Brief Communication

Conditional forebrain deletion of the L-type calcium channel $\text{Ca}_v1.2$ disrupts remote spatial memories in mice

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To determine whether L-type voltage-gated calcium channels (L-VGCCs) are required for remote memory consolidation, we generated conditional knockout mice in which the L-VGCC isoform $\text{Ca}_v1.2$ was postnatally deleted in the hippocampus and cortex. In the Morris water maze, both $\text{Ca}_v1.2$ conditional knockout mice ($\text{Ca}_v1.2^{\text{CKO}}$) and control littermates displayed a marked decrease in escape latencies and performed equally well on probe trials administered during training. In distinct contrast to their performance during training, $\text{Ca}_v1.2^{\text{CKO}}$ mice exhibited significant impairments in spatial memory when examined 30 d after training, suggesting that $\text{Ca}_v1.2$ plays a critical role in consolidation of remote spatial memories.

In recent years, a wealth of experimental evidence has emerged suggesting that activity-dependent changes in neuronal gene expression are regulated in large part by calcium influx via L-VGCCs. For example, calcium influx through L-VGCCs activates calcium-dependent calmodulin kinase IV and Ras/mitogen-activated kinase, which, in turn, phosphorylate the transcription factor CREB at serine 133 (Wu et al. 2001). Once phosphorylated, CREB becomes part of an active transcriptional complex that binds to cAMP-response element DNA sequences to regulate transcription of a number of gene products. Importantly, calcium-mediated activation of CREB has been implicated as a key subcellular signaling cascade in a wide range of behavioral processes including long-term memory consolidation (West et al. 2002; Deisseroth et al. 2003). Given the prominent role that L-VGCCs play in the activation of these signaling cascades, we have begun to examine the impact of altering L-VGCC function on memory consolidation. Consistent with the idea that L-VGCCs are a key component of this process, we have recently demonstrated that deletion of the L-VGCC isoform $\text{Ca}_v1.3$ produces a deficit in consolidation of contextual fear conditioning (McKinney and Murphy 2006). Here we report here that forebrain deletion of the alternate L-VGCC isoform $\text{Ca}_v1.2$ specifically disrupts remote spatial memories while sparing recently acquired spatial memories.

To obtain regionally restricted deletion of $\text{Ca}_v1.2$, mice were generated using a BAC clone containing *Cacna1c* (the mouse $\text{Ca}_v1.2$ gene) obtained by screening a murine strain SV/129J BAC library (Genome Systems Inc.) with a probe representing exon 2 of *Cacna1c*. DNA fragments containing portions of *Cacna1c* were subcloned and mapped. Three adjacent XbaI fragments (4, 0.6, and 1.9 kb) were subcloned into a modified plox vector (Chui et al. 1997) in which the thymidine kinase (TK) cassette had been removed. The MC1-TK cassette (Thomas and Capecchi 1987) was

introduced at the junction of the 3' homology unit and the plasmid vector. The final targeting vector (Fig. 1A) had 4 kb of 5' homology, a *loxP* site introduced into intron 1, a 0.6-kb region containing exon 2, a *loxP*-Neo-*loxP* cassette introduced into intron 2, 1.9 kb of 3' homology, and the MC1-TK cassette.

The targeting vector was linearized and electroporated into R1 embryonic stem (ES) cells (Nagy et al. 1993). Two hundred thirty-two neomycin/ganciclovir-resistant colonies were analyzed by Southern blotting with 5' and 3' probes that were outside the region of homology. Sixteen correctly targeted clones were identified, of which five were amplified. DNA sequence analysis of correctly targeted clones confirmed that exon 2 was flanked by two *loxP* sites in two of the clones. Karyotype analysis performed at the Waisman Center Cytogenetics Laboratory (Madison, WI) identified a karyotypically normal ES clone, which was microinjected into C57BL/6 blastocysts by the University of Wisconsin Biotechnology Center's Transgenic Animal Facility. A male chimeric founder was generated and transmitted the floxed chromosome to his offspring. Mice heterozygous for the floxed $\text{Ca}_v1.2$ exon 2 allele were propagated on a 129SvEv genetic background.

