

The Down Syndrome Critical Region Protein RCAN1 Regulates Long-Term Potentiation and Memory via Inhibition of Phosphatase Signaling

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Regulator of calcineurin 1 (*RCAN1/MCIP1/DSCR1*) regulates the calmodulin-dependent phosphatase calcineurin. Because it is located on human chromosome 21, *RCAN1* has been postulated to contribute to mental retardation in Down syndrome and has been reported to be associated with neuronal degeneration in Alzheimer's disease. The studies herein are the first to assess the role of *RCAN1* in memory and synaptic plasticity by examining the behavioral and electrophysiological properties of *RCAN1* knock-out mice. These mice exhibit deficits in spatial learning and memory, reduced associative cued memory, and impaired late-phase long-term potentiation (L-LTP), phenotypes similar to those of transgenic mice with increased calcineurin activity. Consistent with this, the *RCAN1* knock-out mice display increased enzymatic calcineurin activity, increased abundance of a cleaved calcineurin fragment, and decreased phosphorylation of the calcineurin substrate dopamine and cAMP-regulated phosphoprotein-32. We propose a model in which *RCAN1* plays a positive role in L-LTP and memory by constraining phosphatase signaling.

Key words: calcineurin; protein phosphatase; mental retardation; hippocampus; learning and memory; synaptic plasticity

Introduction

The regulator of calcineurin proteins (RCANs) are a family of small, highly conserved proteins that can bind and inhibit the calcium-regulated protein phosphatase calcineurin (Fuentes et al., 2000; Kingsbury and Cunningham, 2000; Rothermel et al., 2003). The *RCAN1* gene, also known as *DSCR1*, *MCIP1*, or *calcipressin 1*, encodes several protein isoforms through differential promoter usage (Fuentes et al., 1997; Rothermel et al., 2003). Exon 1 (*RCAN1.1*) and exon 4 (*RCAN1.4*) are the predominant isoforms. *RCAN1* is highly expressed in brain, heart, and skeletal muscle and is elevated in the brains Alzheimer's disease (AD) patients (Ermak et al., 2001). Importantly, the *RCAN1* gene is located on the long arm of human chromosome 21, trisomy of which causes Down syndrome.

Calcineurin controls expression of numerous genes, including *RCAN1.4* (Yang et al., 2000; Rothermel et al., 2003) via the transcription factor nuclear activator of T-cells (NFAT). Newly synthesized *RCAN1.4* protein binds calcineurin, thereby forming a

negative regulatory feedback loop (Yang et al., 2000; Rothermel et al., 2003). Although numerous *in vitro* studies demonstrate that RCAN proteins are potent inhibitors of calcineurin activity, there is substantial controversy regarding their actions *in vivo*. Genetic studies from fungi and mice suggest that low levels of RCAN proteins may actually be required to facilitate some calcineurin-dependent signals (Kingsbury and Cunningham, 2000; Vega et al., 2003; Hilioti et al., 2004; Fox and Heitman, 2005; Sanna et al., 2006). Therefore, RCANs may both inhibit and facilitate calcineurin signaling depending on cellular and developmental context.

Calcineurin is intimately involved in many facets of synaptic plasticity and memory formation because of its ability to directly dephosphorylate critical targets in both the presynaptic and postsynaptic compartments of neurons (Mansuy, 2003). Calcineurin also can indirectly promote dephosphorylation of many other proteins by increasing protein phosphatase 1 (PP1) activity. Calcineurin accomplishes this through dephosphorylation of the PP1 inhibitors inhibitor-1 (I-1) and dopamine and cAMP-regulated phosphoprotein-32 (DARPP-32). Behavioral and electrophysiological studies using both genetic and pharmacological methods to alter calcineurin activity indicate that calcineurin is likely a negative regulator of learning, memory, and synaptic plasticity (Yakel, 1997; Winder and Sweatt, 2001; Mansuy, 2003). Finally, dysregulation of calcineurin activity is associated with many forms of brain disease and injury, including Alzheimer's disease and excitotoxic ischemia (Klee et al., 1998; Mansuy, 2003).

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Because of the importance of calcineurin in regulating diverse neuronal processes, there is growing interest in the potential for trisomy of *RCAN1* to contribute to the plethora of phenotypes associated with Down syndrome through its interaction with calcineurin. Thus, understanding how *RCAN1* contributes normally to mechanisms of learning and memory formation is critical to understanding its role in the deficits observed in Down syndrome and Alzheimer's patients. We hypothesized that *RCAN1* is critical for synaptic plasticity and memory because it serves to maintain a balance between neuronal kinase and phosphatase activities. To test this hypothesis, we compared mice with a disruption of the *RCAN1* gene with their wild-type littermates. These experiments provide the first evidence that *RCAN1* serves to positively regulate hippocampus-dependent memory and hippocampal late-phase long-term potentiation (L-LTP) via suppression of calcineurin.

Materials and Methods

RCAN1 knock-out mice. Generation of *RCAN1* knock-out mice has been described previously (Vega et al., 2003). Mouse genotyping was performed by PCR using mutant and wild-type specific primer sets (Vega et al., 2003). PCR products were visualized by agarose gel electrophoresis.

In situ hybridization. A clone specific for *RCAN1.4* transcripts was generated from a 160 bp fragment spanning the 3' untranslated region and coding regions of exon 4 of the mouse *RCAN1* gene (Yang et al., 2000). The plasmid was linearized, gel purified, and used as template for [³⁵S]UTP-labeled sense and antisense riboprobe synthesis (Maxiscript T7/T3 kit; Ambion, Austin, TX). Specific activity of both probes was >1000 Ci/mmol. Two-month-old male C57BL/6 mice were killed and perfused with freshly prepared, chilled 4% paraformaldehyde/DEPC-PBS. Brains were removed and fixed further by immersion in 4% paraformaldehyde/DEPC-PBS overnight at 4°C with gentle agitation. Tissues were stored in DEPC-PBS at 4°C until paraffin embedment and sectioning.

In situ hybridizations were performed according to procedures described previously (Shelton et al., 2000). Briefly, 4 μm paraffin sections mounted on microscope slides were dewaxed, permeabilized, and acetylated before hybridization at 70°C. For hybridization, riboprobes were diluted in a mixture containing 50% formamide, 0.75 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 10 mM NaPO₄, pH 8.0, 10% dextran sulfate, 1× Denhardt's solution, and 0.5 mg/ml tRNA. After hybridization, the sections were rinsed with increasing stringency washes, subjected to RNase A (2 μg/ml, 30 min at 37°C), and dehydrated before dipping in K5 nuclear emulsion gel (Ilford, Poole, UK). Autoradiographic exposure was performed at 3 weeks.

Behavioral studies. For the Morris water maze (MWM), male *RCAN1* knock-out mice and wild-type littermates were used. The pool had dimensions of 48 inch diameter × 36 inch height. Water in the pool was kept at a constant 21°C. The training for the hidden platform version of the MWM consisted of four trials [60 s maximum; intertrial interval (ITI), 30 min] each day for 8 consecutive days. The probe trial was administered 1 h after the completion of training on day 8. The visible platform task consisted of four trials (ITI, 20 min) each day for 2 consecutive days with the escape platform marked by a visible cue and moved randomly between two locations. The trajectories of the mice were recorded with a video tracking system (HVS Image Analyzing VP-200). Data represent mean ± SEM. ANOVA with repeated measures, genotype as between-group measure, day of testing as repeated measure, were used for statistical analysis with *p* < 0.05 as significance criteria.

For fear conditioning, *RCAN1* knock-out and their wild-type littermates (females and males, 2–4 months old) were used. Data obtained from male and female mice were analyzed separately to determine whether there were gender-specific differences in behavior; none were found and the data were pooled. The training sessions for contextual and cued fear conditioning consisted of a 150 s exploration period followed by two conditioned stimulus–unconditioned stimulus (CS–US) pairings separated by 1 min (footshock intensity, 0.5 mA, 2 s duration; tone, 85 db

white noise, 30 s duration). Context tests were performed in the training chamber after 1 and 24 h. Cue tests were performed in an environmentally altered testing chamber either 2 or 24 h after training. Baseline freezing was monitored (3 min) before presentation of the tone (85 db white noise, 3 min duration). For drug injection experiments, mice were injected intraperitoneally with vehicle [30% DMSO, 70% artificial CSF (ACSF)] or vehicle containing drug (5 mg/kg FK506) or (6 μg/kg Calyculin A). Mice were allowed to recover for 60 min before fear conditioning. Effectiveness of the drug injection was determined by phosphatase activity analysis (supplemental Fig. 2a, available at www.jneurosci.org as supplemental material). Data represent mean ± SEM. One-way ANOVA was used for statistical analysis with *p* < 0.05 as significance criteria.

Electrophysiology. Transverse hippocampal slices (400 μm) were prepared from age-matched mice (12–24 weeks of age) using conventional techniques. Slices were maintained at 30°C in an interface chamber perfused with oxygenated ACSF containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 D-glucose, 2 CaCl₂, and 1 MgCl₂ (2 ml/min). When indicated, ACSF was supplemented with FK506 (A.G. Scientific, San Diego, CA) and Calyculin A (Axxora, San Diego, CA). Extracellular field EPSPs (fEPSPs) were evoked by stimulation of Schaeffer collateral pathway afferents and were measured by recording in stratum radiatum of area CA1. Stable baseline synaptic transmission was established for 20–30 min with a stimulus intensity of 40–50% of the maximum fEPSP before LTP-inducing high-frequency stimulation (HFS). Stimulus intensity of the HFS was matched to the intensity used in the baseline recordings. LTP was induced by either one or four trains (5 min intertrain interval) of 100 Hz HFS for 1 s. Data were collected and presented as the average slope of the fEPSP from six individual traces collected over 2 min and then normalized to baseline recordings of fEPSPs. Hippocampal slices from *RCAN1* knock-out and wild-type mice were prepared simultaneously and placed in a chamber outfitted with dual-recording equipment, thereby minimizing day-to-day variability in slice preparations and recordings. Wild-type control LTP and *RCAN1* knock-out control LTP data were recapitulated in Figure 4a–d. When specified, ACSF was supplemented with either FK506 (10 μM) or Calyculin A (750 nM). One-way ANOVA and *post hoc* tests were used for electrophysiological data analysis with *p* < 0.05 as significance criteria.

