Neuronal L-Type Calcium Channel Signaling to the Nucleus Requires a Novel CaMKII α -Shank3 Interaction

Tyler L. Perfitt,¹ Xiaohan Wang,² Matthew T. Dickerson,¹ Jason R. Stephenson,^{1,2} Terunaga Nakagawa,^{1,2,3} David A. Jacobson,¹ and Roger J. Colbran^{1,2,4}

¹Department of Molecular Physiology and Biophysics, ²Vanderbilt Brain Institute, ³Center for Structural Biology, and ⁴Vanderbilt-Kennedy Center for Research on Human Development, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0615

The activation of neuronal plasma membrane Ca²⁺ channels stimulates many intracellular responses. Scaffolding proteins can preferentially couple specific Ca²⁺ channels to distinct downstream outputs, such as increased gene expression, but the molecular mechanisms that underlie the exquisite specificity of these signaling pathways are incompletely understood. Here, we show that complexes containing CaMKII and Shank3, a postsynaptic scaffolding protein known to interact with L-type calcium channels (LTCCs), can be specifically coimmunoprecipitated from mouse forebrain extracts. Activated purified CaMKII α also directly binds Shank3 between residues 829 and 1130. Mutation of Shank3 residues ⁹⁴⁹Arg-Arg-Lys⁹⁵¹ to three alanines disrupts CaMKII binding *in vitro* and CaMKII association with Shank3 in heterologous cells. Our shRNA/rescue studies revealed that Shank3 binding to both CaMKII and LTCCs is important for increased phosphorylation of the nuclear CREB transcription factor and expression of c-Fos induced by depolarization of cultured hippocampal neurons. Thus, this novel CaMKII-Shank3 interaction is essential for the initiation of a specific long-range signal from LTCCs in the plasma membrane to the nucleus that is required for activity-dependent changes in neuronal gene expression during learning and memory.

Key words: calcium; CaMKII; ion channel; scaffolding protein; transcriptional regulation

Significance Statement

Precise neuronal expression of genes is essential for normal brain function. Proteins involved in signaling pathways that underlie activity-dependent gene expression, such as CaMKII, Shank3, and L-type calcium channels, are often mutated in multiple neuropsychiatric disorders. Shank3 and CaMKII were previously shown to bind L-type calcium channels, and we show here that Shank3 also binds to CaMKII. Our data show that each of these interactions is required for depolarization-induced phosphorylation of the CREB nuclear transcription factor, which stimulates the expression of c-Fos, a neuronal immediate early gene with key roles in synaptic plasticity, brain development, and behavior.

Introduction

Neuronal depolarization stimulates Ca²⁺ influx and multiple intracellular signaling pathways that are essential for normal brain

functions, including excitation-transcription (E-T) coupling. Ca²⁺-dependent phosphorylation of the nuclear transcription factor CREB at Ser¹³³ stimulates the transcription of immediate early genes encoding multiple proteins (e.g., c-Fos, BDNF, homer1a) that play key roles in learning and memory (Dolmetsch, 2003; Flavell and Greenberg, 2008; Benito et al., 2011; Bading, 2013). Disruptions in activity-dependent gene expression are associated with multiple neuropsychiatric disorders (Ebert and Greenberg, 2013; Gallo et al., 2018) that have been linked to mutations in Ca²⁺ signaling proteins, including L-type calcium channels (LTCCs) and CaMKII (Nyegaard et al., 2010; Pinggera et al., 2015, 2017; Dick et al., 2016; Limpitikul et al., 2016; Pinggera and Striessnig, 2016; Kury et al., 2017; Stephenson et al., 2017; Akita et al., 2018; Chia et al., 2018; Cohen et al., 2018; Moon et al., 2018;

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Correspondence should be addressed to Roger J. Colbran at roger.colbran@vanderbilt.edu.

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Proietti Onori et al., 2018). For example, Timothy syndrome is caused by mutations in the $\text{Ca}_v1.2$ LTCC $\alpha1$ subunit that can disrupt neuronal E-T coupling (Li et al., 2016), contributing to neurobehavioral symptoms of this complex disorder, including autism spectrum disorder (ASD). Recent studies have shown that initiation of LTCC-dependent E-T coupling requires recruitment of multiple CaMKII holoenzymes to a nanodomain close to LTCCs (Wheeler et al., 2008; Ma et al., 2014; Wang et al., 2017), and that E-T coupling is disrupted by a CaMKII mutation linked to intellectual disability (Cohen et al., 2018). However, molecular mechanisms underlying the organization and function of this LTCC nanodomain remain incompletely understood.

CaMKII has critical roles in neuronal signaling and plasticity. Ca^{2+} /calmodulin (CaM) binding to 12-subunit CaMKII holoenzymes stimulates intersubunit autophosphorylation at Thr286 (in CaMKII α), a key mechanism underlying learning and memory (for review, see Lisman et al., 2012; Hell, 2014; Shonesy et al., 2014). Thr286-autophosphorylated CaMKII can remain autonomously active after the initial Ca^{2+} influx dissipates and Ca^{2+} /CaM dissociates (Lai et al., 1986; Miller and Kennedy, 1986; Miller et al., 1988), leading to sustained phosphorylation of downstream targets. Activated CaMKII also interacts with a number of other synaptic proteins. For example, activated CaMKII binding to NMDAR GluN2B subunits is important for CaMKII targeting to dendritic spines and for normal synaptic plasticity (Strack and Colbran, 1998; Bayer et al., 2001, 2006; Halt et al., 2012). In addition, densin-180 can bind to both $\text{Ca}_v1.3$ LTCCs and CaMKII, thereby suppressing Ca^{2+} -dependent inactivation of $\text{Ca}_v1.3$ and facilitating overall Ca^{2+} entry via LTCCs (Jenkins et al., 2010; Jiao et al., 2011). A recent study also showed that CaMKII binding to mGlu5 metabotropic glutamate receptors modulates the mobilization of intracellular Ca^{2+} stores (Marks et al., 2018). This emerging evidence supports the hypothesis that direct interactions of CaMKII with CaMKII-associated proteins (CaMKAPs) are important for synaptic signaling.

Our recent proteomics study identified Shank3 as one of the most abundant proteins in CaMKII complexes isolated from a Triton X-100/deoxycholate-solubilized synapse-enriched subcellular fraction (Baucum et al., 2015). Canonically, Shank3 contains an N-terminal ankyrin repeat domain, SH3 and PDZ domains, a proline-rich region with binding sites for homer and cortactin, and a C-terminal SAM domain that mediates oligomerization (Naisbitt et al., 1999). Diverse mutations in Shank3 are strongly linked to ASD and schizophrenia (Leblond et al., 2014; Soler et al., 2018), while a chromosomal deletion causes haploinsufficiency of the *SHANK3* gene in 22q13 deletion syndrome (Phelan-McDermid syndrome), another neurodevelopmental disorder associated with ASD (Harony-Nicolas et al., 2015). Indeed, knockdown of Shank3 expression in cultured hippocampal neurons reduces dendritic spine formation and mEPSC frequency (Verpelli et al., 2011), and several *Shank3* mutant mouse lines display different combinations of deficits in synaptic transmission, social behavior, and learning (for review, see Monteiro and Feng, 2017). The Shank3 PDZ domain can bind to a C-terminal PDZ binding motif in $\text{Ca}_v1.3$ LTCCs, and deletion of this PDZ binding motif disrupts $\text{Ca}_v1.3$ clustering in neuronal dendrites and LTCC-dependent E-T coupling (Zhang et al., 2005). Therefore, we hypothesized that direct interactions of Shank3 with LTCCs and CaMKII are important for CaMKII function within the LTCC nanodomain that is required for neuronal E-T coupling.

Here we identify a novel binding site for CaMKII in Shank3 and show that CaMKII activation, either by Ca^{2+} /CaM binding or Thr286 autophosphorylation, is required for this interaction. Using site-directed mutagenesis, we identified three residues in Shank3 that are critical for this interaction. Mutation of these residues in full-length Shank3 disrupts coimmunoprecipitation and colocalization with CaMKII. In addition, this Shank3 mutation disrupts LTCC/CaMKII-dependent E-T coupling to CREB and subsequent c-Fos expression in hippocampal neurons.