Experimental animals were generated by crossing heterozygous floxed $\text{Ca}_v1.2$ mice ($\text{Ca}_v1.2^{\text{f/+}}$ mice) with transgenic mice that expressed Cre-recombinase (Chen et al. 2006). Cre-recombinase expression was regulated by the calcium calmodulin kinase II α (CaMKII α) promoter, which reaches peak expression levels postnatally (Kelly et al. 1987; Sugiura and Yamauchi 1992), and restricted Cre-recombinase expression to glutamatergic neurons of the forebrain (Chen et al. 2006). These mice (termed here the CaMK-Cre mice) were maintained on a C57BL/6 background (10+ generations). Offspring from the F_1 cross that were heterozygous floxed and Cre-positive (i.e., $\text{Ca}_v1.2^{\text{f/+}}$, CaMK-Cre^{Cre/+}) were then intercrossed (nonsibling) with mice heterozygous floxed and Cre-negative (i.e., $\text{Ca}_v1.2^{\text{f/+}}$, CaMK-Cre^{+/+}) to achieve the following genotypes: conditional knockout mice ($\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{Cre/+}), wild-type mice ($\text{Ca}_v1.2^{\text{f/+}}$, CaMK-

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Figure 1. Generation and characterization of $\text{Ca}_v1.2^{\text{cKO}}$ mice. (A) Exon 2 of the $\text{Ca}_v1.2$ wild-type gene (*Cacna1c*) is flanked by 2 XbaI restriction sites that were eliminated in the targeting vector by the insertion of a loxP site (arrowhead) in the 5'-flanking region of exon 2 and a neomycin cassette (Neo) that was flanked on both ends by loxP sites (arrowheads) introduced into the 3' adjacent intron (intron 2). The targeting construct also carried the MC1 thymidine kinase (MC1-TK) cassette, which was used for negative selection with ganciclovir. Successful homologous recombination resulted in a floxed allele in which both exon 2 and the neomycin cassette would be flanked by loxP sites and therefore eliminated when exposed to Cre-recombinase. (B) Verification of conditional deletion of $\text{Ca}_v1.2$ exon 2 by RT-PCR. RNA samples from $\text{Ca}_v1.2^{\text{cKO}}$ mice and littermate controls were isolated from the cerebellum (Cb), cortex (Ctx; Left and Right hemispheres), and hippocampus (Hc). RNA was then reverse-transcribed, and the resulting cDNA was amplified by PCR (using primers P1 and P2 in A) and separated by electrophoresis on an agarose gel. Deletion of exon 2 in the $\text{Ca}_v1.2^{\text{cKO}}$ mice (lower band; -Ex 2) was detected in the cortex and hippocampus, whereas no such deletion was seen in control littermates (upper band; +Ex 2). (C) Western blot analysis of membrane fractions isolated from hippocampus, cortex, and cerebellum using an anti- $\text{Ca}_v1.2$ antibody reveals dramatically decreased detection of $\text{Ca}_v1.2$ in the hippocampus and cortex of the $\text{Ca}_v1.2^{\text{cKO}}$ mice with no detectable decrease in the cerebellum. Blots were also probed with anti-NrCAM to control for protein loading.

Cre^{+/+}, floxed controls ($\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{+/+}), and Cre controls ($\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{Cre/+}), all on an F2 129Sv:C57Bl/6 hybrid background. For ease of reading, conditional knockout mice ($\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{Cre/+}) are referred to as $\text{Ca}_v1.2^{\text{cKO}}$ throughout the remainder of the text.

RT-PCR experiments were carried out using brain tissue from $\text{Ca}_v1.2^{\text{cKO}}$ and control ($\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{+/+}) littermates. Mice were anesthetized and decapitated, and their brains were dissected in ice-cold PBS to recover the cerebellum, hippocampus, and cortex. RNA was extracted using Trizol (Invitrogen) per the manufacturer's instructions and quantified by spectrophotometer. Total RNA (2 μg) was reverse-transcribed using a SuperScript III First-Strand Synthesis System (Invitrogen), and the resulting cDNA was used as the PCR template. The PCR product was amplified using primers flanking exon 2. The sequences for RT-PCR primers were as follows: forward (P1) (Fig. 1A) CGGTGC-TAAATCTTGGGAAGGG, and reverse (P2) (Fig. 1A) CCAACCAT-TGCGGAGGTAAGC.

