological and genetic evidence, both loss of function and gain of function, linking NMDAR-dependent LTP in the hippocampal CA1 region to certain types of learning (Morris et al., 1986a; McHugh et al., 1996; Tsien et al., 1996; Tang et al., 1999). However, a description of hippocampal synaptic plasticity and memory merely based on NMDAR-mediated processes falls short in various aspects, as shown in this study and by others (Impey et al., 1996; Borroni et al., 2000; Rampon and Tsien, 2000; Woodside et al., 2004). Thus, LTP can also be induced in a Ca_v1.x channel-dependent, NMDAR-independent manner in the CA1 region (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Cavus and Teyler, 1996; Morgan and Teyler, 1999). In particular, activity of Ca_v1.x channels has been shown to be necessary for induction of L-LTP by strong stimulus protocols (Impey et al., 1996; Raymond and Redman, 2002). In addition, biochemical processes, like the activation of ERK and the subsequent phosphorylation of CREB, have been linked to Ca_v1.x channel activity (Impey et al., 1996; Mermelstein et al., 2000; West et al., 2001; Wu et al., 2001). Therefore, we hypothesized that NMDAR-independent, L-type Ca²⁺ channel-dependent synaptic plasticity might, in parallel to NMDAR-activated processes, be necessary for spatial learning in the behaving animal.

Although the pharmacological analysis of cultured cells and slice preparations has provided much insight into the regulation and function of L-type Ca²⁺ channels in neurons (for review, see Hofmann et al., 1999; Striessnig, 1999; Moosmang et al., 2005), their significance *in vivo* is far less clear because of several problems associated with the currently available pharmacological tools. Most importantly, all L-type Ca²⁺ channel blockers affect the function of the cardiovascular system to some extent, which may have substantial input on the function of the CNS. In fact, a number of investigations demonstrate that pharmacological block of L-type calcium channels may enhance learning through changes in cerebral blood flow (Deyo et al., 1989; Deyo and Hittner, 1995). Conversely, other studies show that the pharmacological block of neuronal L-type Ca²⁺ channels impairs memory (e.g., amygdala-dependent conditioned fear memory) (Bauer et al., 2002). Moreover, available pharmacological tools do not allow separation of the individual function from the various members of the L-type Ca²⁺ channel family (e.g., Ca_v1.2 and Ca_v1.3). The interpretation of *in vivo* data are further complicated because all known classes of Ca²⁺ channel blockers can also block other voltage-gated channels at higher concentrations (Galper and Catterall, 1979; Ragsdale et al., 1991; Rampe et al., 1993; Diocot et al., 1995; Ishibashi et al., 1995).

Therefore, we decided to use a genetic approach to analyze the role of NMDAR-independent hippocampal synaptic plasticity *in vivo*. We created a second-generation knock-out mouse strain using the Cre/loxP technique and Nex-Cre mice (Schwab et al., 2000). This approach yielded a mouse line (Ca_v1.2^{HCKO} mice) deficient in the Ca_v1.2 protein, mainly in the hippocampal formation and cerebral cortex. The deletion of the *CACNA1C* gene became effective only after the first weeks of postnatal development; there was a normal level of hippocampal Ca_v1.2 protein in 2- to 3-week-old Ca_v1.2^{HCKO} mice, whereas the protein was absent in adult mice. Hippocampal pyramidal cells undergo maximal dendritic growth during this period, and their cellular organization and synaptic connections are principally established at this age (Wong and Ghosh, 2002). Thus, Ca_v1.2^{HCKO} mice provide a model that should be devoid of developmental disturbances in the brain architecture (Redmond et al., 2002; Aizawa et al., 2004). This assumption is supported by our findings that control and Ca_v1.2^{HCKO} mice show no differences in the anatomical properties of the brain and in their characteristics of basal synaptic transmission in the hippocampus.

Our combined electrophysiological, biochemical, and behavioral analysis of the Ca_v1.2^{HCKO} mice provides strong evidence that NMDAR-independent synaptic plasticity in the hippocampal CA1 region is functionally linked to hippocampus-dependent spatial learning. The following findings support this conclusion. First, NMDAR-dependent LTP was not altered in the hippocampal CA1 region of Ca_v1.2^{HCKO} mice (i.e., defects observed in other forms of synaptic plasticity were selective). Second, NMDAR-independent L-LTP induced by various paradigms was defective in the Schaffer collateral/CA1 pathway of Ca_v1.2^{HCKO} mice. Furthermore, the protein synthesis inhibitor anisomycin decreased NMDAR-independent LTP_K 2 h after treatment with TEA in the control mice to the level observed in the mutants. These results clearly suggest that Ca_v1.2^{HCKO} mice are deficient in protein synthesis-dependent L-LTP, which has been explicitly linked to long-term memory (Kandel, 2001; Martin and Morris, 2002). Third, the defect of protein synthesis-dependent synaptic plasticity in the Ca_v1.2^{HCKO} mice was accompanied by reduced activation of MAPK cascade signaling. Fourth, this impairment of the MAPK pathway was paralleled by diminished CRE-

dependent transcription in the CA1 region after L-LTP induction. Fifth, as a consequence, the mutant mice were severely impaired in two different spatial learning tasks. They showed inferior performance in a well established discriminatory water-maze task (Arns et al., 1999; Steckler et al., 1999; Kleppisch et al., 2003) and in a more complex labyrinth-maze task (Adelsberger et al., 2005).