Immunoblotting. Soluble protein extracts were prepared by homogenizing the tissue samples in ice-cold buffer [in mM: 50 Tris-HCl, pH 7.5, 150 KCl, 1 DTT, 1 EDTA, 1× complete protease inhibitor cocktail III (Sigma-Aldrich, St. Louis, MO), 1× phosphatase inhibitor cocktail I (Sigma-Aldrich); 1× phosphatase inhibitor cocktail II (Sigma-Aldrich)]. Soluble cytoplasmic and pellet fractions were prepared by ultracentrifugation (100,000 × *g* for 45 min at 4°C) using a Beckman Coulter (Fullerton, CA) Optima TL ultracentrifuge. Proteins were resolved on SDS-polyacrylamide gels, and immunoblotting was performed using standard techniques. A rabbit polyclonal antiserum raised against RCAN1 that detects both isoforms 1.1 and 1.4 was diluted 1:1000 in 10% milk-TTBS (Tween 20 Tris-buffered saline) and used to detect RCAN isoforms 1.1 and 1.4 (a generous gift from Myogen, Denver, CO) (Bush et al., 2004). Phospho-(Thr-34) DARPP-32 and total DARPP-32 antibodies (Cell Signaling Technologies, Beverly, MA) were diluted 1:1000. Calcineurin A (CnA) and Calcineurin B (CnB) antibodies (Abcam, Cambridge, MA) were diluted 1:1000. β-Actin antibody was diluted 1:5000. Laminin AC (Cell Signaling Technology) and voltage-dependent anion channel (VDAC) antibodies (Novus Biologicals, Littleton, CO) were diluted 1:1000. Anti-rabbit and anti-goat HRP tagged antibodies (Promega, Madison, WI) were diluted 1:5000. All antibodies used were diluted in 0.2% I-Block (Tropix, Foster City, CA). All blots were developed using enhanced chemiluminescence detection (GE Healthcare, Fairfield, CT). The bands of each Western blot were quantified from film exposures (Eastman Kodak, Rochester, NY) in the linear range (determined by multiple exposures) for each antibody and normalized initially by total protein and subsequently via densitometry using a desktop scanner and NIH ImageJ software. Data represent mean ± SEM. A Student's *t* test was used for Western blot analysis with *p* < 0.05 as significance criteria.

For fractionation of organelles, hippocampi from three animals were pooled, weighed, and homogenized on ice in 5–10 vol of extraction

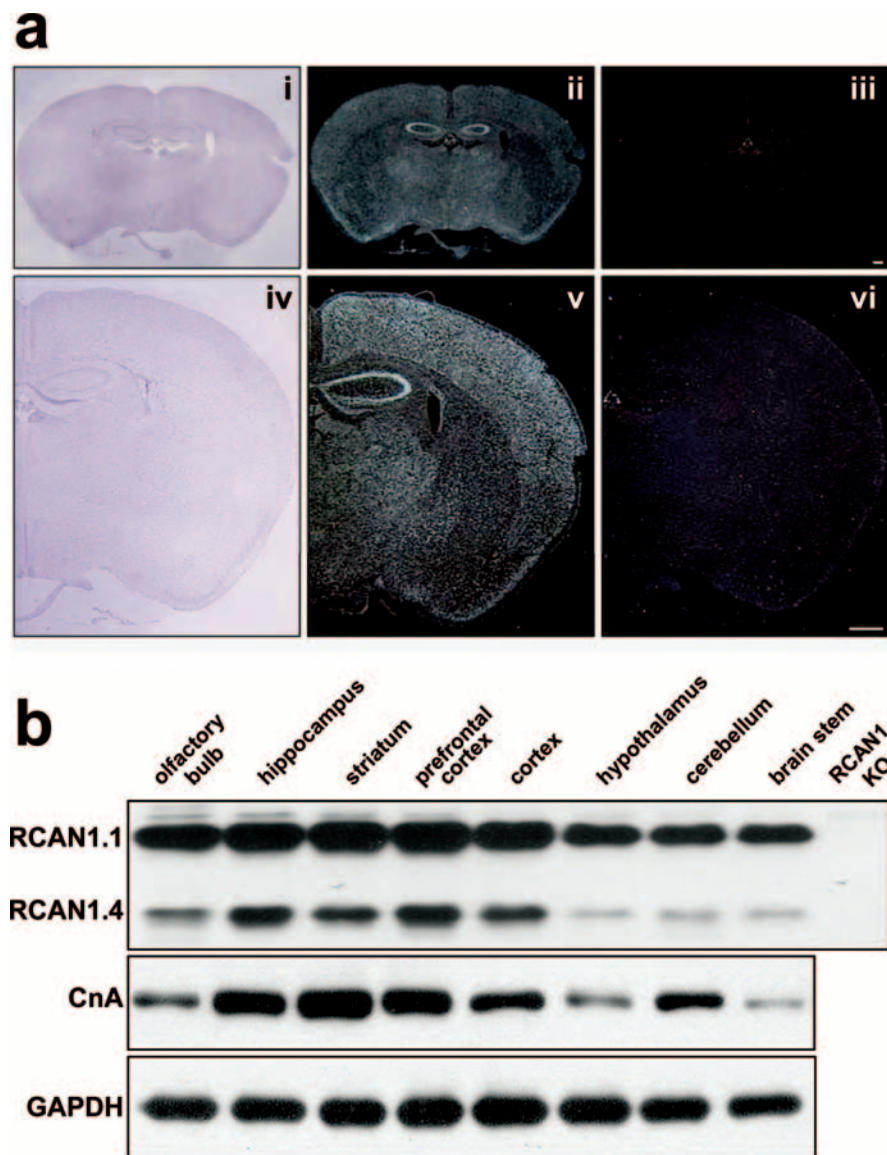


Figure 1. Analysis of *RCAN1.4* mRNA and protein levels in the mouse brain. **a**, *In situ* hybridization using an antisense and sense *RCAN1.4* probes and Nissl staining of sectioned mouse brains. Nissl staining from coronal sections of a wild-type *RCAN1* mouse for reference (*i*, *iv*). In wild-type mice, an antisense *RCAN1.4* riboprobe shows that *RCAN1.4* is expressed throughout the cortex and is highly enriched in the hippocampus and cortex (*ii*, *v*). A sense probe failed to generate any detectable signal (*iii*, *vi*). Hybridization using an antisense *RCAN1.4* probe did not generate signal in *RCAN1* knock-out mice (data not shown). Scale bar, 300 μ m. **b**, Western blot analysis using antibodies to *RCAN1* and calcineurin-A (CnA)-specific antibodies (60 kDa). Both *RCAN1.1* (38 kDa, top band) and *RCAN1.4* (26 kDa, bottom band) are recognized by the *RCAN1* antibody. *RCAN1.1* and *RCAN1.4* were present in all regions of the mouse brain examined; *RCAN1.4* is enriched in tissue in which calcineurin expression is the highest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 37 kDa) is provided as a loading control. In *RCAN1* knock-out (KO) mice, *RCAN1.1* and *RCAN1.4* are completely absent.

buffer (in mM: 4 HEPES, pH 7.4, 320 sucrose, 1 DTT, 1 orthovanadate, and 1 \times complete protease inhibitor cocktail) using a 2 ml Kontes Dounce tissue grinder (A and B pestle, 20 strokes each). The homogenate was centrifuged 3 min at 800 \times g, 4°C. The nuclear-enriched pellet (P1) was washed twice with extraction buffer. The supernatant then was centrifuged for 55 min at 17,000 \times g, 4°C. The mitochondrial-enriched pellet (P2) was washed twice. The supernatant from P2 contained the soluble cytoplasmic fraction.

Calcineurin activity assay. Hippocampal tissue was harvested from wild-type and *RCAN1* knock-out mice (pooled samples contain at least three whole hippocampi) collected from mice aged (12–24 weeks) at identical times during the day to reduce variability because of possible circadian regulation of calcineurin activity. Soluble protein extracts were

prepared by homogenizing the tissue samples in ice-cold lysis buffer containing protein inhibitor cocktail III (Sigma-Aldrich). Soluble cytoplasmic and pellet protein fractions were prepared by ultracentrifugation (100,000 \times g for 45 min at 4°C). The pelleted fraction was resuspended in assay buffer. Excess sample phosphate was removed using freshly prepared columns containing Desalting Resin (Bio-Rad, Hercules, CA). Protein extracts were quantified and equal protein amounts were incubated for 30 min after general instructional guidelines for the Biomol Green QuantiZyme Assay System (Biomol, Plymouth, PA). Liberated phosphate was colorimetrically measured using a KC Junior plate reader at λ 620. The resulting calcineurin activity was plotted as arbitrary units, and data represented as means \pm SEM. Student's *t* test was used for Western blot analysis with *p* < 0.05 as significance criteria.