Immunoblotting was carried out using membrane fractions from $\text{Ca}_v1.2^{\text{cKO}}$ and control ($\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{+/+}) littermates. Tissue samples from the cortex, hippocampus, and cerebellum were microdissected in ice-cold PBS and homogenized in HSE buffer (10 mM HEPES, 350 mM sucrose, and 5 mM EDTA at pH 7.4) containing Complete Protease Inhibitor (Roche Diagnostics). The homogenate was then centrifuged for 5 min at 2000g at 4°C. The supernatant was removed and centrifuged again at 100,000g for 1 h to obtain membrane fractions. The resulting pellet was resuspended in ice-cold HSE buffer containing protease inhibitors. Protein content was determined by Bradford assay (Bio-Rad), using bovine serum albumin (BSA) as a standard. Membrane fractions (50 μg) were solubilized in Laemmli buffer, separated on a 7.5% SDS-PAGE gel, and transferred to PVDF membranes (Bio-Rad) overnight. Blots were probed with an anti- $\text{Ca}_v1.2$ antibody (1:200; Alomone Labs, Jerusalem, Israel, product # ACC-022, Lot# AN-03) whose epitope is located in the N terminus of the protein (residues 2–15). Blots were also probed with an NrCAM-specific antibody (1:40,000; ABCAM), which served as the loading control. Incubation with the primary antibody was

followed by washing with PBS-Tween and incubation with a horseradish peroxidase-conjugated anti-rabbit secondary (Bio-Rad; 1:5000). Blots were washed and immunoreactivity was visualized with an enhanced chemiluminescence detection system (ECL Plus; Amersham).

The water maze experiment was carried out as previously described (McKinney and Murphy 2006; McKinney et al. 2007). Mice were maintained on a 12-h light/dark cycle with access to food and water ad libitum. Approximately equal numbers of male and females were used, and the experimenter was kept blind to the genotype. All experiments were conducted in accordance with the guidelines set forth by the University of Michigan Committee on Use and Care of Animals. Mice were given two trials per day for 14 d, which ended either when the mouse climbed onto the platform or when 60 sec had elapsed. To assess the stability of newly acquired spatial memories during training, probe trials (in which the platform was removed) were administered on the morning of days 4, 7, 10, and 13 prior to the start of training for that day. The mouse was started in a position opposite the platform location and allowed to swim for 60 sec. To examine remote spatial memory, mice were subjected to an additional probe trial 30 d following training. As a control for motivation, swimming ability, and sensory perception required during the spatial version of the water maze, mice were run in the visible-platform version of the water maze on the day following the remote memory probe. In this version, a distinct local cue (a flag) was fixed to the center of the hidden platform by a Plexiglas rod. Mice were given four visible-platform trials in blocks of two with an interblock interval of 1 h.

For the water maze experiments, all four genotypes ($\text{Ca}_v1.2^{\text{cKO}}$, $n = 8$; $\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{+/+}, $n = 6$; $\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{+/+}, $n = 6$; and $\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{Cre/+}, $n = 6$) were used. A post hoc repeated measures ANOVA of the three control genotypes ($\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{+/+}; $\text{Ca}_v1.2^{\text{f/f}}$, CaMK-Cre^{+/+}; and $\text{Ca}_v1.2^{\text{+/+}}$, CaMK-Cre^{Cre/+}) revealed no significant difference in latency to reach the platform during training ($F_{(2,16)} = 2.9$; $P > 0.05$, for the effect of genotype). Similarly, percent time spent in training quadrant during the probe trials was not significantly

different ($F_{(2,15)} = 0.22$; $P > 0.05$, for the effect of genotype, day 13 probe). Therefore the three genotypes are presented in aggregate (labeled as "Controls").