Materials and Methods

Animals. All mice were housed on a 12 h light-dark cycle with food and water *ad libitum*. *Camk2a*^{−/−} (referred to as CaMKII α -KO) and *Camk2a*^{tm2Sva} (RRID:MGI:3764491, referred to as CaMKII α ^{T286A}) mice on a C57B/6J background were described previously (Giese et al., 1998; Marks et al., 2018). WT and homozygous littermates were generated using a HetxHet breeding strategy. Both male and female mice age P28–P30 were used for biochemical studies. All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were performed following the National Institutes of Health's *Guide for the care and use of laboratory animals*.

Antibodies used. The following antibodies were used for immunoblotting at the indicated dilutions: mouse anti-CaMKII α 6G9 (Thermo Fisher Scientific; catalog #MA1-048, RRID:AB_325403, 1:5000), pT286 CaMKII α (Santa Cruz Biotechnology; catalog #sc-12886-R, RRID:AB_2067915, 1:3000), mouse anti-Shank3 (University of California at Davis/National Institutes of Health NeuroMab Facility; catalog #N367/62, RRID:AB_2315920, 1:2000), rabbit monoclonal anti-Shank3 (D5K6R) (Cell Signaling Technology; catalog #64555, RRID:AB_2799661, 1:3000), goat anti-GST (GE Healthcare Life Sciences; catalog #27-4577-01, 1:5000), polyclonal goat CaMKII antibody (RRID:AB_2631234, 1:5000) (McNeill and Colbran, 1995), mouse anti-PSD-95 (NeuroMab, catalog #75-028, RRID:AB_2292909, 1:50,000), mouse anti-GFP (Vanderbilt Antibody and Protein Resource; catalog #1C9A5, 1:3000), mouse anti-HA (BioLegend; catalog #901503, RRID:AB_2565005, 1:3000), HRP-conjugated anti-rabbit (Promega; catalog #W4011, RRID:AB_430833, 1:6000), HRP-conjugated anti-mouse (Promega; catalog #W4021, RRID:AB_430834, 1:6000), HRP-conjugated anti-goat (Abcam; catalog #Ab6741, RRID:AB_955424, 1:3000), IR dye-conjugated donkey anti-mouse 800CW (LI-COR Biosciences; catalog #926-32213, RRID:AB_621847, 1:10,000), and IR dye-conjugated donkey anti-goat 680LT (LI-COR Biosciences; catalog #926-68024, RRID:AB_10706168, 1:10,000).

DNA constructs. A construct encoding GFP-Shank3 was a gift from Dr. Craig Garner (Stanford University). The construct expressing shRNA (pLL3.7) was a gift from Dr. Luk Van Parijs (Massachusetts Institute of Technology). This plasmid was modified to replace the CMV promoter with a 0.4 kb fragment of the mouse CaMKII promoter (designated as pLLCK) that is primarily active only in excitatory neurons (Dittgen et al., 2004). Control shRNA (5'-TCGCTTGGGCGAGAGTAAG-3') was designed following Boudkkazi et al. (2014). Shank3 shRNA (5'-GGAAAGT CACCAGAGGACAAGA-3') was designed following Verpelli et al. (2011). Sequences encoding shRNA were inserted into pLLCK at HpaI and XhoI restriction sites. For Ca^{2+} imaging studies using fura-2, the sequence encoding GFP in pLLCK was replaced with the sequence encoding the red fluorophore mApple. The Shank3 shRNA-resistant construct (Shank3^R) was designed by introducing 6 "silent" nucleotide mutations in the target site that do not alter amino acid sequence at Arg¹¹⁸⁷, Lys¹¹⁸⁸, Ser¹¹⁸⁹, and Pro¹¹⁹⁰. The mAp-Shank3 construct was generated by inserting Shank3 cDNA into pmApple-C1 construct at Bgl-III and EcoRI restriction sites. The Shank3 shRNA-resistant construct containing a deletion of the PDZ domain (mAp-Shank3^R Δ PDZ) was generated by in-frame PCR deletion of the entire 270 bp region encoding 572^{Iso}-Val⁶⁶¹ from mAp-Shank3^R.

Rat $\text{Ca}_v1.3$ complete coding sequence (GenBank accession number AF370010) was a gift from Dr. Diane Lipscombe (Brown University). A plasmid encoding $\text{Ca}_v1.3$ -CTD with an N-terminal HA-tag (HA- $\text{Ca}_v1.3$ -CTD, for coimmunoprecipitation) was made by inserting rat $\text{Ca}_v1.3$ cDNA

encoding ¹⁴⁶⁹Met-Leu²¹⁶⁴ into the pCGNh vector, a gift from Dr. Winship Herr (Université de Lausanne). All constructs were confirmed by DNA sequencing.

Mouse forebrain homogenization and subcellular fractionation. Forebrains were dissected and fractionated as previously described (Baucum et al., 2015; Stephenson et al., 2017). Briefly, P28–P30 mice were anesthetized with isoflurane, decapitated, and forebrains were quickly dissected, cut in half down the midline, and a half forebrain was immediately homogenized in an isotonic buffer (150 mM KCl, 50 mM Tris HCl, pH 7.5, 1 mM DTT, 0.2 mM PMSF, 1 mM benzimidazole, 1 μ M pepstatin, 10 mg/L leupeptin, 1 μ M microcystin). The homogenate (~5 ml) was rotated end-over-end at 4°C for 30 min, at which point an aliquot of the “whole forebrain” input was collected. Samples were then centrifuged at 100,000 $\times$ g for 1 h. After removing the supernatant (cytosolic S1 fraction), the pellet was resuspended in the isotonic buffer containing 1% (v/v) Triton X-100, triturated until homogeneous, and then rotated end-over-end at 4°C for 30 min. Homogenates were then centrifuged at 10,000 $\times$ g for 10 min, and the supernatant (Triton-soluble membrane S2 fraction) was removed. The second pellet (Triton-insoluble synaptic P2 fraction) was resuspended in isotonic buffer containing 1% Triton X-100 and 1% deoxycholate and then sonicated. The P2 fraction was then mixed with 4 $\times$ SDS-PAGE sample buffer or used for immunoprecipitation studies (see below).

Recombinant mouse CaMKII α and GST-tagged protein purification. Expression and purification of recombinant mouse CaMKII α have been described previously (McNeill and Colbran, 1995). GST-Shank3 constructs were created by PCR amplification of the relevant cDNA fragments for insertion between EcoRI and BamHI restriction sites in pGEX6P-1. GST-GluN2B was described previously (Strack et al., 2000). The vectors encoding GST fusion proteins were transformed into BL21 (DE3) pLysS bacteria cells, and proteins were purified as previously described (Robison et al., 2005a).

CaMKII autophosphorylation and GST cosedimentation assays. CaMKII α (1.25 μ M subunit) was incubated on ice for 90 s with 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 0.5 mM CaCl₂, 1 μ M CaM, and 1 mM DTT, with or without 400 μ M ATP (T286 autophosphorylated or basal, respectively), and reactions were terminated with 45 mM EDTA. Separate reactions incubated CaMKII α (1.25 μ M subunit) with 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 0.5 mM CaCl₂, 1 μ M CaM, 1 mM DTT, with no EDTA or ATP added (Ca/CaM). The reaction was then diluted 10-fold using 1 $\times$ GST pulldown buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl; 1% (v/v) Triton X-100), supplemented with 10 mM magnesium acetate and 0.5 mM CaCl₂ for Ca/CaM incubations. CaMKII α (125 nM subunit) was incubated with GST or GST-fusion protein (125 nM) and Pierce Glutathione Agarose beads (Thermo Fisher Scientific, catalog #16101, 10 μ l packed resin). Reactions were rocked for 1 h at 4°C. Beads were washed three times with GST buffer, supplemented as described above where appropriate. Proteins were eluted with 20 mM glutathione, pH 8.0, for 10 min (Sigma Millipore).

Western blot analysis. Samples were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membrane (Protran). The membrane was blocked in blotting buffer containing 5% nonfat dry milk, 0.1% Tween 20, in TBS (20 mM Tris, 136 mM NaCl) at pH 7.4 for 30 min at room temperature. The membrane was incubated with primary antibody (see dilutions above) in blotting buffer for 1 h at room temperature or overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature, washed again, and then visualized using enzyme-linked chemiluminescence using the Western Lightening Plus-ECL, enhanced chemiluminescent substrate (PerkinElmer) and visualized using Premium x-ray Film (Phenix Research Products) exposed to be in the linear response range. Images were quantified using ImageJ software (RRID:SCR_003070). Signals detected in the negative control lanes were used as background value and were subtracted from experimental lanes. Secondary antibodies conjugated to infrared dyes (LI-COR Biosciences) were used for development with an Odyssey system (LI-COR Biosciences).