Protein phosphatase-1 activity assay. Hippocampal lysates were prepared in the same way as for calcineurin assays. Protein extracts were quantified, and equal protein amounts were incubated for 30 min following general instructional guidelines for the EnzCheck Phosphatase Assay kit, using DiFUMP as a substrate (Invitrogen, Carlsbad, CA). Liberated fluorescent phosphatase substrate was measured using a Synergy2 microplate reader using an excitation 360 λ and emission 460 λ . PP1 activity was determined by subtracting total phosphatase activity from phosphatase activity after 20 min preincubation of lysate with 100 nM nuclear inhibitor of protein phosphatase 1 (EMD Biochemicals, San Diego, CA). The resulting PP1 activity was plotted as fold of wild-type, and data represented as means \pm SEM. Single-factor ANOVA analysis was used for analysis with *p* < 0.05 as significance criteria.

Results

RCAN1.4 is most abundant in areas of the brain in which calcineurin is most abundant

Although it is well established that *RCAN1* is highly expressed in the brain, no studies to date have distinguished between the various *RCAN1* isoforms. We were specifically interested in determining the pattern of expression for *RCAN1.4* because this transcript is under the control of calcineurin via a cluster of NFAT sites in the promoter directly upstream of exon 4 (Yang et al., 2000). By monitoring *RCAN1.4* protein levels, we sought to determine

in which regions of the brain the *RCAN1*/calcineurin feedback loop is active. *In situ* hybridization using an oligonucleotide antisense probe specific for *RCAN1.4* revealed that *RCAN1.4* mRNA is highly expressed in the mouse brain and is particularly enriched in hippocampal pyramidal neurons (Fig. 1*aii*,*av*). A sense probe for the same sequence produced no signal (Fig. 1*aiii*,*avi*). Brain sections from wild-type and *RCAN1* knock-out mice were examined at different ages during postnatal development. No gross anatomical difference between wild-type and *RCAN1* knock-out mice was detected (supplemental Fig. 1*a*,

available at www.jneurosci.org as supplemental material). RCAN1 protein levels were assessed by Western blots using an antibody that detects both the RCAN1.1 and RCAN1.4 isoforms. RCAN1.1 protein was abundant throughout the brain, with highest levels in the cortex and hippocampus (Fig. 1*b*). RCAN1.4 was less abundant than RCAN1.1 and more variable region to region. RCAN1.4 protein levels were enriched in the hippocampus striatum, cortex, and prefrontal cortex (supplemental Fig. 1*b,d*, available at www.jneurosci.org as supplemental material). Notably, the levels of RCAN1.4 protein correlated with the level of the catalytic subunit of calcineurin, CnA. Regions of the brain with the highest levels of CnA also contained the highest levels of RCAN1.4 (Fig. 1*b*). Brain extracts from *RCAN1* knock-out mice lacked both RCAN1 isoforms but contained normal levels of CnA, suggesting that, although RCAN1.4 protein levels are dependent on calcineurin, calcineurin levels are independent of *RCAN1* (supplemental Fig. 1*c*, available at www.jneurosci.org as supplemental material).

RCAN1 knock-out mice exhibit impaired spatial learning and memory

To test whether *RCAN1* is involved in learning and memory, *RCAN1* knock-out mice and their wild-type littermates were trained in the hidden platform version of the MWM, which measures the ability to learn the relationship between distal environmental cues and the location of an escape platform obscured by opaque water (Morris, 1984). During the acquisition phase of the task, both *RCAN1* knock-out mice and their wild-type littermates exhibited decreasing escape latencies throughout the MWM training, indicating that both groups of mice learned the location of the escape platform. However, escape latencies of the *RCAN1* knock-out mice were significantly longer than their wild-type littermates (Fig. 2*a*). This impairment was not attributable to differences in swim speed or impaired vision, as assessed by visual platform on days 9 and 10 (Fig. 2*a*). Moreover, *RCAN1* knock-out mice performed similar to wild-type littermates in motor performance and learning as determined by the rotating rod test (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). These results indicate that the *RCAN1* knock-out mice have a spatial learning impairment rather than a deficit in gross sensorimotor ability.

To assess spatial memory, the *RCAN1* mutant mice were tested in a probe trial in which the escape platform was removed from the maze. In the probe test, *RCAN1* knock-out mice did not demonstrate a specific preference for the target quadrant (Fig. 2*b*). In addition, the *RCAN1*-deficient mice exhibited a significant reduction in the number of platform crossings when compared with their wild-type littermates (Fig. 2*c*). The combined results from MWM training and probe testing indicate compromised spatial learning and memory in the mice lacking *RCAN1*.

RCAN1 knock-out mice display normal contextual fear memory but reduced cued fear memory that is rescued by inhibition of calcineurin

To determine how the loss of *RCAN1* affects associative learning and memory, we tested the mice using a conditioned fear paradigm. In this paradigm, mice display memory for environmental context or an emotionally neutral CS (white noise tone) resulting from its temporal association with an aversive US (footshock) through a stereotypical behavioral response known as “freezing” (Phillips and LeDoux, 1992).

For contextual and cued conditioning, *RCAN1* knock-out mice and their wild-type littermates were trained with a pair of

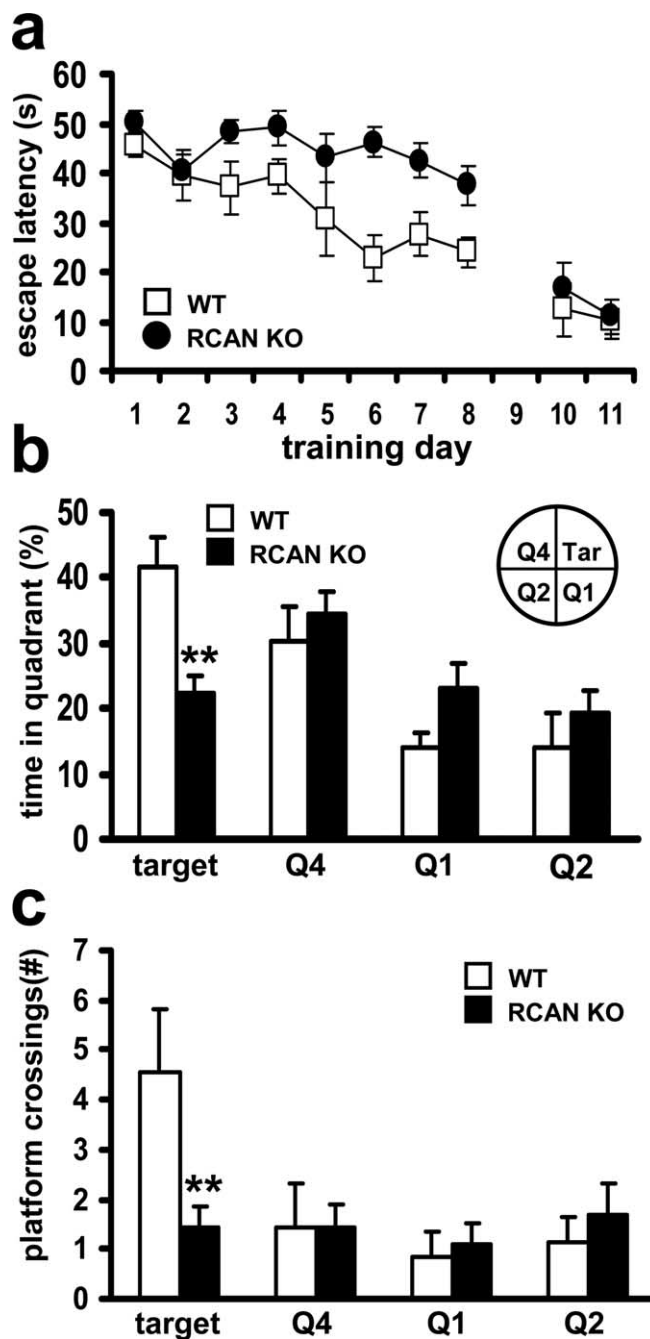


Figure 2. The *RCAN1* knock-out mice display impaired spatial learning and memory. *a*, Escape latency times in hidden platform Morris water maze training. For acquisition (days 1–8), $p < 0.01$, ANOVA; for visible platform performance (days 10–11), $p > 0.05$, ANOVA. *b*, The mean time spent in each maze quadrant (Q) during the probe test (** $p < 0.0001$, ANOVA). *c*, The mean number of platform crossings (equivalent location) for each quadrant during probe test. Wild-type (WT) mice, $n = 9$; *RCAN1* knock-out mice (RCAN KO), $n = 11$ (** $p < 0.0001$, ANOVA).