Results from a typical RT-PCR experiment are presented in Figure 1B. Deletion of exon 2 in the $\text{Ca}_v1.2^{\text{cKO}}$ mice was readily detected within the cortex and hippocampus when compared to control mice. Conversely, the deletion of exon 2 was negligible within the cerebellum in the $\text{Ca}_v1.2^{\text{cKO}}$ mice. This pattern of deletion is consistent with previous results obtained with this line of Cre-recombinase transgenic mice (Chen et al. 2006). Western blot analysis of membrane fractions isolated from hippocampus, cortex, and cerebellum from $\text{Ca}_v1.2^{\text{cKO}}$ and control mice is presented in Figure 1C. Deletion of exon 2 resulted in substantial loss of immunoreactivity in both the hippocampus and cortex but not cerebellum in $\text{Ca}_v1.2^{\text{cKO}}$ mice. Similar results were obtained with an antibody produced against residues 818–835 of $\text{Ca}_v1.2$ (Hell et al. 1993).

To examine the impact of deletion of $\text{Ca}_v1.2$ on spatial learning and memory, $\text{Ca}_v1.2^{\text{cKO}}$ and control mice were subjected to the hidden-platform version of the Morris water maze (Morris 1981; Morris et al. 1982). During the 14 d of training, the average latency to reach the platform in both groups decreased with no significant difference between the two groups (Fig. 2A), demonstrating that $\text{Ca}_v1.2^{\text{cKO}}$ and control mice acquire the hidden-platform version of the Morris water maze equally well.

Probe trials were administered throughout training (on days 4, 7, 10, and 13) to assess retention of recently acquired spatial memories. The results from the probe trial given on day 13 (probe 4) are presented in Figure 2B. During the probe trial on day 13, both groups spent >50% time searching in the quadrant where the platform was previously located (training quadrant TQ in Fig. 2B); however, there was no significant difference in the amount of time that the $\text{Ca}_v1.2^{\text{cKO}}$ mice spent in the TQ compared to their control littermates.

The percent time spent in the TQ during each of the probes is presented in Figure 2C. After 14 d of training, mice were left undisturbed for 30 d. Following this 30-d period, a single remote memory probe was administered. While the performance of the control mice during the remote memory probe remained similar to that observed during the last memory probe administered during training, the $\text{Ca}_v1.2^{\text{cKO}}$ mice spent significantly less time searching in the quadrant where the platform was previously located.

Although the $\text{Ca}_v1.2^{\text{cKO}}$ mice exhibited normal water maze performance during the training phase, it could be argued that the spatial memory deficit that we observed after 30 d might have been due to a change in motivation or swimming ability. This seems highly unlikely. We did not observe any significant thigmotactic behavior in the $\text{Ca}_v1.2^{\text{cKO}}$ mice during the remote