Cell culture, transfection, and lysis. HEK293T cells (ATCC; catalog #CRL-3216, RRID:CVCL_0063) were cultured and maintained in DMEM containing 10% FBS, L-glutamine, and 1% penicillin/streptomycin

at 37°C in 5% CO₂. Cells plated on 10 cm dishes were transfected with 5–10 μ g of DNA. After 24–48 h, cells were rinsed in ice-cold PBS and lysed in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM benzimidazole, 10 μ g/ml leupeptin, and 10 μ M pepstatin).

Immunoprecipitation. Mouse forebrain fractions or HEK293T cell lysates were incubated with mouse anti-CaMKII α (Thermo Fisher Scientific, catalog #MA1-048), rabbit anti-Shank3 (Bethyl, catalog #A304-178A RRID:AB_2621427), or rabbit anti-GFP (Thermo Fisher Scientific, catalog #A-11222 RRID:AB_221569) and rocked end-over-end at 4°C for 1 h with 10 μ l prewashed Dynabeads Protein G (Thermo Fisher Scientific, catalog #10002D, for mouse) or Protein A (Thermo Fisher Scientific, catalog #10001D, for rabbit). HEK293T lysates were supplemented with 1.5 mM CaCl₂ and 1 μ M calmodulin (final concentrations) to partially activate CaMKII during immunoprecipitation. The beads were isolated magnetically and washed three times using lysis buffer before eluting proteins using 2 $\times$ SDS-PAGE sample buffer. Inputs and immune complexes were immunoblotted side by side as indicated (see Western blot analysis).

Colocalization studies. *STHdh*^{Q7/Q7} cells (RRID:CVCL_M590) (Trettel et al., 2000), a gift from Dr. Aaron Bowman (Vanderbilt University), were cultured and maintained in DMEM containing 10% FBS, L-glutamine, 1% penicillin/streptomycin, and 400 μ g/ml G418 (Mediatech) at 33°C in 5% CO₂. Cells were plated onto 15 mm coverslips pretreated with poly-D-lysine in 12-well plates and transfected with 3 μ g of DNA overnight. Media was then removed and cells were incubated in serum-free DMEM containing either 0.49% DMSO (Pierce) or a differentiation medium (serum-free DMEM supplemented with 10 ng/ml fibroblast growth factor) (Promega), 240 μ M isobutylmethylxanthine (Sigma Millipore), 20 μ M 12-O-tetradecanoylphorbol-13-acetate (Sigma Millipore), 48.6 μ M forskolin (Sigma Millipore), and 5 μ M DA (Sigma Millipore). After 8–14 h, cells were fixed in ice-cold 4% PFA–4% sucrose in 0.1 M PB, pH 7.4, for 3 min and –20°C methanol for 10 min. Coverslips were mounted on microscope slides using ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific, catalog #P36931). Images were collected using a Carl Zeiss 880 inverted confocal microscope using 63 $\times$ objective. Thresholding and intensity correlation analysis to compare normalized pixel intensities in each color channel were performed using ImageJ as previously described (Baucum et al., 2010). GFP and mApple channels were automatically thresholded before calculating the intensity correlation quotient (ICQ). This method interprets ICQ values in the following ranges: 0 < ICQ $\leq$ 0.5, dependent overlap of fluorescent signals; ICQ = 0, random overlap of fluorescent signals; and 0 > ICQ $\geq$ –0.5, segregation of fluorescent signals (Li et al., 2004).

Primary hippocampal neuronal cultures and neuronal stimulation assay. Dissociated hippocampal neurons were prepared from E18 Sprague Dawley rat embryos, as previously described (Shanks et al., 2010). Sex of the embryos was not determined. Neurons were transfected at 7–9 DIV using Lipofectamine 2000 following the manufacturer's directions (Thermo Fisher Scientific). A total of 1 μ g of DNA was transfected for each well of a 12-well plate for 2–3 h before switching back to conditioned media. At DIV 13–14, neurons were preincubated for 2 h with 5K Tyrode's solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.5, ~313 mOsm) with 1 μ M TTX, 10 μ M APV, and 50 μ M CNQX to suppress intrinsic neuronal activity by blocking sodium channels, NMDARs and AMPARs, respectively. Neurons were then treated with either 5K Tyrode's or 40K Tyrode's solution (adjusted to 40 mM KCl and 115 mM NaCl, with all three inhibitors present) for 90 s. For the analysis of pCREB levels, neurons were immediately fixed using ice-cold 4% PFA–4% sucrose in 0.1 M PB, pH 7.4, for 3 min and –20°C methanol for 10 min. For the analysis of c-Fos expression, Tyrode's solution was aspirated after 90 s and replaced with conditioned media for 3 h before fixation. Fixed neurons were washed three times with PBS, permeabilized with PBS + 0.2% Triton X-100, and then incubated with blocking solution for 1 h: 1 $\times$ PBS, 0.1% Triton X-100 (v/v), 2.5% BSA (w/v), 5% normal donkey serum (w/v), 1% glycerol (v/v). Neurons were then incubated with blocking solution overnight with primary antibodies: rabbit anti-pCREB (Cell Signaling Technology, catalog #9198, RRID:AB_2798432, 1:1000) or rabbit anti-c-Fos (EMD

Millipore, catalog #ABE457, RRID:AB_310107, 1:500), and mouse anti-CaMKII α 6G9 (Thermo Fisher Scientific, catalog #MA1-048, 1:1000). The following day, neurons were washed three times in PBS + 0.2% Triton X-100 and then incubated with blocking solution for 1 h with secondary antibodies: donkey anti-rabbit 647 AlexaFluor-647 (Thermo Fisher Scientific, catalog #A-31573, RRID:AB_10891079) and donkey anti-mouse AlexaFluor-546 (catalog #A-10036, RRID:AB_2534012). Neurons were washed with PBS three times and mounted on slides using Prolong Gold Antifade Mountant with DAPI.

Neuronal imaging and quantification. The experimenter was blinded to the transfection conditions by coding the culture dishes before microscopy and image analysis. Images were collected using a Carl Zeiss 880 inverted confocal microscope with a 40 $\times$ /1.30 Plan-Neofluar oil lens. The binocular lens was used to identify transfected neurons based on EGFP expression from the shRNA construct. In experiments with mAp-Shank3 rescue, mAp expression was also confirmed in EGFP-positive cells. The DAPI channel was then used to focus on the z-plane that yielded the highest DAPI signal (one that presumably runs through the nuclei) for image acquisition. Images were then collected in all channels, and MetaMorph Microscope Automation and Image Analysis Software (Molecular Devices, RRID:SCR_002368) was used to quantify pCREB or c-Fos signals. Briefly, nuclei were identified by thresholding the DAPI channel to create and select the nuclear ROIs. The ROIs were then transferred to other channels to measure the average pCREB or c-fos intensity. ROIs were collected from transfected (EGFP-positive) neurons and nearby nontransfected (EGFP-negative) neurons. The relative intensity was calculated as $[(\text{channel}^x - \text{channel}^{5K})/(\text{channel}^{40K} - \text{channel}^{5K})]$, where channel^x is the signal being calculated, and channel^{5K} and channel^{40K} are the average signals of the 5K and 40K conditions in that batch of cultured neurons, respectively. Sample size was calculated based on previous experimental design (Wang et al., 2017). Data shown were collected from images of the indicated total number of neurons from 3 to 5 independent cultures.