CS–US stimulations during exploration of a new environment (context). *RCAN1*-deficient mice exhibited comparable performance in both short-term memory (STM) and long-term memory (LTM) for the training context (Fig. 3*a*), which suggests that *RCAN1*-deficient mice have normal associative learning and unaltered contextual fear memory. In contrast to contextual conditioning, the *RCAN1* knock-out mice demonstrated a deficit in both short- and long-term cued memory (Fig. 3*b*). The memory impairment was more pronounced during STM testing but was

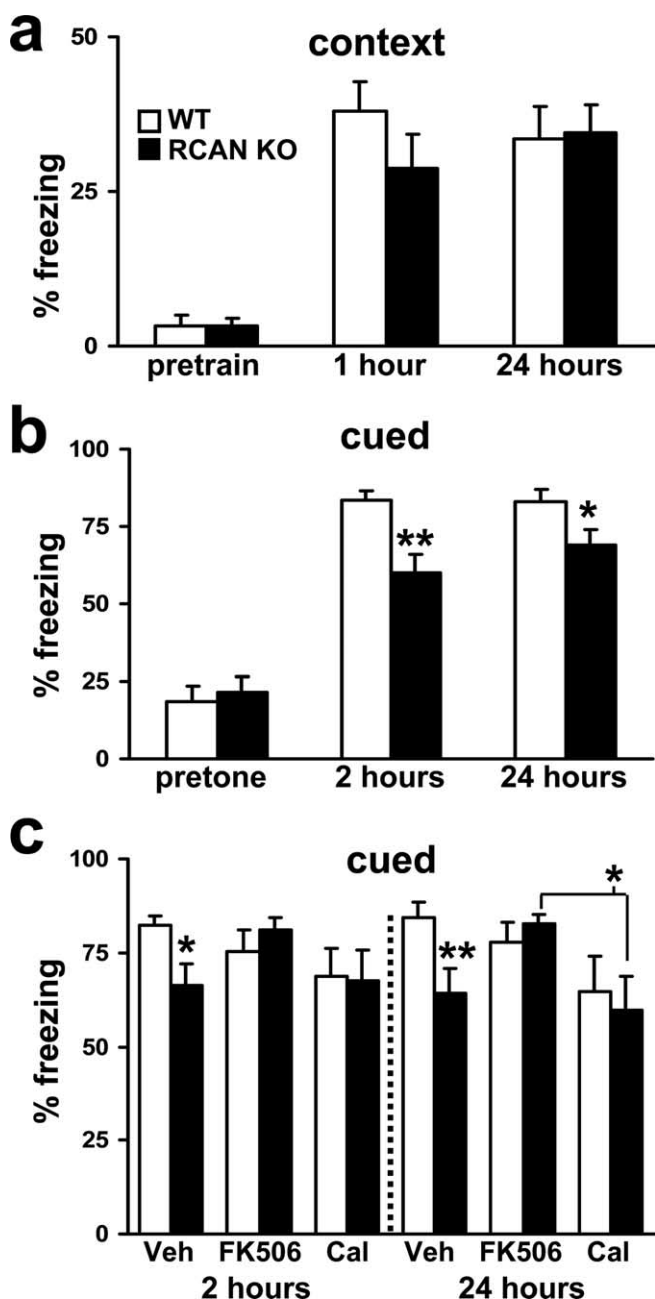


Figure 3. *RCAN1* knock-out mice display cued associative fear memory deficits. **a**, Mean freezing behavior of wild-type and *RCAN1* knock-out mice. Both genotypes performed similarly during training and had similar freezing behavior in both short-term (1 h) and long-term (24 h) contextual memory tests ($p > 0.05$, ANOVA). **b**, *RCAN1* knock-out mice displayed impaired and short-term (2 h) and long-term (24 h) cued fear memory but had similar levels of pretone freezing ($p > 0.05$, ANOVA). Wild-type (WT) mice, $n = 22$; *RCAN1* knock-out mice (RCAN KO), $n = 16$ (* $p < 0.05$, ** $p < 0.01$, ANOVA). **c**, Inhibition of calcineurin rescues deficits in both STM and LTM for cued conditioning in the *RCAN1* knock-out mice. Vehicle-injected (Veh): wild-type (WT) mice, $n = 10$; *RCAN1* knock-out mice (RCAN KO), $n = 10$. FK506-injected (FK506): WT, $n = 9$; RCAN KO $n = 8$. Calyculin A-injected (Cal): WT, $n = 8$; RCAN KO, $n = 8$ (* $p < 0.05$, ** $p < 0.01$, ANOVA).

still evident 24 h later during LTM testing (Fig. 3b). Because RCAN1 is a potent inhibitor of calcineurin activity, we examined fear memory after pretraining with the calcineurin inhibitor FK506. Injection of FK506 60 min before fear conditioning rescued the deficits in both STM and LTM observed in *RCAN1* mutant mice, returning the levels of freezing to those observed in

wild-type mice (Fig. 3c). Because calcineurin can regulate PP1 via phosphorylation of either DARPP or I-1, we also examined whether pretraining PP1 blockade would also rescue the memory deficits observed in *RCAN1* knock-out mice. In contrast, injection of the PP1/protein phosphatase 2A (PP2A) inhibitor Calyculin A did not rescue either the STM or LTM deficits displayed by the *RCAN1* mutant mice (Fig. 3c). Interestingly, injection of Calyculin A significantly reduced LTM for cued conditioning in wild-type mice (Fig. 3c). The effectiveness of the drugs to inhibit phosphatase activity *in vivo* was confirmed by phosphatase activity assays from hippocampal extracts (supplemental Fig. 2a, available at www.jneurosci.org as supplemental material). These results indicate that *RCAN1* plays a role in associative fear memory. Moreover, the impairments in fear memory caused by the deletion of *RCAN1* can be ameliorated by inhibiting calcineurin activity, which suggests that the memory deficits observed in *RCAN1* mutant mice are not exclusively developmental in nature. Altogether, these findings indicate that *RCAN1* is required for the formation and consolidation of some forms of memory but is dispensable for others.

Deletion of *RCAN1* does not alter basal synaptic transmission but blunts paired-pulse facilitation in hippocampal area CA1

We next investigated synaptic plasticity at the Schaffer collateral-CA1 synapse in hippocampal slices from *RCAN1* mutant mice. Because calcineurin can influence presynaptic plasticity via regulation of synaptic vesicle release and uptake (Wang and Kelly, 1997), it is possible that the loss of *RCAN1* affects basal synaptic function. We found that basal synaptic transmission, measured as synaptic output in response to a stimulatory input, was normal in *RCAN1* knock-out mice (Fig. 4a). We next examined whether *RCAN1* was required for normal paired-pulse facilitation (PPF), a form of presynaptic plasticity evoked by two temporally linked stimuli (Katz and Miledi, 1968). PPF was reduced at several time intervals in the *RCAN1*-deficient mice compared with wild-type littermates (Fig. 4b). This reduction in PPF was similar to that observed in a previous study examining the role of calcineurin in hippocampal synaptic plasticity (Winder et al., 1998). In this report, overexpression of a constitutively active form of the calcineurin catalytic subunit resulted in alterations in synaptic plasticity, some of which could be rescued via pharmacological blockade of phosphatase activity with Calyculin A. To assess whether the alterations in PPF observed in the *RCAN1* knock-out mice occur through similar mechanisms, we attempted to rescue the reduction in PPF with either Calyculin A or FK506. We found that Calyculin A reversed the reduced PPF in *RCAN1*-deficient mice (Fig. 4c). Direct inhibition of calcineurin activity with FK506 failed to rescue the PPF phenotype in the *RCAN1* mutant mice (Fig. 4c). The effectiveness of the drugs to inhibit phosphatase activity in slices was confirmed by phosphatase activity assays from hippocampal slices (supplemental Fig. 2b, available at www.jneurosci.org as supplemental material). The short-term plasticity deficits exhibited by the *RCAN1* knock-out mice appear to be insensitive to pharmacological inhibition of calcineurin activity, but they may be acutely rescued by an inhibitor of downstream phosphatases regulated by calcineurin through DARPP-32 dephosphorylation.

RCAN1 knock-out mice exhibit impaired L-LTP in hippocampal area CA1

LTP can be divided into at least two components: early-phase (E-LTP) and L-LTP. A single 100 Hz train of HFS elicits E-LTP, an enhancement of synaptic transmission that requires neither