Figure 2. $\text{Ca}_v1.2^{\text{cKO}}$ mice exhibit disrupted remote spatial memories. (A) Mice were trained for two trials a day for 14 d. The average time to reach the hidden platform (latency in seconds) is plotted for each training day. The latency to reach the platform in both groups decreased as training progressed, reaching an average escape latency of 16.0 ± 3.6 sec for the $\text{Ca}_v1.2^{\text{cKO}}$ mice and 17.5 ± 3.2 sec for the controls. There was a main effect of training day on the latency to find the hidden platform ($F_{(13,325)} = 11.7$; $p < 0.0001$); however, there was no main effect of genotype on latency ($F_{(1,25)} = 0.24$; $p > 0.05$) and no interaction between training day and genotype ($F_{(13,325)} = 0.86$; $p > 0.05$). (B) A 60-sec probe trial completed before the start of training on day 13 reveals that both $\text{Ca}_v1.2^{\text{cKO}}$ and control littermate mice spend a significant percentage of time during the trial searching in the quadrant where the platform was previously located (TQ; training quadrant) with the $\text{Ca}_v1.2^{\text{cKO}}$ mice spending $57\% \pm 3.7\%$ time in the TQ and control mice searching $57.6\% \pm 8.7\%$ time in the TQ. However, there was no significant difference in the amount of time that the $\text{Ca}_v1.2^{\text{cKO}}$ mice spent in the TQ compared to their control littermates ($F_{(1,24)} = 0.20$; $p > 0.05$; One-Way ANOVA). The dashed line (25%) represents random or "chance" performance; (AR) adjacent right; (AL) adjacent left; (OP) opposite. (C) The percent time that the $\text{Ca}_v1.2^{\text{cKO}}$ mice spent searching in the training quadrant (TQ) during each of the four probes completed during training (on days 4, 7, 10, and 13) was not significantly different when compared to control littermates. However, when tested 30 d later (remote memory probe), the $\text{Ca}_v1.2^{\text{cKO}}$ mice exhibit a degraded search strategy when compared to their littermate controls. In contrast to the performance of the control mice during the remote memory probe ($56.8\% \pm 2.7\%$ time in TQ), which remained similar to that observed during the last memory probe administered during training, the $\text{Ca}_v1.2^{\text{cKO}}$ mice spent significantly less time ($41.7\% \pm 4.2\%$) in the TQ when compared to control littermates ($F_{(1,23)} = 9.4$; $P < 0.01$; One-Way ANOVA). All data are presented as mean $\pm$ SEM.

memory probe (Fig. 3A). Similarly, the average swim speed for control mice and $\text{Ca}_v1.2^{\text{cKO}}$ mice was not significantly different during the remote memory probe (Fig. 3B). Finally, when both groups were subsequently tested in a nonspatial version of the Morris water maze in which the escape platform was clearly marked, the $\text{Ca}_v1.2$ knockout mice and their control littermates exhibited similar escape latencies (Fig. 3C), suggesting that the deficit that we observed during the remote memory test was not likely due to a nonspecific performance issue in the $\text{Ca}_v1.2$ knockout mice.

The principle finding of this study is that forebrain deletion of $\text{Ca}_v1.2$ significantly disrupts remote spatial memories while leaving recently acquired spatial memories (i.e., spatial memories assessed during training) intact. The exact mechanism by which this remote memory deficit arises remains unclear; however, recent *in vitro* experiments demonstrating that $\text{Ca}_v1.2$ plays an obligate role in activation of CREB (Weick et al. 2003, but see also Zhang et al. 2006) lead us to hypothesize that the deficits in long-term memory in the $\text{Ca}_v1.2^{\text{cKO}}$ mice might result from an inability to fully activate CREB. This hypothesis would be con-

Figure 3. $Ca_v1.2^{cKO}$ mice are not thigmotactic, have normal swim speeds, and exhibit normal performance in the visible platform version of the water maze. (A) $Ca_v1.2^{cKO}$ mice exhibited no significant thigmotactic behavior during the remote memory probe, spending $20.0 \pm 2.8\%$ of the trial within 10 cm of the wall, a value that was almost identical to that observed in control littermates ($20.0 \pm 3.2\%$; $F_{(1,23)} = 0.021$; $P > 0.05$; One-Way ANOVA). (B) During the remote memory probe, the average swim speed for control mice (20.8 ± 0.7 cm/sec) and $Ca_v1.2^{cKO}$ mice (22.6 ± 1.0 cm/sec) was not significantly different ($F_{(1,23)} = 2.3$; $P > 0.05$; One-Way ANOVA). (C) During the visible-platform version of the Morris water maze completed following the remote memory probe, both the $Ca_v1.2$ knockout mice and their control littermates exhibited minimal escape latencies (6.5 ± 0.9 and 7.8 ± 0.8 sec, respectively) that were not significantly different from each other ($F_{(1,23)} = 0.94$; $P > 0.05$). All data are presented as mean $\pm$ SEM.