Neuronal Ca^{2+} imaging. Dissociated rat hippocampal neurons were cultured in 35 mm glass-bottom dishes (Cellvis, catalog #D35-10-1.5-N) coated with 2.5 $\mu\text{g}/\text{ml}$ laminin (Roche Diagnostics) and 37.5 $\mu\text{g}/\text{ml}$ poly-L-lysine (Sigma Millipore), as described previously (Sala et al., 2003). Cultures were transfected with a total of 2.5 μg of DNA/dish after DIV 8, as described above. To allow for Ca^{2+} imaging using fura-2, shRNA constructs were reengineered to coexpress an mApple fluorescent reporter rather than GFP. Transfected neurons were imaged on DIV 12–14. Cells were incubated for 30 min at 37°C in 5% CO_2 in conditioned culture medium (see above) supplemented with 2 μM fura-2 acetoxymethyl ester AM (Thermo Fisher Scientific, catalog #F1221) and then incubated in 5K Tyrode's solution with TTX, APV, and CNQX (see above) for 5 min at 37°C. Cells were incubated in 2.5 ml solution and perfused at a flow rate of 2 ml/min at 32°C with identical buffer for at least 300 s; then flow was changed to 40K Tyrode's solution with TTX, APV, and CNQX for at least 150 s. Intracellular Ca^{2+} was measured every 5 s as a ratio of fura-2 emission (510 nm) by excitation at 340 and 380 nm (F_{340}/F_{380}) using an Eclipse Ti2 microscope (Nikon) equipped with an epifluorescence illuminator (Sutter Instrument), a Prime 95B equipped with 25 mm CMOS sensors camera (Photometrics Scientific), and Elements software (Nikon; RRID:SCR_014329). Transfected (mApple-positive) cell somas were selected as ROIs using Elements software (Nikon), and Ca^{2+} responses were quantified as a function of time by the change in fura-2 fluorescence ratio above baseline ($\Delta F = (F_{340}/F_{380})/(F_{340}/F_{380})_{\text{baseline}}$). Daily averages from 12 to 90 transfected control and Shank3 shRNA cells were generated from four independent biological replicates. Areas under the curve of the averaged fura-2 responses during the first 90 s of stimulation were quantified as an indicator of the change in intracellular Ca^{2+} for each replicate.

Statistics. Statistical tests and parameters are indicated in figure legends. Differences were considered significant if $p \leq 0.05$. Sample sizes for each experiment are based on previously published studies from our laboratory and standards in the field. The experimenter was blind to the transfection condition when analyzing the levels of pCREB or c-Fos for Figures 6–8. Data are mean $\pm$ SEM with individual data points indicated.

Results

CaMKII α and Shank3 interact in the mouse forebrain

Our previous proteomics study detected numerous peptides originating from the Shank3 protein in immunoprecipitated CaMKII complexes isolated from solubilized synaptic fractions of mouse forebrain (Baucum et al., 2015). To extend this observation, we first compared the distribution of Shank3 and CaMKII across cytosolic (S1), Triton-soluble membrane (S2), and Triton-insoluble synaptic (P2) fractions isolated from mouse forebrain extracts. The Shank3 antibody detected two major bands in whole mouse forebrain extracts: the expected ~ 180 kDa band, plus an ~ 125 kDa band. Both Shank3 bands were undetectable in S1 or S2 fractions and were relatively enriched in the P2 fraction, similar to other synaptic proteins, such as PSD-95 (Fig. 1A, left). Since Shank3 undergoes complex transcriptional and post-transcriptional regulation through intragenic promoters and alternative splicing (Wang et al., 2011; Waga et al., 2014), the two bands may represent different Shank3 variants. In contrast, similar levels of CaMKII were detected in the S2 and P2 fractions, with lower levels in S1, consistent with our prior studies (Gustin et al., 2011). Thus, a subpopulation of CaMKII and essentially all of the Shank3 are present in mouse forebrain subcellular fractions enriched in synaptic proteins in WT mice.

To further investigate the association of Shank3 with CaMKII α , synaptic P2 fractions were isolated in parallel from WT or CaMKII α -KO littermates (as a negative control), solubilized by sonication in sodium deoxycholate plus Triton X-100 to at least partially disrupt the postsynaptic density (see Materials and Methods), and incubated with a control IgG antibody or a CaMKII α -specific monoclonal antibody. Immunoblotting confirmed that CaMKII α -KO mice do not express CaMKII α , and revealed that the levels of Shank3 in whole forebrain lysates (both major bands) and the distribution of Shank3 between the S1, S2, and P2 fractions was similar in WT and CaMKII α -KO mice (Fig. 1A,B, left). Shank3 was readily detected in CaMKII α complexes isolated from WT mouse forebrain, but not from CaMKII α -KO mouse forebrain, and not in any samples isolated using a control IgG (Fig. 1C). Interestingly, the ratio of the higher and lower molecular weight Shank3 bands was consistently higher in CaMKII immune complexes than in the P2 input, perhaps suggesting that CaMKII preferentially interacts with the larger Shank3 protein. Together, these data demonstrate that Shank3 is specifically associated with CaMKII α complexes in mouse brain extracts.

We next tested for reciprocal coimmunoprecipitation of CaMKII α with complexes isolated using a Shank3 antibody. Since interactions between CaMKII and other proteins are often enhanced by Thr286 autophosphorylation, we compared the association of CaMKII α with Shank3 in solubilized synaptic P2 fractions isolated from WT mice and CaMKII α ^{T286A} mice. The knock-in mutation of Thr286 to Ala in CaMKII α ^{T286A} mice prevents CaMKII regulation by Thr286 autophosphorylation (Giese et al., 1998). Immunoblotting confirmed that whole forebrain lysates from WT and CaMKII α ^{T286A} mice contain similar total levels of CaMKII α and Shank3 (both major bands) (Fig. 1B, right). Moreover, the distribution of Shank3 between S1, S2, and P2 fractions was similar in WT and CaMKII α ^{T286A} mice (Fig. 1A), although CaMKII α was partially redistributed from P2 to S2 fractions in CaMKII α ^{T286A} mice (Fig. 1A), as previously reported (Gustin et al., 2011). Shank3 immune complexes isolated from WT and CaMKII α ^{T286A} mice contained similar levels of Shank3, but there was a substantial reduction in levels of coimmunoprecipitated CaMKII α from CaMKII α ^{T286A} compared with WT tis-

Figure 1. Reciprocal coimmunoprecipitation of Shank3 and CaMKIIα from mouse forebrain extracts. **A**, Whole forebrain lysates (Input), cytosolic (S1), membrane-associated (S2), and synaptic (P2) subcellular fractions from WT, CaMKIIα-KO, and CaMKIIα^{T286A} mice were immunoblotted for localization of Shank3 (Cell Signaling Technology antibody), PSD-95, and CaMKIIα. The Shank3 antibody detected two bands (open and solid arrowheads) that are primarily localized in P2 fractions, which were also enriched for CaMKIIα. **B**, The upper and lower Shank3 bands in whole forebrain lysates from WT and CaMKIIα-KO mice (left) or WT and CaMKIIα^{T286A} mice (right) were quantified. Signals were corrected for protein loading based on Ponceau-stained membranes and normalized to the levels of the upper Shank3 band in WT samples. **C**, Synaptic (P2) fractions from WT or CaMKIIα-KO mouse forebrains were immunoprecipitated using control IgG or CaMKIIα-specific antibodies, and immunoblotted using Shank3 (Cell Signaling Technology antibody) and pan-CaMKII antibodies. Arrows indicate CaMKIIα and CaMKIIβ bands. CaMKIIα and coimmunoprecipitated Shank3 were not detected in samples isolated from CaMKIIα-KO mice. **D**, Synaptic (P2) fractions from WT or CaMKIIα^{T286A} mouse forebrains were immunoprecipitated using control IgG or Shank3 (Bethyl) antibodies, and immunoblotted using Shank3 (Cell Signaling Technology) and CaMKIIα antibodies. The levels of coprecipitated CaMKIIα from CaMKIIα^{T286A} mice were significantly reduced (93 ± 4% reduction in lane 6 compared with lane 5, $n = 3$, $p < 0.001$, one-sample Student's t test with equal variance compared with theoretical value of 100). All immunoblots are representative of ≥3 biological replicates.

sue (Fig. 1D, compare CaMKIIα signal in lanes 5 and 6) (93 ± 4% reduced compared with WT, $p = 0.0008$, one-sample Student's t test with equal variance compared with the theoretical normalized WT value of 100, $n = 3$). In combination, these data indicate that Shank3 interacts directly or indirectly with CaMKII in deoxycholate-solubilized synaptic fractions of mouse brain, and that this association is regulated by Thr286 autophosphorylation of CaMKII.