The gene inactivation in Ca_v1.2^{HCKO} mice was not limited strictly to the hippocampus but occurred also in the cerebral cortex. We cannot completely rule out an impact of the loss of Ca_v1.2 protein in cortical areas on behavioral functions. However, differences between control and Ca_v1.2^{HCKO} mice were detected exclusively in tests designed to reveal defects in learning and memory associated with the hippocampus (e.g., the water maze task) (Arns et al., 1999; Morris, 2003). However, both genotypes performed equally in a series of behavioral control tests that are supposed to depend at least partially on a normal function of the cortex.

How might Ca_v1.2 channels support NMDAR-independent LTP and spatial learning? We found that Ca_v1.2 channel-mediated Ca²⁺ influx activates the protein kinase ERK and CREB-dependent transcription under conditions leading to L-LTP. It has been reported that LTP-inducing high-frequency stimulation activates ERK in the CA1 area, and that the MAPK kinase (MEK) inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] blocks both ERK activation and NMDAR-dependent LTP (English and Sweatt, 1996, 1997). ERK activity is also required for LTP in the dentate gyrus (Coogan et al., 1999), the amygdala (Schafe et al., 2000), and NMDAR-independent forms of LTP in the CA1 area (Kanterewicz et al., 2000). The role of ERK for long-term memory in behaving animals has been characterized using spatial-learning and fear-conditioning (Schafe et al., 2000) paradigms. Training in the Morris water maze leads to activation of ERK in the hippocampus of the corresponding animals (Blum et al., 1999). Moreover, intrahippocampal infusion of ERK inhibitors impairs the performance in this task (Selcher et al., 1999). Studies in invertebrates and rodents indicate that CREB-dependent transcription is essential for both L-LTP (Bourchuladze et al., 1994; Deisseroth et al., 1996; Barco et al., 2002) and many forms of learning and memory (for review, see Lonze and Ginty, 2002). Phosphorylation of CREB at Ser¹³³ leads to recruitment of other transcription machinery to CREs to regulate gene transcription (Lonze and Ginty, 2002). As such, it is thought that CREB phosphorylation at Ser¹³³ is an important step in the induction of gene expression critical for memory. We show that the prolonged phosphorylation of CREB at Ser¹³³ is impaired in Ca_v1.2^{HCKO} mice. This phosphorylation can also be prevented by inhibitors of the ERK cascade (Wu et al., 2001). In conclusion, we propose that the defects of LTP and memory in the Ca_v1.2^{HCKO} mice are the consequence of impaired activation of ERK and defective CREB-dependent transcription, which are normally coupled specifically to a Ca_v1.2 channel-mediated intracellular signal.

The outstanding role of NMDARs for synaptic plasticity and memory formation has been explained with their unique property: a voltage-dependent block by Mg²⁺ allows them to function as Hebbian coincidence detectors (Bliss and Collingridge, 1993). Can Ca_v1.2 channels serve a similar function? Ca_v1.2 channel-mediated dendritic Ca²⁺ influx has been proposed to arise from strong depolarization caused by backpropagating action potentials (APs) (Spruston et al., 1995; Yuste and Denk, 1995; Magee and Johnston, 1997) that are likely to occur during the induction of NMDAR-independent LTP. In this scenario, the Ca_v1.2 chan-

nel might facilitate the invasion of antidromic APs into distal dendritic structures in an activity-dependent manner (Magee and Johnston, 1997) (i.e., they could also serve as coincidence detectors). However, electrophysiological characteristics of $Ca_v1.x$ channels favor Ca^{2+} entry associated with EPSPs as opposed to APs, which is reflected in a much stronger L-type Ca^{2+} channel-dependent CREB phosphorylation in response to synaptic activity (Mermelstein et al., 2000). In line with these findings, $Ca_v1.2$ may support synaptic potentiation, independent of postsynaptic action potential firing, by contributing to regenerative local dendritic spikes (Golding et al., 2002). Future studies (e.g., dendritic Ca^{2+} imaging in $Ca_v1.2^{HCKO}$ mice) will elucidate the precise way in which $Ca_v1.2$ can function as a Hebbian coincidence detector.

In conclusion, our findings show that NMDA receptor-independent, $Ca_v1.2$ L-type Ca^{2+} channel-dependent synaptic plasticity significantly contributes to spatial learning in the behaving mouse.

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