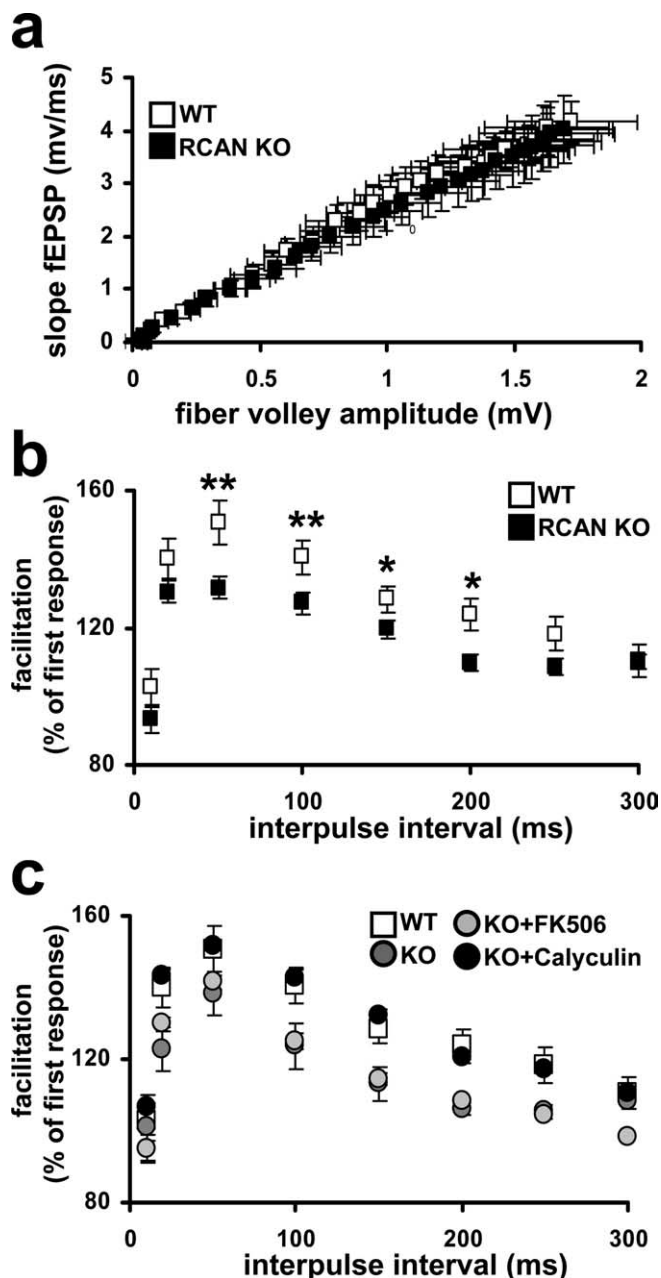


Figure 4. *RCAN1* knock-out mice have normal basal synaptic transmission but have deficits in PPF. **a**, Input versus output plot shows that wild-type and *RCAN1* knock-out mice had comparable fEPSP slopes evoked by increasing stimulation. Wild-type (WT) mice, *RCAN1* knock-out mice (RCAN KO), $n = 24$ slices, 16 mice per genotype ($p > 0.05$, Student's t test). **b**, *RCAN1* knock-out mice had reduced PPF compared with their wild-type littermates. The percentage facilitation, determined by the ratio of the second fEPSP to the first fEPSP, is shown at interpulse intervals from 10 to 300 ms. Wild-type (WT) mice, *RCAN1* knock-out mice (RCAN KO) $n = 22$ slices, 14 mice per genotype ($*p < 0.05$, $**p < 0.01$, ANOVA). **c**, PPF deficits in *RCAN1* knock-out mice are rescued by pretreatment with the PP1 inhibitor Calyculin A but not the calcineurin inhibitor FK506. Wild-type (WT) mice, *RCAN1* knock-out mice (KO), *RCAN1* knock-out mice pretreated with 10 μM FK506 (KO+FK506), *RCAN1* knock-out mice pretreated with 750 nM Calyculin A (KO+Calyculin), $n = 16$ slices, 8 mice per genotype or treatment (KO+Calyculin compared with WT at all time points, $p > 0.05$, ANOVA).

protein kinase A (PKA) activity nor new RNA and protein synthesis. The more robust and stable L-LTP that can be elicited by four trains of HFS requires PKA activity and both RNA and protein synthesis (Huang et al., 1996). Previous studies using pharmacological or genetic manipulation of calcineurin activity have

shown variable effects on E-LTP (Winder et al., 1998; Zhuo et al., 1999; Malleret et al., 2001; Zeng et al., 2001). However, it is generally accepted that calcineurin acts to gate the transition of E-LTP to more enduring forms such as L-LTP that are dependent on PKA (Wang and Kelly, 1996; Winder et al., 1998; Malleret et al., 2001).

First, we examined the effects *RCAN1* deletion on E-LTP induced with one train of HFS. This pattern of stimulation elicited E-LTP in *RCAN1* knock-out mice indistinguishable from that in wild-type littermates (Fig. 5*a*). To test whether the loss of *RCAN1* altered L-LTP, we stimulated hippocampal slices with four spaced trains of HFS. L-LTP in the *RCAN1*-deficient mice was significantly impaired, in both the initial amplitude and the overall duration of potentiation, when compared with their wild-type littermates (Fig. 5*b*). This result also is consistent with findings from studies of L-LTP in mice with increased expression of constitutively active calcineurin (Winder et al., 1998). We next investigated whether the observed LTP deficit could be rescued by pharmacological blockade of protein phosphatases. Hippocampal slices were preincubated with either FK506 or Calyculin A, and then L-LTP was elicited using four trains of HFS. As with PPF, we found that the calcineurin-specific inhibitor FK506 failed to rescue the L-LTP impairments in the *RCAN1* knock-out mice (Fig. 5*c*). Furthermore, FK506 also impaired L-LTP in wild-type mice (Fig. 5*c*). Conversely, Calyculin A pretreatment restored L-LTP in the *RCAN1* knock-out mice to levels of potentiation observed in wild-type mice. In contrast to its effect on LTP in slices from *RCAN1* knock-out mice, Calyculin A produced L-LTP deficits in wild-type slices similar to those observed in FK506-treated slices (Fig. 5*d*). Reduced L-LTP in wild-type hippocampal slices by FK506 and Calyculin A was not entirely unexpected, because a reduction in L-LTP has been observed in previous studies using calcineurin and PP1 inhibitors (Lu et al., 1996; Winder et al., 1998). Together, our findings closely resemble the results obtained from transgenic mice with increased calcineurin activity. This implies that *RCAN1* facilitates long-lasting synaptic plasticity through inhibition of calcineurin.

Specific pools of calcineurin are more active in the hippocampus of *RCAN1* knock-out mice

The preceding behavioral and electrophysiological studies suggest that calcineurin activity is increased in the hippocampus of *RCAN1* knock-out mice. To assess whether the capacity for calcineurin signaling is altered in *RCAN1* mutant mice, we used enzymatic assays to measure calcineurin activity in extracts made from hippocampal slices. This assay was performed at saturating levels of calcium and calmodulin to fully activate all of the calcineurin in the extract. Thus, this assay is a measure of maximal potential activity in the tissue rather than the actual *in vivo* activity. We found an increase in hippocampal calcineurin activity in total cell lysates from *RCAN1* knock-out animals compared with wild-type mice (Fig. 6*a*). Because calcineurin can be localized to specific subcellular domains, we performed fractionation studies in which organelles and membranes were separated from cytoplasm by centrifugation. We found that soluble cytoplasmic calcineurin activity was not significantly different in the *RCAN1*-deficient mice compared with wild-type mice (Fig. 6*a*). In contrast, calcineurin activity was elevated in the pellet fraction of lysates from *RCAN1* knock-outs. This finding supports a model in which *RCAN1* primarily functions as an inhibitor of calcineurin activity in the hippocampus. Moreover, it suggests that the action of *RCAN1* may be selective for specific subcellular pools of calcineurin.

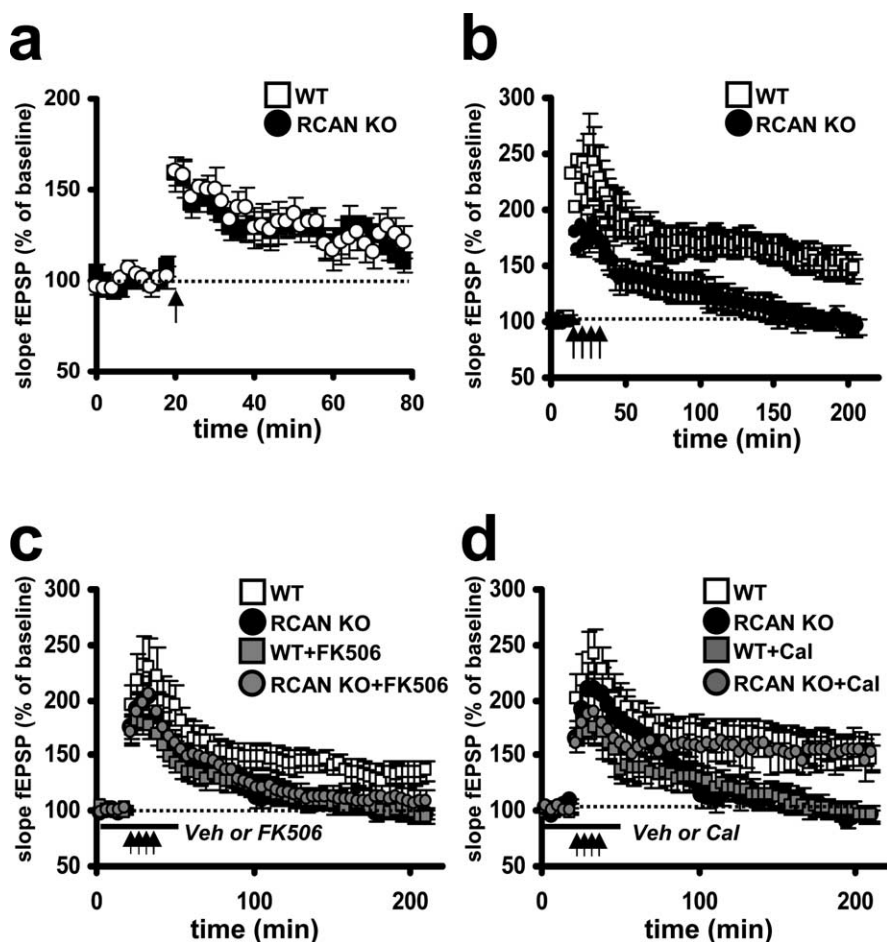


Figure 5. *RCAN1* knock-out mice express normal E-LTP but have deficits in L-LTP. *a*, A single train of HFS (100 Hz for 1 s) evoked similar levels of E-LTP in wild-type and *RCAN1* knock-out mice that decayed to baseline levels after 80 min. Wild-type (WT) mice, *RCAN1* knock-out mice (*RCAN1* KO), $n = 14$ slices, 9 mice per genotype ($p > 0.05$, ANOVA). *b*, Four trains of HFS (100 Hz trains for 1 s, intertrain interval of 5 min) evoked L-LTP in wild-type mice that persisted at least 3 h but did not evoke L-LTP in the *RCAN1* knock-out mice. Wild-type (WT) mice, *RCAN1* knock-out mice (*RCAN1* KO), $n = 18$ slices, 11 animals per genotype ($p < 0.001$, ANOVA). *c*, The L-LTP deficit in *RCAN1* knock-out mice was not rescued by pretreatment with the calcineurin inhibitor FK506. Pretreatment with FK506 also impaired L-LTP in wild-type slices. Wild-type (WT) mice, *RCAN1* knock-out mice (*RCAN1* KO), wild-type mice pretreated with $10 \mu\text{M}$ FK506 (WT+FK506), *RCAN1* knock-out mice pretreated with $10 \mu\text{M}$ FK506 (*RCAN1* KO+FK506), $n = 12$ slices, 7 mice per genotype or treatment ($p < 0.01$, WT vs WT + FK506; $p > 0.05$, WT + FK506 vs *RCAN1* KO; ANOVA). *d*, The L-LTP deficit in *RCAN1* knock-out mice was rescued by pretreatment with the protein phosphatase-1 inhibitor Calyculin A. Pretreatment with Calyculin A also resulted in impaired L-LTP in wild-type mice. Wild-type (WT) mice, *RCAN1* knock-out mice (*RCAN1* KO), wild-type mice pretreated with 750 nM Calyculin A (WT+Cal), *RCAN1* knock-out mice pretreated with 750 nM Calyculin A (*RCAN1* KO+Cal), $n = 16$ slices, 10 animals per genotype or treatment ($p < 0.001$, WT vs WT + Cal; $p > 0.05$, WT vs *RCAN1* KO + Cal; ANOVA). Veh, Vehicle.