T286-autophosphorylated CaMKIIα directly binds to Shank3 (829–1130)

To determine whether CaMKIIα directly interacts with Shank3, we first expressed and purified a series of six nonoverlapping GST fusion proteins spanning the full length of Shank3 (Figs. 2A,B). Some of the purified proteins contained proteolytic degradation fragments, and the full-length proteins are denoted by asterisks in Figure 2B. Since coimmunoprecipitation of CaMKIIα with Shank3 from brain extracts was strongly reduced in the absence of Thr286 autophosphorylation, each purified GST fusion protein was incubated with purified Thr286-autophosphorylated CaMKIIα, and complexes were isolated using glutathione agarose. A GST fusion protein containing the CaMKII binding domain of the NMDAR GluN2B subunit (residues 1260–1309), a well-

Figure 2. T286-phosphorylated CaMKIIα specifically binds to Shank3 (829–1130). **A**, Domain structure of full-length Shank3 and the six GST-Shank3 fusion proteins used in these studies that span the entire Shank3 protein. Gray boxes represent canonical Shank3 domains. Residue numbers are listed in parentheses. ANK, Ankyrin repeats domain; SH3, Src homology 3 domain; PDZ, PSD95/Dlg1/zo-1 domain; PRR, proline-rich region containing binding sites for Homer and Cortactin; SAM, sterile α motif involved in multimerization of Shank3. **B**, Glutathione agarose cosedimentation assay shows that preactivated (Thr286-autophosphorylated) CaMKIIα specifically binds to GST-Shank3 #4 (829–1130) and positive control GST-GluN2B (1260–1309). *Full-length GST fusion proteins on the GST immunoblot. **C**, Glutathione agarose cosedimentation assay shows that, in the absence of Ca²⁺/CaM binding or Thr286 autophosphorylation, CaMKIIα (Basal) does not bind to GST-Shank3 #4; *in vitro* binding of CaMKIIα is partially supported by Ca²⁺/CaM binding (Ca/CaM), and maximally enhanced by pT286 autophosphorylation. Bar graph represents levels of each form of CaMKII bound to GST-Shank3 #4 (or the GST negative control) (mean ± SEM) relative to levels of pT286-CaMKIIα binding to GST-GluN2B. Immunoblots are representative of three or four biological replicates.

established CaMKAP (Strack and Colbran, 1998), was used as a positive control. Similar amounts of activated CaMKII α bound to a GST-Shank3 fusion protein containing residues 829–1130 (Fig. 2, GST-Shank3 #4) and to GST-GluN2B, but there was no consistently detectable interaction with any other Shank3 fragment (Fig. 2B).

CaMKII is initially activated by Ca²⁺/CaM binding alone followed by Thr286 autophosphorylation, which is sufficient to maintain the activated conformation, even following dissociation of Ca²⁺/CaM. Since CaMKAPs display distinct preferences for binding to various active and inactive conformations of CaMKII, we tested the binding of different CaMKII α conformations to GST-Shank3 (829–1130) in parallel (Fig. 2C). There was no detectable binding of inactive CaMKII to GST-Shank3 (829–1130) (Fig. 2C), suggesting that CaMKII activation is essential for binding to this fragment. Inactive CaMKII α also did not bind to any other GST-Shank3 proteins compared with GST negative control under these conditions (data not shown). GST-Shank3 (829–1130) bound to the active conformations of CaMKII α induced by Ca²⁺/CaM binding alone or by Thr286 autophosphorylation, but Ca²⁺/CaM binding alone only partially supported binding (23 $\pm$ 5%; mean $\pm$ SEM). In combination, these data show that activated CaMKII α can directly bind to a central, poorly characterized domain in the Shank3 protein, and that Thr286 autophosphorylation significantly enhances the interaction.

A Shank3 tribasic residue motif is required for CaMKII binding

Residues 829–1130 of Shank3 connect the canonical PDZ domain to the proline-rich motif, but the functional role(s) of this region is poorly understood (Naisbitt et al., 1999; Tu et al., 1999). To identify CaMKII binding determinants within this domain, we initially generated and purified three smaller GST-Shank3 fusion proteins (Fig. 3A). Similar amounts of Thr286-autophosphorylated CaMKII bound to GST-Shank3 (931–1014) (protein 4b in Fig. 3A) and GST-Shank3 (829–1130), but there was no detectable interaction with GST-Shank3 proteins containing residues 829–930 (protein 4a) or residues 1015–1130 (protein 4c) (Fig. 3B). Examination of the amino acid sequence of Shank3 residues 931–1014 revealed a region sharing some similarity with recently characterized CaMKII binding domains in the N-terminal domain of the LTCC Ca_v1.3 α subunit (Wang et al., 2017) and the C-terminal domain (CTD) of mGlu5 (Marks et al., 2018) (Fig. 3A). Since mutation of the tribasic residue motifs in Ca_v1.3 (⁸³Arg-Lys-Arg⁸⁵) and mGlu5 (⁸⁶⁶Lys-Arg-Arg⁸⁶⁸) prevented CaMKII α binding, we mutated the conserved Shank3 ⁹⁴⁹Arg-Arg-Lys⁹⁵¹ motif to Ala residues within GST-Shank3 (829–1130). This RRR/AAA mutation essentially abrogates binding of Thr286-autophosphorylated CaMKII α (Fig. 3C). These data indicate that ⁹⁴⁹Arg-Arg-Lys⁹⁵¹ in Shank3 is essential for the direct binding of Thr286-autophosphorylated CaMKII *in vitro*.

The RRR/AAA mutation of full-length Shank3 disrupts binding to CaMKII but not to Ca_v1.3

To determine whether the ⁹⁴⁹Arg-Arg-Lys⁹⁵¹ motif is essential for association of CaMKII with full-length Shank3, we generated the RRR/AAA mutant in GFP-tagged full-length Shank3 (GFP-Shank3-AAA). CaMKII α was coexpressed with GFP, GFP-Shank3-WT, or GFP-Shank3-AAA in HEK293T cells. Excess Ca²⁺/CaM was added to the cell lysates to activate CaMKII α , and a GFP antibody was used for immunoprecipitation. Similar robust GFP protein signals were detected in samples immunoprecipitated from each lysate; CaMKII α was detected in immune complexes con-

Figure 3. Characterization of the CaMKII binding motif in Shank3. **A**, Top, Diagram of 3 truncations used to map the CaMKII interaction site within GST-Shank3 #4 (829–1130). Bottom, Sequence alignment of human Shank3 residues 941–959 with the corresponding Shank3 residues in other species and the CaMKII binding domain in the N-terminal domain of the *Rattus norvegicus* Ca_v1.3 α 1 subunit (Wang et al., 2017) and the C-terminal tail of the *Rattus norvegicus* mGlu5 (Marks et al., 2018). Black represents conserved residues. Gray represents dissimilar residues. Red box represents the conserved tribasic residue motif. **B**, Glutathione agarose cosedimentation assay comparing binding of activated CaMKII α to GST-Shank3 #4 (829–1130) and 3 nonoverlapping fragments (4a, 4b, and 4c). *Full-length GST fusion proteins. GST-Shank3 #4 (829–1130) and #4b (931–1014) bind similar amounts of pT286-autophosphorylated CaMKII α , but there is no detectable binding to the other Shank3 fragments. **C**, Mutation of amino acids ⁹⁴⁹RRR to AAA in GST-Shank3 #4 (829–1130) blocks CaMKII α binding in glutathione agarose cosedimentation assay (98 $\pm$ 4% reduced compared with WT, $n = 3$, $p < 0.001$, one-sample Student's t test with equal variance compared with theoretical value of 100). All immunoblots are representative of three biological replicates.

taining GFP-Shank3-WT, but not GFP-negative control or GFP-Shank3-AAA complexes (Fig. 4A). Thus, the ⁹⁴⁹Arg-Arg-Lys⁹⁵¹ motif in Shank3 is required for CaMKII α association with the full length protein.

Figure 4. A Shank3^{949RRK951} to AAA mutation disrupts association with CaMKIIα but does not with the Ca_v1.3 CTD. **A**, Soluble fractions of HEK293T cells expressing CaMKIIα with GFP or GFP-Shank3 (WT or ^{949RRK951} to AAA mutant) were immunoprecipitated using a GFP antibody. Coprecipitation of CaMKIIα with GFP-Shank3-AAA is significantly reduced by 95 ± 10% compared with GFP-Shank3-WT. ***p < 0.001 (one-sample Student's *t* test with equal variance compared with a theoretical value of 100). **B**, Soluble fractions of HEK293T cells expressing GFP or GFP-Shank3 (WT or AAA) with the HA-tagged CTD of the Ca_v1.3 α1 subunit (HA-Ca_v1.3-CTD) were immunoprecipitated as in **A**. The AAA mutation has no significant effect on the coprecipitation of HA-Ca_v1.3-CTD (*p* = 0.84). All immunoblots are representative of four biological replicates. Error bars indicate the mean ± SEM.