sistent with a recent report using a similar line of $Ca_v1.2$ knockout mice in which deletion of $Ca_v1.2$ resulted in a disruption in CREB signaling in the hippocampus and a deficit in spatial learning (Moosmang et al. 2005). Somewhat in contrast to this previous report, we find that deletion of $Ca_v1.2$ did not result in significant impairments in spatial learning or 24-h memory. This apparent contradiction may reflect differences in the pattern of deletion imposed by the promoter that was used to drive the expression of Cre-recombinase. In the Moosmang et al. (2005) study, Cre-recombinase was driven by the endogenous regulatory sequences of NEX, a gene that encodes the neuronal basic helix-loop-helix protein (Goebbels et al. 2006). Alternatively, our failure to observe spatial learning deficits and/or 24-h memory impairments may reflect the substantial differences in the behavioral tasks used. Spatial learning and memory in the Moosmang et al. (2005) study were assessed using visible platforms in a discriminatory water maze task and the labyrinth maze, whereas the

hidden-platform version of the water maze was used in the present study.

We have previously demonstrated that deletion of $Ca_v1.3$ (the other predominate L-type calcium channel isoform found in brain) results in a consolidation deficit in 24-h memory for fear conditioning but does not alter 24-h memories in the hidden-platform version of the water maze (McKinney and Murphy 2006). Although in our previous report we did not specifically examine remote spatial memory in the $Ca_v1.3$ mice, our new data raise the intriguing possibility that these two different isoforms may play differential roles in specific behaviors and do so on different time scales. In light of the fact that the L-type calcium channel isoforms $Ca_v1.2$ and $Ca_v1.3$ have rather striking differences in cellular localization (Hell et al. 1993) and biophysical properties (Xu and Lipscombe 2001; Helton et al. 2005), it might be reasonable to posit that they play different functional roles in behavior. In addition, it has recently been reported that the two different isoforms exhibit differential signaling to pCREB, with $Ca_v1.3$ more efficient at low levels of activity and $Ca_v1.2$ more efficient at high levels of activity (Zhang et al. 2006), further suggesting a functional dissociation. This notion is further supported by preliminary data suggesting that, in contrast to $Ca_v1.3$, deletion of $Ca_v1.2$ does not alter consolidation of contextual conditioned fear memories when assessed 24 h after training (B.C. McKinney and G.G. Murphy, unpubl.). The hippocampus plays a vital role in the acquisition of new memories; however, once consolidated, these memories become hippocampal-independent. Lesions of the hippocampus made shortly after contextual fear conditioning disrupt recent memories, while lesions at later time points (~ 30 d later) leave remote memories intact (Kim and Fanselow 1992; Anagnostaras et al. 1999). Similar experiments have been conducted using the water maze and post-training lesions (e.g., Clark et al. 2005) or hippocampal inactivation (e.g., Broadbent et al. 2006). In contrast to the results obtained with contextual fear conditioning, hippocampal damage/inactivation does not result in a temporally graded amnesia in the water maze; damage/inactivation of the hippocampus produces deficits in water maze performance regardless of when the training occurred. The resolution of a temporal gradient for spatial memories in the water maze may be confounded by the role that the rodent hippocampus is thought to play in navigation itself (Whishaw et al. 1997). Thus, the $Ca_v1.2^{cKO}$ mice described herein might prove to be a useful tool in this regard, in that deletion of $Ca_v1.2$ disrupts remote memories exclusively.

At present little is known regarding the extrahippocampal structures that subserve remote spatial memories. However, a recent report demonstrating that expression of remote memories preferentially activates c-fos in the anterior cingulate cortex (ACC) coupled with the observation that transient inactivation of the ACC results in profound impairments in the expression of remote memories while leaving recent memories intact suggest that the ACC might be a cortical site of consolidation for remote spatial memories (Teixeira et al. 2006). The ACC is also known to exhibit multiple forms of synaptic plasticity that have been previously ascribed as cellular correlates of learning and memory. Interestingly, synaptic plasticity in the ACC is blocked by L-type calcium channel antagonists (Wei et al. 1999; Liauw et al. 2005). Taken collectively, these results lead to the intriguing hypothesis that the remote memory impairments that we have observed in the $Ca_v1.2^{cKO}$ mice may be due to altered synaptic plasticity within the ACC, and our future experiments will seek to test this hypothesis.

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