We proceeded to determine whether the increase in calcineurin activity in the *RCAN1* knock-out mice was a result of changes in either the abundance or subcellular location of calcineurin protein with Western blot analysis. The full-length, 60 kDa CnA subunit was present in both the soluble cytosolic and pellet (containing both mitochondrial and nuclear compartment) fractions at comparable levels in wild-type and *RCAN1* knock-out mice (supplemental Fig. 1c, available at www.jneurosci.org as supplemental material) (Fig. 6b). Therefore, the elevation in calcineurin activity in the hippocampus from *RCAN1* mutant mice is not because of a corresponding change in the abundance of the catalytic subunit of calcineurin but more likely attributable to a loss of inhibition by RCAN1. We also noticed an increase in a 48 kDa constitutively active fragment of CnA that can be generated by calpain cleavage (Wu et al., 2004; Shioda et al., 2006) was present in the hippocampus of *RCAN1*

mutant mice. No difference in the abundance of the 48 kDa fragment of CnA was observed in the soluble supernatant fraction between *RCAN1* mutant mice and wild-type mice. However, in the pellet fraction, we observed increased levels of the 48 kDa isoform in the *RCAN1*-deficient mice (Fig. 6b). The increased abundance of the 48 kDa isoform in *RCAN1* knock-out mice does suggest that, in addition to inhibiting calcineurin activity, *RCAN1* may also help protect full-length CnA from cleavage by calpain. Although RCAN1 protein was present in both the supernatant and pellet fractions from wild-type mice (Fig. 6c), calcineurin activity and CnA cleavage was increased only in the pelleted fraction, which is enriched for nuclei and mitochondria (Fig. 6b). We further fractionated these subcellular compartments using differential centrifugation of whole hippocampus pooled from several animals to assess the location of RCAN1 and calcineurin proteins. The purity of the fractionation was verified using antibodies for nuclear-specific Laminin AC and mitochondrial-specific VDAC (Fig. 6d). CnA was present in both the soluble and mitochondrial-enriched fractions but was absent from nuclei. RCAN1.1 and RCAN1.4 shared the same pattern of distribution. These results demonstrate that RCAN1 and calcineurin proteins are available to interact in either the cytoplasm or mitochondria of the hippocampus. The lack of either calcineurin or RCAN1 in the nuclear-enriched fraction in Figure 6d suggests that the elevated calcineurin activity measured in Figure 6a is primarily from calcineurin localized in mitochondria. Previous studies have shown that both calcineurin (Shibasaki et al., 1997) and RCAN (Chang and Min, 2005) proteins can localize to mitochondria. Furthermore, substantial experimental data indicate that mitochondrial activities can influence synaptic plasticity (Ben-Shachar and Laifenfeld, 2004; Jonas, 2006; Mattson, 2006). Whether RCAN1 and calcineurin localized to mitochondria are involved in this process remains to be determined.

Phosphorylation of DARPP-32 is decreased in the hippocampus of *RCAN1*-deficient mice

Calcineurin activity promotes dephosphorylation of a number of proteins important for synaptic plasticity either directly or indirectly by releasing repression of PP1 activity (Yakel, 1997; Klee et al., 1998; Snyder et al., 1998). We examined the phosphorylation state of a direct target of calcineurin, DARPP-32. PKA-phosphorylated forms of DARPP-32 and the closely related I-1 inhibit PP1 activity. Calcineurin dephosphorylates both DARPP-32 and I-1, releasing repression of PP1 and promoting dephosphorylation of PP1 substrates (Hemmings et al., 1984; Snyder et al., 1998; Bibb et al., 1999). Because we detected ele-

vated CnA activity in hippocampal lysates from *RCAN1* knock-out mice, we posited that PP1 activity would also be elevated in *RCAN1* knock-out mice. Indeed, PP1 activity was elevated in hippocampal lysates from the *RCAN1* mutant mice (Fig. 7*a*). In preparations in which cytoplasmic, mitochondrial, and nuclear compartments were isolated, elevated PP1 activity was detected primarily in the mitochondrial fraction (Fig. 7*a*). This finding is consistent with the levels of calcineurin activity in the *RCAN1* knock-out mice after crude fractionation (Fig. 6*a*). We then separated hippocampal lysates into crude supernatant and pellet (mixture of cytoplasmic/nuclear) fractions and then quantified total and phosphorylated DARPP-32. Total DARPP-32 protein levels were similar in *RCAN1* knock-out and wild-type lysates. In contrast, there was a significant reduction in the level of DARPP-32 phosphorylated at threonine-34, the calcineurin dephosphorylation site, in both the soluble and crude pellet fractions of hippocampal lysates from *RCAN1* mutant mice (Fig. 7). The decreased DARPP-32 phosphorylation is consistent with an increase in calcineurin activity and a derepression of PP1 activity in the hippocampus of the *RCAN1*-deficient mice. Repression of this downstream elevation in PP1 activity may be the mechanism through which Calyculin A rescues the L-LTP impairment in the *RCAN1* knock-out mice (Fig. 5*d*).

Discussion

In this study, we used mice with a genetic disruption of the *RCAN1* locus to assess the role of *RCAN1* in learning and memory. *RCAN1* knock-out mice lack expression of all known *RCAN1* transcripts and their encoded proteins, including the *RCAN1.1* and *RCAN1.4* isoforms that are abundant in the hippocampus. We found that the *RCAN1* knock-out mice displayed deficits in learning and memory for several tasks, as well as hippocampal L-LTP. Biochemical analysis of hippocampal lysates from the *RCAN1* knock-out mice revealed that they have increased enzymatic activity of both calcineurin and PP1 and decreased phosphorylation of the calcineurin substrate DARPP-32, consistent with an elevation in calcineurin activity in the hippocampus of *RCAN1* knock-out mice. The findings presented here represent, to the best of our knowledge, the first studies demonstrating a critical role for *RCAN1* in the proper manifestation of memory and L-LTP.

We found that the *RCAN1* knock-out mice have pronounced spatial learning and memory deficits in the MWM task (Fig. 2) and STM and LTM impairments after associative cued fear conditioning (Fig. 3). These deficits were similar to those found in