To test the specificity of the RRK/AAA mutation in Shank3, we performed a similar coimmunoprecipitation experiment using an HA-tagged CTD of the L-type calcium channel Ca_v1.3 α subunit, which can interact with the nearby PDZ domain (residues 572–661) of Shank3 (Zhang et al., 2005). Lysates of HEK293T cells expressing HA-Ca_v1.3-CTD and GFP, GFP-Shank3-WT, or GFP-Shank3-AAA were immunoprecipitated using a GFP antibody. Similar amounts of the HA-Ca_v1.3-CTD coimmunoprecipitated with both GFP-Shank3-WT and GFP-Shank3-AAA immune complexes, but not with GFP alone (Fig. 4B). Thus, the interaction of Ca_v1.3 with Shank3 is not affected by mutation of the ^{949Arg-Arg-Lys951} motif, indicating that this mutation does not have broader (nonspecific) effects on the association of other proteins with GFP-Shank3.

The RRK/AAA mutation of GFP-Shank3 disrupts colocalization with mApple-CaMKIIα

To investigate whether Shank3 interacts with CaMKIIα in intact cells, we performed a series of colocalization experiments in

STHdh^{+/+} cells. This striatal progenitor cell line can be pharmacologically induced to initiate a neuronal differentiation program (see Materials and Methods), extending neurite-like outgrowths containing microtubule-associate protein 2, a dendritic marker (Trettel et al., 2000). However, *STHdh*^{+/+} cells do not express detectable levels of endogenous Shank3 or CaMKIIα before or after differentiation (Fig. 5A, left, middle). Therefore, they represent an excellent heterologous cell model to explore the colocalization of Shank3 and CaMKIIα in a neuron-like environment, without potentially confounding effects of high levels of endogenous CaMKIIα, Shank3, or other CaMKAPs that might compete for the interactions of expressed proteins.

Since our *in vitro* studies indicate that CaMKII activation and Thr286 autophosphorylation are a critical modulator of Shank3 binding, we first investigated colocalization of GFP-Shank3-WT with mAp-CaMKIIα-WT, mAp-CaMKIIα-T286D (phospho-mimetic), or mAp-CaMKIIα-T286A (phospho-null) in fixed, differentiated cells. GFP-Shank3-WT (green channel) was concentrated in puncta in the soma and in neurite-like outgrowths (Fig. 5B), but the appearance of the mApple fluorescence (magenta) varied with the CaMKIIα Thr286 mutation. Signals from mAp-CaMKIIα-WT and CaMKIIα-T286D displayed punctate characteristics in the soma and in neurite-like outgrowths that appeared to partially overlap with GFP-Shank3-WT punctae, whereas the signal from mAp-CaMKIIα-T286A was generally diffuse throughout the cell, and the limited number of visible punctae did not align with GFP-Shank3-WT punctae (Fig. 5B, inset, neurite-like outgrowths). To provide an unbiased assessment of the colocalization of the mApple and GFP signals across multiple cells, we analyzed images of whole transfected cells using image correlation analysis, which quantifies colocalization on an ICQ scale from −0.5 (mutually exclusive signals) to 0.5 (perfect colocalization), with 0 representing random overlap (Li et al., 2004). The ICQ values for overlap of GFP-Shank3-WT with mAp-CaMKIIα-WT, mAp-CaMKIIα-T286D, and mAp-CaMKIIα-T286A were 0.29 ± 0.02, 0.36 ± 0.02, and 0.17 ± 0.02, respectively (Fig. 5C). These data confirm that mAp-CaMKIIα-WT and mAp-CaMKIIα-T286D significantly colocalized with GFP-Shank3-WT in these cells, whereas the colocalization of mAp-CaMKIIα-T286A with GFP-Shank3-WT was much weaker (*p* < 0.0001). The unexpectedly robust colocalization of mAp-CaMKIIα-WT with GFP-Shank3-WT may be explained by the observation that mAp-CaMKIIα-WT is significantly phosphorylated at Thr286 in *STHdh*^{+/+} cells under these conditions (Fig. 5A, right). Together, these data show that Shank3 colocalization with CaMKII in intact cells is sensitive to Thr286 modification, commensurate with our biochemical data (Fig. 2C).

If colocalization of mAp-CaMKIIα-WT with GFP-Shank3 requires the direct interaction that we identified *in vitro*, the RRK/AAA mutation in the central domain of Shank3 should interfere with colocalization. Therefore, a second series of studies compared the colocalization of mAp-CaMKIIα-WT with GFP-Shank3-WT or GFP-Shank3-AAA; as an additional negative control, we examined the colocalization of soluble mApple with GFP-Shank3-WT (Fig. 5D). GFP-Shank3-WT and GFP-Shank3-AAA adopted a punctate localization pattern similar to that observed in Figure 5B. However, mAp-CaMKIIα-WT overlapped with GFP-Shank3-WT punctae, but not GFP-Shank3-AAA (Fig. 5D, compare top to center). The soluble mApple diffusely filled the cell, with minimal overlap with GFP punctae (Fig. 5D, bottom). This qualitative assessment was confirmed by image correlation anal-

Figure 5. Shank3^{949RRK951} to AAA mutation disrupts colocalization of activated CaMKII α . **A**, Immunoblots of undifferentiated, differentiated, and transfected/differentiated *STHdh*^{+/-} cells, with a WT mouse forebrain lysate as positive control. Shank3 and CaMKII α are not expressed in nontransfected *STHdh*^{+/-} cells, and transfected mApple-tagged CaMKII α (mAp-CaMKII α) is T286-phosphorylated. **B**, Representative images of differentiated *STHdh*^{+/-} cells expressing GFP-Shank3-WT with mAp-CaMKII α -WT (left), mAp-CaMKII α -T286D (middle), or mAp-CaMKII α -T286A (right). Inset, Regions (dashed line box) of the processes containing punctate GFP signals (arrowheads) that overlap with mApple signal from CaMKII α -WT and -T286D, but not -T286A. **C**, Intensity correlation analysis quantifying the colocalization of GFP and mAp signals in transfected and differentiated *STHdh*^{+/-} cells in **B**. Each data point represents an ICQ value from a single cell, with 7–12 cells analyzed from each of three independent cultures/transfections. ICQ values for GFP-Shank3-WT and either mAp-CaMKII α -WT (mean $\pm$ SEM: 0.29 ± 0.02) or mAp-CaMKII α -T286D (0.36 ± 0.02) were significantly more colocalized compared with mAp-CaMKII α -T286A (0.17 ± 0.02) (one-way ANOVA, $F_{(2,71)} = 21.35, p < 0.0001$). Tukey's *post hoc* test: *** $p < 0.001$, **** $p < 0.0001$. **D**, Representative images of differentiated *STHdh*^{+/-} expressing GFP-Shank3-WT or GFP-Shank3-AAA with soluble mAp or mAp-CaMKII α -WT. Inset, Expanded regions of the processes, as in **B**. **E**, Intensity correlation analysis quantifying the colocalization of GFP and mAp signals in transfected and differentiated *STHdh*^{+/-} cells in **C**. GFP-Shank3-WT and mAp-CaMKII α -WT (0.31 ± 0.02) are significantly more colocalized than GFP-Shank3-WT and mAp (0.07 ± 0.01) or GFP-Shank3-AAA and mAp-CaMKII α (0.09 ± 0.02) (one-way ANOVA, $F_{(2,70)} = 39.55, p < 0.0001$). Tukey's *post hoc* test: **** $p < 0.0001$. Scale bars: **B**, **D**, 2.5 μ m.

ysis, with ICQ values for overlap of 0.31 ± 0.02 , 0.07 ± 0.01 , and 0.09 ± 0.02 , respectively (Fig. 5E). These data indicate that the colocalization of mAp-CaMKII α -WT with GFP-Shank3-WT punctae in intact cells is disrupted by the RRR/AAA mutation that we have shown disrupts the direct interaction of CaMKII with Shank3 *in vitro*.

Effects of Shank3 overexpression on LTCC signaling to the nucleus

Previous studies indicate that Shank3 may be critical for optimal LTCC signaling that increases Ser¹³³ phosphorylation of the CREB transcription factor in the nucleus (Zhang et al., 2005). This pathway can be initiated by local Ca²⁺ influx, without re-

Figure 6. Effects of Shank3 overexpression on LTCC signaling to the nucleus. **A**, Shank3 was immunoprecipitated from soluble fractions of HEK293T cells expressing HA-Ca_v1.3-CTD with mAp-Shank3^R-WT or mAp-Shank3^R-ΔPDZ. Immunoblots (representative of three biological replicates) demonstrate that HA-Ca_v1.3-CTD coimmunoprecipitates with mAp-Shank3^R-WT, but not with mAp-Shank3^R-ΔPDZ. **B**, Schematic of experimental protocols. Primary hippocampal neurons were transfected (see below) and then incubated to stimulate LTCC signaling to the nucleus (see Materials and Methods). Neurons were either fixed and stained using DAPI and pSer133-CREB antibodies after a 90 s depolarization (top: for **C**), or incubated for an additional 3 h in conditioned media before fixation and staining with DAPI and c-Fos antibodies (bottom: for **D**). **C**, Overexpression of mAp-Shank3-WT, but not mAp-Shank3-AAA or mAp-Shank3-ΔPDZ, increases the levels of pCREB staining relative to nontransfected neurons under basal and depolarized conditions (two-way ANOVA with 2 factors [Mutant, Stimulation]: Mutant, $F_{(3,179)} = 17.86, p < 0.0001$; Stimulation, $F_{(1,179)} = 1108, p < 0.0001$; Interaction, $F_{(3,179)} = 4.442, p < 0.01$). Dunnett's multiple-comparisons test: $**p < 0.01, ****p < 0.0001$. **D**, The expression of c-Fos is not affected by overexpression of mAp-Shank3-AAA, or mAp-Shank3-ΔPDZ (two-way ANOVA with 2 factors [Mutant, Stimulation]: Mutant, $F_{(3,166)} = 2.152, p = 0.12$; Stimulation, $F_{(1,166)} = 830.1, p < 0.0001$; Interaction, $F_{(3,166)} = 0.3284, p = 0.82$, Dunnett's multiple-comparisons test). Error bars indicate mean $\pm$ SEM. Superimposed data points indicate values from single cells accumulated from three to five independent neuronal cultures/transfections. Images below the bar graphs are of representative nuclei for each condition. Scale bars, 5 μ m.

quiring global increases in Ca²⁺, and requires CaMKII recruitment to this LTCC nanodomain (Wheeler et al., 2008; Ma et al., 2014; Wang et al., 2017). Since Shank3 interacts with several other proteins, in addition to the CTD of Ca_v1.3 LTCCs (via its PDZ domain) (Zhang et al., 2005) and CaMKII (current observation), any effects of manipulating Shank3 expression on signaling to the nucleus may involve multiple mechanisms. The role of CaMKII binding to Shanks may be revealed by comparing the effects of expressing Shank3-WT and Shank3-AAA (which cannot bind CaMKII). As a complementary tool, we also generated a Shank3 mutant lacking the entire PDZ domain (Shank3-ΔPDZ) that is unable to interact with the CTD of Ca_v1.3 (Fig. 6A). Comparing the effects of expressing Shank3-WT and Shank3-ΔPDZ in neurons should reveal the role of Shank3 PDZ domain binding to Ca_v1.3.

We initially compared the effects of overexpressing WT or mutated Shank3 proteins on LTCC/CaMKII-dependent signaling to the nucleus in cultured hippocampal neurons using a well-established stimulation paradigm (Fig. 6B, top) (Wheeler et al., 2008; Ma et al., 2014; Li et al., 2016; Wang et al., 2017). Intrinsic neuronal activity was blocked by preincubation in 5 mM K⁺ Tyrode's solution (5K) containing APV and CNQX (to block acti-

vation of NMDA- and AMPA-type glutamate receptors, respectively) and TTX (to inhibit voltage-dependent sodium channels). Neurons were then depolarized by replacing the solution with 40 mM K⁺ Tyrode's solution (40K), also containing APV, CNQX, and TTX for 90 s. It is well established that this treatment robustly increases nuclear staining detected using a phospho-Ser¹³³-specific CREB antibody (pCREB intensity) that can be completely disrupted by the selective LTCC blocker nimodipine (10 μ M) (Wheeler et al., 2012; Wang et al., 2017). To determine whether this depolarization also induces gene expression, the depolarization buffer was replaced with conditioned media after 90 s, and neurons were fixed 3 h later to immunostain them for expression of the c-Fos immediate early gene (Fig. 6B, bottom). The brief neuronal depolarization is sufficient to induce a robust increase in c-Fos protein staining 3 h later (Fig. 6D), which can be completely blocked by preincubation with 10 μ M nimodipine (T.L.P. and R.J.C., unpublished observation).

To identify transfected neurons, Shank3-WT, -AAA, and -ΔPDZ were overexpressed as mApple-tagged proteins. Approximately 3 d after transfection, cultures were fixed under basal (5K) conditions or after the 90 s 40K depolarization (see above) and examined by confocal microscopy to quantify pCREB signals

Figure 7. Shank3 knockdown disrupts pCREB signaling and c-Fos expression. **A**, Validation of Shank3 shRNA and mApple-Shank3 shRNA-resistant (mAp-Shank3^R) expression vectors. Expression of shRNA, mAp-Shank3, and mAp-Shank3^R in HEK293T cells. Lysates of cells expressing (as indicated above) a control shRNA or Shank3 shRNA, along with mAp-Shank3 constructs with the WT shRNA target sequences (mAp-Shank3-WT and mAp-Shank3-AAA) or contain “silent” mutations that confer shRNA resistance (mAp-Shank3^R constructs) were immunoblotted for Shank3 (NeuroMab antibody). **B**, DIV13 primary hippocampal neurons transfected at DIV10 with Shank3 shRNA (GFP⁺) and stained for Shank3 (magenta; CST antibody) and CaMKIIα (white; a marker of excitatory neurons). Neurons expressing the Shank3 shRNA contain substantially reduced levels of Shank3 (reduced by $91 \pm 2\%$ relative to nearby nontransfected excitatory neurons). **** $p < 0.0001$ (one-sample unpaired Student’s *t* test with equal variance compared with a theoretical value of 100). Error bars indicate mean $\pm$ SEM. Each data point represents a single cell accumulated from three independent neuronal cultures/transfections. Scale bars, 20 μ m. **C**, Shank3 knockdown has little effect on global Ca^{2+} influx. Left, fura-2-loaded hippocampal neurons transfected with control shRNA (nRNA) or Shank3 shRNA were equilibrated with Tyrode’s solution containing 5 mM KCl for 3–5 min and switched (black arrow) to Tyrode’s solution containing 40 mM KCl for 90 s. The graph plots mean $\pm$ SEM $\Delta F/F0$ values for the last 30 s of the equilibration period and during depolarization from four independent experiments (12–90 cells per replicate). The data were analyzed using a two-way repeated-measures ANOVA: Factor 1 (time), $F_{(1,342,8,049)} = 32.63$, $p = 0.0003$; Factor 2 (control/shRNA), $F_{(1,6)} = 1.703$, $p = 0.2398$; Interaction, $F_{(35,210)} = 0.8281$, $p = 0.7423$. Right, Comparison of average areas under the curve from each independent experiment revealed no statistically significant difference ($n = 4$, $p = 0.13$; paired Student’s *t* test with equal variance). **D**, The robust increase in pCREB levels following a brief depolarization (as in Fig. 6A) is significantly reduced in cells expressing the Shank3 shRNA (red bar), but not control shRNA (gray bar) (5K vs 40K: unpaired Student’s *t* test with equal variance, **** $p < 0.0001$; 40K stimulations: one-way ANOVA, $F_{(2,98)} = 38.17$, $p < 0.0001$; Tukey’s *post hoc* test, **** $p < 0.0001$). **E**, Similarly, the robust increase in c-Fos expression 3 h after the brief depolarization is significantly reduced in cells expressing Shank3 shRNA (red bar), but not control shRNA (gray bar) (5K vs 40K: unpaired Student’s *t* test with equal variance, **** $p < 0.0001$; 40K stimulations: one-way ANOVA, $F_{(2,90)} = 9.990$, $p < 0.001$, Tukey’s *post hoc* test, *** $p < 0.001$). Error bars indicate mean $\pm$ SEM. Each data point represents a single cell accumulated from three independent neuronal cultures/transfections. Images below the bar graphs are of representative nuclei for each condition. Scale bars, 5 μ m.