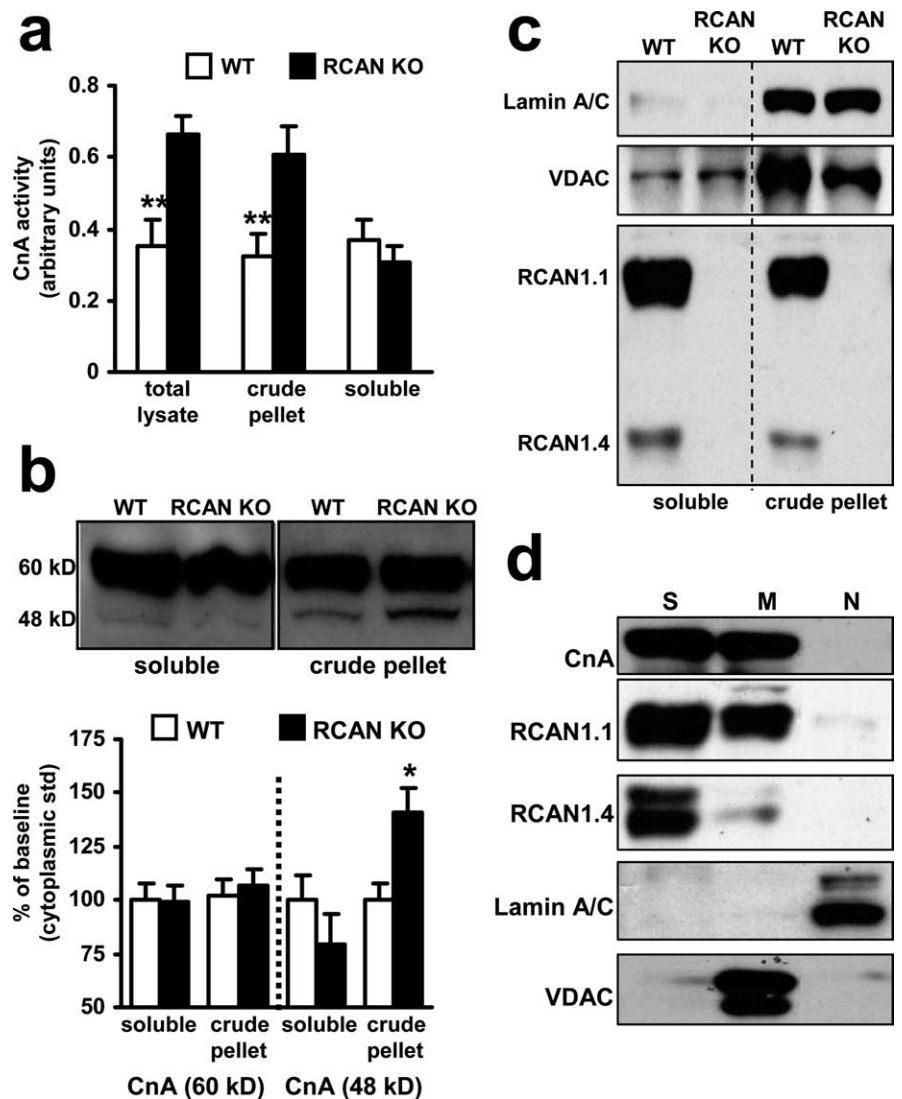


Figure 6. *RCAN1* knock-out mice exhibit elevated calcineurin activity. **a**, Calcineurin activity is upregulated in hippocampal extracts from *RCAN1* knock-out mice compared with wild-type mice. Total lysate, $n = 3$ both genotypes (** $p < 0.01$, Student's t test); crude pellet fraction (nuclear/mitochondrial enriched), $n = 9$ both genotypes (** $p < 0.01$, Student's t test); soluble fraction (cytoplasmic), $n = 11$ both genotypes ($p > 0.05$, Student's t test). **b**, Western blot analysis of calcineurin isoforms in hippocampal extracts. There was no difference in calcineurin levels between wild-type and *RCAN1* knock-out mice with respect to the 60 kDa isoform. A 48 kDa calcineurin isoform was present in greater levels in crude pellet (nuclear/mitochondrial enriched) extracts (represented as percentage of wild-type soluble cytoplasmic levels for each isoform). $n = 9$, each genotype, wild-type (WT) mice, *RCAN1* knock-out mice (RCAN KO) (* $p < 0.05$, ANOVA). **c**, *RCAN1.1* and *1.4* are absent from the pellet and soluble fractions of *RCAN1* knock-out mice after crude lysate fractionation. Mitochondrial (VDAC) and nuclear [Laminin AC (Laminin A/C)] proteins are both present in the crude pellet fraction. **d**, Organelle fractionation of hippocampi from wild-type mice: soluble cytoplasmic fraction (S), mitochondrial-enriched fraction (M), and nuclear-enriched fraction (N). CnA, *RCAN1.1*, and *RCAN1.4* were found in both the soluble and mitochondrial-enriched fractions. The nuclear protein Laminin AC and the mitochondrial protein VDAC were used to confirm the purity of the fractionation. Extracts in **c** were isolated without phosphatase inhibitors so that calcineurin activity could be measured. Extracts in **d** were isolated with phosphatase inhibitors, so phosphorylation of *RCAN1.4* was preserved, resulting in the observed doublet.

mice with inducible, hippocampal-restricted overexpression of constitutively active calcineurin (Mansuy et al., 1998a,b) and the direct opposite of learning behaviors of animals in which calcineurin was inhibited by either transgenic expression of a calcineurin inhibitory domain or application of antisense oligonucleotides (Ikegami and Inokuchi, 2000; Malleret et al., 2001). This suggests that *RCAN1* provides a constraint on calcineurin activity during learning and memory and that this constraint is absent in the *RCAN1* knock-out mice. Importantly, the cued fear memory deficit displayed by *RCAN1* knock-out mice was rescued by

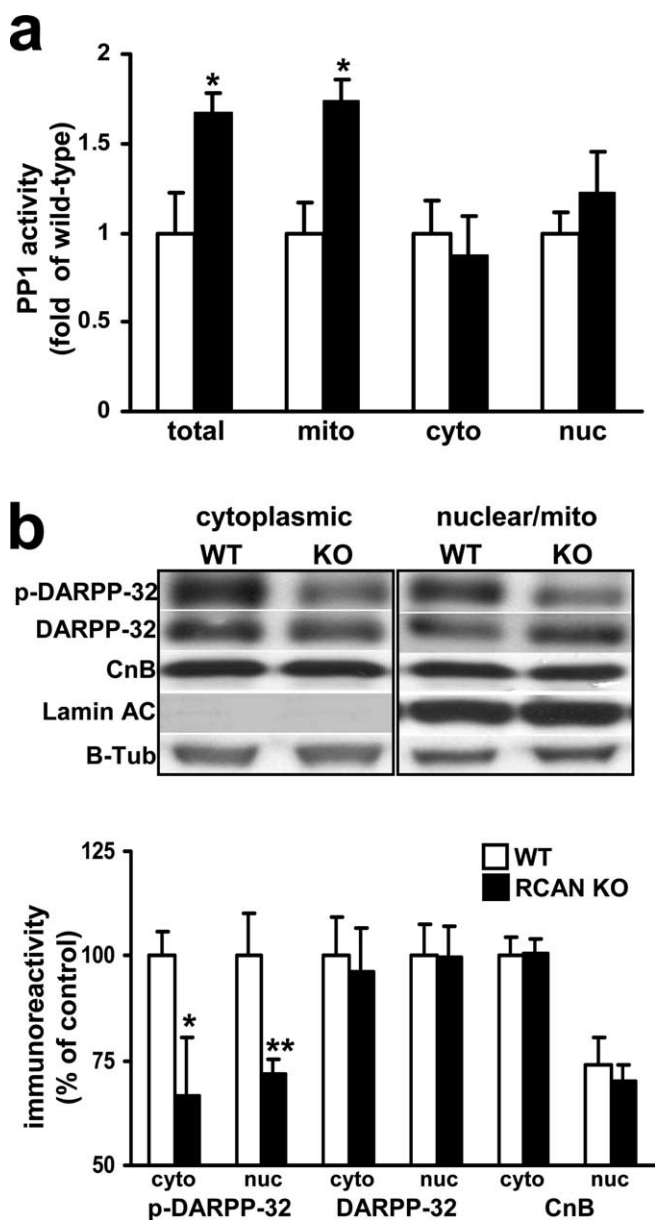


Figure 7. *RCAN1* knock-out mice exhibit elevated PP1 activity and decreased DARPP-32 phosphorylation. **a**, PP1 activity is upregulated in hippocampal extracts from *RCAN1* knock-out mice compared with their wild-type littermates. Total lysate (tot), $n = 5$ for both genotypes ($*p < 0.05$); mitochondrial fraction (mito), $n = 5$ for both genotypes ($*p < 0.05$); cytoplasmic soluble fraction (cyto), $n = 4$ both genotypes [$p > 0.05$, nuclear (nuc), $n = 4$ both genotypes, Student's *t* test]. **b**, DARPP-32 phosphorylation is decreased in *RCAN1* knock-out mice. Western blot analyses from crude hippocampal pellet extracts (enriched for the nuclear and cytoplasmic compartments). Proteins examined are involved in calcineurin regulation or are downstream targets of calcineurin activity. p-DARPP-32, DARPP phosphorylated at Thr-34; DARPP, total DARPP; CnB, calcineurin regulatory subunit; Lamin AC, nuclear Laminin protein AC; B-Tub, β -tubulin. $n = 9$, each genotype, wild-type (WT), *RCAN1* knock-out mice (RCAN KO) ($*p < 0.05$, $**p < 0.01$, cytoplasm; $p < 0.01$, nuclear; ANOVA).

acutely inhibiting calcineurin. This finding argues against a developmental defect as the primary cause of the learning and memory deficits displayed by the *RCAN1* knock-out mice. Interestingly, the *RCAN1*-deficient mice did not exhibit alterations in contextual fear memory (Fig. 3*a*). Inducible expression of constitutively active calcineurin produced similar results (Mansuy et al., 1998*a,b*), arguing that calcineurin activation does not influence contextual fear memory. Together, these findings suggest

that proper calcineurin/*RCAN1* interactions are required for multiple types of learning and memory.

Synaptic plasticity is thought to underlie the acquisition and storage of information (Martin et al., 2000) and therefore is often used as a cellular model of learning and memory. The *RCAN1* knock-out mice exhibited deficits in specific forms of short- and long-term synaptic plasticity. PPF, a form of short-term plasticity dependent on calcineurin activity (Wang and Kelly, 1997), was reduced in *RCAN1* knock-out mice (Fig. 4*c*). However, *RCAN1* apparently is not required for the proper manifestation of all forms of short-term plasticity. E-LTP, a form of LTP that does not depend on either new PKA activity or protein synthesis (Huang et al., 1996), was normal in the *RCAN1* knock-out mice (Fig. 5*a*) similar to transgenic mice that overexpress a constitutively active calcineurin (Winder et al., 1998). These findings further support the concept that *RCAN1* and calcineurin are involved in some, but not all, functional aspects of synaptic plasticity.

The *RCAN1* knock-out mice also displayed significant deficits in L-LTP, a form of synaptic plasticity dependent on PKA, *de novo* gene expression, and protein translation (Huang et al., 1996; Abel et al., 1997) (Fig. 5*b*), corroborating studies demonstrating L-LTP deficits in mice that overexpress a constitutively active calcineurin (Winder et al., 1998) and enhancement of LTP acquisition when calcineurin is inhibited (Ikegami and Inokuchi, 2000; Malleret et al., 2001). Our findings are consistent with a model in which *RCAN1* constrains calcineurin activity to promote L-LTP. Whether newly synthesized *RCAN1* proteins or existing pools of *RCAN1* perform these functions will require additional investigation, as will the relative contributions of *RCAN1.1* and *RCAN1.4* to L-LTP.

It is generally accepted that kinase activities promote synaptic plasticity, whereas phosphatases inhibit synaptic plasticity. The ability of Calyculin A, a PP1/PP2A inhibitor, to rescue both the PPF and L-LTP deficits is consistent with the idea that the *RCAN1* knock-out mice have elevated phosphatase activities and argues against a purely developmental defect as the cause of the deficits we observe in the *RCAN1*-deficient mouse. Decreased phosphorylation of the PP1 inhibitor DARPP-32, a calcineurin substrate (Fig. 7), suggests that loss of *RCAN1* can lead to both a direct elevation in calcineurin activity and an indirect derepression of PP1 activity.

In contrast to our cued memory experiments, FK506, a direct calcineurin inhibitor, failed to rescue the PPF and L-LTP deficits. Furthermore, FK506 treatment actually blocked L-LTP in wild-type mice (Fig. 5*c*). This may simply be attributable to differences in phosphatase signaling in specific brain regions. Cued fear memory is primarily an amygdala-dependent task, whereas our synaptic plasticity studies were performed in area CA1 of the hippocampus. Studies using pharmacological inhibitors of calcineurin (FK506 and cyclosporine A) have produced a spectrum of effects on both short- and long-term plasticity (Wang and Stelzer, 1994; Lu et al., 1996; Wang and Kelly, 1996; Ikegami and Inokuchi, 2000).

The failure of FK506 to rescue the synaptic plasticity deficits in *RCAN1* mutant mice may be because the drug concentration was insufficient or had secondary effects unrelated to calcineurin activity. However, we propose two alternative explanations that are more intriguing. One is that *RCAN1* functions are not limited to calcineurin inhibition. Another possibility is that *RCAN1* does not have the same effect on all cellular pools of calcineurin. This is supported by our findings that the enzymatic activity of calcineurin and the 48 kDa isoform were elevated only in the pellet

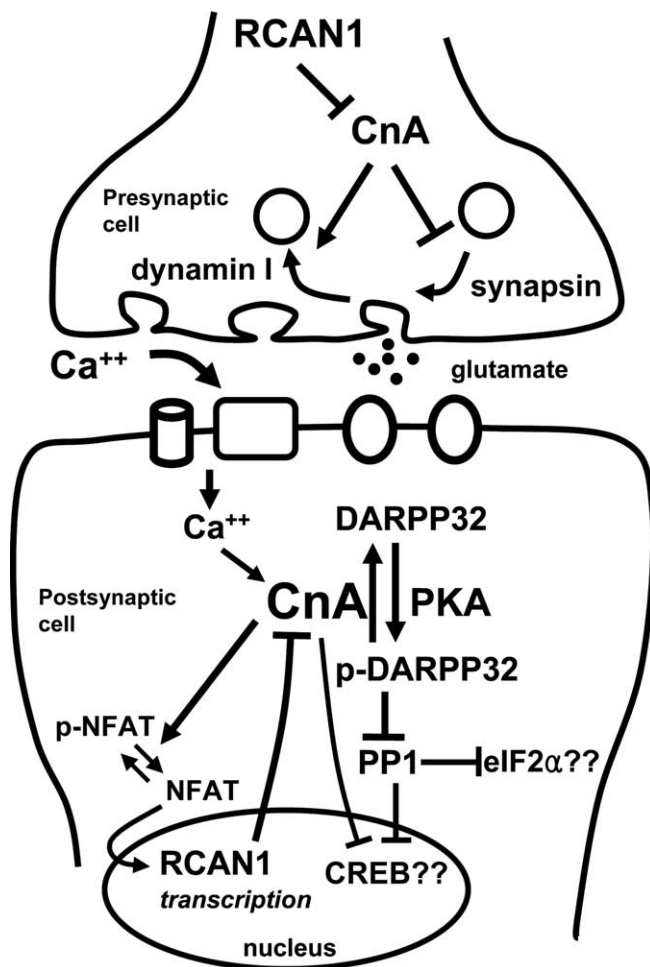


Figure 8. Schematic model of presynaptic and postsynaptic calcineurin (CnA) signaling. Direct substrates of CnA include dynamin I, synapsin, NFAT, DARPP-32, and I-1. In addition, CnA can promote PP1 activity through dephosphorylation of the PP1 inhibitors I-1 and DARPP-32. Through this mechanism, CnA activity could increase the rate of inactivation of critical effectors such as the translation initiation factor eIF2 α and the transcription factor cAMP response element-binding protein (CREB). RCAN1 helps maintain the phosphorylation of these targets by constraining CnA activity.

fraction in hippocampal extracts from *RCAN1* knock-out mice (Fig. 6). Calcineurin has numerous intracellular targets in addition to DARPP-32 (Fig. 8). Which of these targets are influenced by the presence of *RCAN1* is still unknown. Inhibition by FK506 presumably would act on the entire cellular pool of calcineurin. Mice with a forebrain-specific knock-out of the CnB regulatory subunit have impaired LTP, working memory deficits, and a generalized perturbation of brain functions (Zeng et al., 2001; Miyakawa et al., 2003), suggesting that, although calcineurin negatively modulates learning, memory, and synaptic plasticity, complete loss of calcineurin activity is not beneficial for cognitive function. It seems likely that there are mechanisms in place to maintain the proper balance in kinase and phosphatase activities at the synapse. Our studies suggest that *RCAN1* is an important component of this regulatory network and acts by placing a constraint on calcineurin activity.

Similar conclusions that calcineurin activity is elevated in *RCAN1* knock-out mice have been drawn from studies of T-cell activation (Ryeom et al., 2003). Conversely, studies of cardiac hypertrophy in *RCAN1* knock-out mice by our group and others (Vega et al., 2003; Sanna et al., 2006) suggest that calcineurin

activity is lower in the hearts of these animals. A model in which the *RCAN1* gene product can be conditionally knocked out or restored in adult mice will be required to fully delineate the positive and negative effects of *RCAN1* on calcineurin/PP1 signaling cascades and their contribution to LTP and memory.

The *RCAN1/DSCR1* gene is located on the long arm of human chromosome 21. There has been substantial speculation that trisomy of this locus contributes to learning and memory deficits in individuals with Down syndrome as well as accelerating the onset of an Alzheimer's-like dementia. Based on the studies we present here, one might predict that trisomy of *RCAN1/DSCR1* would increase inhibition of calcineurin signaling and improve learning and memory, which is clearly not the case in Down syndrome. Thus, perturbation of calcineurin activity, regardless of direction, may upset a critical balance in kinase and phosphatase activities sufficient to disrupt optimal neuronal function. *Drosophila* display learning deficits with either overexpression or deletion of the *RCAN1* homolog (Chang et al., 2003), demonstrating that any alteration in *RCAN1* likely perturbs essential learning processes. It is unlikely that trisomy of *RCAN1* alone causes Down syndrome. Rather, it is postulated that several genes, including intersectin-1 and the kinase *DYRK1A*, cooperate with *RCAN1/DSCR1* to amplify gene-dosage imbalances (de la Luna and Estivill, 2006; Gwack et al., 2006; Keating et al., 2006). Indeed, our results using FK506 blockade of elevated calcineurin activity in *RCAN1* mutants strongly suggest the critical need for dynamically balancing neuronal kinase and phosphatase pathways for proper cognitive function.

Overexpression of *RCAN1* also has been observed in AD patients (Ermak et al., 2001; Harris et al., 2007), but the impact of this on calcineurin activity is not clear. Calcineurin activity has been reported to increase (Liu et al., 2005) and decrease (Gong et al., 1994; Lian et al., 2001) in AD patients. Decreased calcineurin activity has been proposed to result in increased tau phosphorylation, leading to the formation of neurofibrillary tangles (Ermak et al., 2001; Harris et al., 2007). Evidence for increased calcineurin activity in AD was provided by direct enzymatic assays of human AD tissue (Liu et al., 2005) and of tissue from mouse models of AD (Norris et al., 2005). Moreover, recent findings indicate that acute blockade of calcineurin activity improves memory and cognitive function in AD model mice (Dineley et al., 2007). Cleavage of calcineurin by calpain increases levels of the constitutively active 48 kDa CnA isoform in response to ischemic injury and in AD (Wu et al., 2004; Liu et al., 2005; Shioda et al., 2006). An increase in this cleaved form of CnA in the hippocampus of *RCAN1* knock-out mice (Fig. 6b) suggests that *RCAN1* may be involved in proteolytic processing of calcineurin.

Calcineurin activity also has been implicated in the pathologies of ischemic brain injury and neurotoxicity (Liu et al., 2005; Norris et al., 2005; Shioda et al., 2006). These processes involve elevated levels of oxidative stress, calcium, and calcineurin activity (Norenberg and Rao, 2007). Oxidative stress is associated with both Down syndrome and AD (Perry et al., 2000; Lott et al., 2006), and it was demonstrated recently that overexpression of *RCAN1* renders neurons more susceptible to oxidative stress (Porta et al., 2007). Mitochondria play an important role in oxidative stress (Norenberg and Rao, 2007), and *RCAN1*, in addition to regulating calcineurin, may be important for normal mitochondrial function. Perturbation of the *Drosophila* homolog of *RCAN1*, *sarah*, resulted in mitochondrial abnormalities and elevated levels of reactive oxygen species (Chang and Min, 2005). Our finding of increased levels of the 48 kDa isoform of CnA from mitochondrial-associated subcellular fractions may pro-

vide for a mechanistic link between oxidative stress and calcineurin function.

In summary, our findings strongly suggest that RCAN1 facilitates synaptic plasticity and memory by constraining phosphatase signaling via inhibition of calcineurin and its downstream target PP1. Thus, RCAN1 represents an important potential therapeutic target for the treatment of numerous neurological disorders whose pathologies involve the dysregulation of calcineurin.

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