in DAPI-stained nuclei of mApple- and CaMKIIα-expressing neurons relative to CaMKIIα-expressing neurons in nontransfected cultures. The overexpression of mAp-Shank3-WT resulted in a small but significant increase in pCREB intensity in both unstimulated and depolarized neurons relative to nontransfected neurons (Fig. 6C, compare green and black bars). Notably, this increase of pCREB intensity was not detected in neurons overexpressing similar levels of mAp-Shank3-AAA or mAp-Shank3-ΔPDZ (Fig. 6C, blue and purple bars). However, the modest increases in pCREB intensity following overexpression of mAp-Shank3-WT were not sufficient to enhance c-Fos expression in unstimulated or depolarized neurons (Fig. 6D). Together, these data indicate that, under these conditions, the overexpression of Shank3 has relatively limited effects on LTCC signaling to the nucleus, perhaps because these cells contain significant levels of endogenous Shank3.

Shank3 is required for LTCC-dependent CREB phosphorylation and gene expression

To further investigate the role of Shank3 in LTCC signaling to the nucleus, we adopted an shRNA knockdown approach to suppress the expression of endogenous Shank3. First, we confirmed that a previously used shRNA targeting Shank3 (Vercelli et al., 2011) essentially completely suppressed the expression of mAp-Shank3-WT or mAp-Shank3-AAA in HEK293 cells, whereas a control shRNA (Boudkkazi et al., 2014) has no effect (Fig. 7A, lanes 2 and 3). Moreover, the Shank3 shRNA has no effect on expression of an shRNA-resistant forms of Shank3 (mAp-Shank3^R) (Fig. 7A, lanes 6–8). Furthermore, staining for Shank3 protein in CaMKII-positive primary hippocampal neurons was reliably decreased in neurons expressing the Shank3 shRNA relative to nearby non-

Figure 8. Rescue of pCREB signaling and c-Fos expression after Shank3 shRNA knockdown. The Shank3 shRNA construct was transfected alone or with shRNA-resistant mAp-Shank3^R-WT (green bar), mAp-Shank3^R-AAA (blue bar), or mAp-Shank3^R-ΔPDZ (purple bar). Neurons were depolarized for 90 s, and the levels of pCREB (**A**) and c-Fos (**B**) were determined (see Materials and Methods) in transfected (colored bars) and nearby nontransfected (black bars) neurons. **A**, The expression of mAp-Shank3^R-WT, but not mAp-Shank3^R-AAA or mAp-Shank3^R-ΔPDZ, rescued signaling to pCREB relative to shRNA alone. In addition, neurons expressing Shank3-WT had significantly higher pCREB signal relative to nearby, nontransfected neurons (two-way ANOVA with 2 factors [Mutant, Transfection]: Mutant, $F_{(3,156)} = 10.14$, $p < 0.0001$; Transfection, $F_{(1,156)} = 22.70$, $p < 0.0001$; Interaction, $F_{(3,156)} = 12.29$, $p < 0.0001$, comparison between mutants: Dunnett's multiple-comparisons test, **** $p < 0.0001$; comparison between transfected/nontransfected cells: Sidak's multiple-comparisons test, * $p < 0.05$). **B**, The expression of mAp-Shank3^R-WT, but not mAp-Shank3^R-AAA or mAp-Shank3^R-ΔPDZ, rescued signaling to increase c-Fos expression relative to shRNA alone (two-way ANOVA with 2 factors ([Mutant, Transfection]: Mutant, $F_{(3,150)} = 13.80$, $p < 0.0001$; Transfection, $F_{(1,150)} = 26.52$, $p < 0.0001$; Interaction, $F_{(3,150)} = 8.149$, $p < 0.0001$, comparison between mutants: Dunnett's multiple-comparisons test, **** $p < 0.0001$; comparison between transfected/nontransfected cells: Sidak's multiple-comparisons test). Error bars indicate mean $\pm$ SEM. Each data point represents a single cell, accumulated from four or five independent neuronal cultures/transfections. Images below the bar graphs are of representative nuclei for each condition. Scale bars, 5 μ m.

transfected neurons (Fig. 7B). Thus, this shRNA effectively suppresses Shank3 expression in neurons.

Deletion of five C-terminal amino acids from Cav1.3 prevents binding to Shank3 but has little impact on LTCC gating, or on whole-cell LTCC current amplitude or current–voltage relationship in heterologous cells and in cultured neurons, or on total Cav1.3-LTCC-mediated neuronal Ca²⁺ influx (Zhang et al., 2005). However, the impact of neuronal Shank3 knockdown on LTCC-mediated Ca²⁺ influx has not been determined. Therefore, we used fura-2 to assess somatic Ca²⁺ responses during a 90 s, 40K depolarization as used for studying E-T coupling (see above). Comparison of average Ca²⁺ responses ($\Delta F/F_0$) over time in neurons expressing control or Shank3 shRNA from four independent experiments revealed no statistically significant difference (Fig. 7C, left). Similarly, Shank3 knockdown had no statistically significant impact on total Ca²⁺ influx, as estimated from average areas under the curve from each experiment (Fig. 7C, right). However, there was a trend for a small reduction of Ca²⁺ influx in Shank3 shRNA neurons. Together, these data indicate that knockdown of Shank3 expression results in little effect on global Ca²⁺ influx via LTCCs under these conditions.

Neuronal cultures were then transfected to express either control or Shank3 shRNA and depolarized for 90 s. While control shRNA had no effect on pCREB intensity (Fig. 7D, gray bar), neurons expressing Shank3 shRNA had a significant, if partial, reduction in pCREB intensity relative to nontransfected neurons and control shRNA-expressing neurons (Fig. 7D, red bar). This decrease in pCREB intensity in neurons expressing the Shank3 shRNA was mirrored by a parallel decrease of c-Fos expression when assessed 3 h after the brief depolarization

(Fig. 7E, red bar), whereas c-Fos was robustly expressed in nontransfected neurons and neurons expressing control shRNA 3 h after stimulation. Thus, Shank3 expression appears to be necessary for the full extent of both CREB phosphorylation as well as c-Fos expression following a brief neuronal depolarization.

Rescue of shRNA effects by Shank3-WT, but not Shank3-AAA or Shank3-ΔPDZ

We then investigated whether Shank3 shRNA-induced suppression of E-T coupling could be rescued by Shank3 reexpression from shRNA-resistant constructs (mAp-Shank3^R). Neurons were transfected to express Shank3 shRNA alone or Shank3 shRNA plus either mAp-Shank3^R-WT, mAp-Shank3^R-AAA, or mAp-Shank3^R-ΔPDZ. Following depolarization, pCREB staining was quantified in neurons expressing the shRNA, and in nearby nontransfected neurons within the same dish, to provide an internal control for each experiment with a similar robust increase of pCREB staining intensity under all conditions (Fig. 8A, black bars). As seen in Figure 7D, Shank3 shRNA expression significantly, if partially, reduced the pCREB signal (Fig. 8A, red bar), but expression of mAp-Shank3^R-WT rescued pCREB intensity to a level slightly but significantly higher than that in nontransfected neurons (Fig. 8A, green bar). However, the level of pCREB staining was not rescued by expression of either mAp-Shank3^R-AAA or mAp-Shank3^R-ΔPDZ (Fig. 8A, blue and purple bars). Moreover, analysis of parallel neuronal cultures 3 h following the brief depolarization revealed that partial suppression of c-Fos protein expression by the Shank3 shRNA was rescued by mAp-Shank3^R-WT, but not by mAp-Shank3^R-

AAA or mAp-Shank3^R-ΔPDZ (Fig. 8B). However, c-Fos expression in neurons expressing both the Shank3 shRNA and mAp-Shank3^R-WT was not significantly different from c-Fos levels in nearby nontransfected neurons. Together, these findings significantly extend previous studies by showing that the Shank3 PDZ domain is important for LTCC signaling to the nucleus, presumably by binding to Ca_v1.3 (Zhang et al., 2005), and that CaMKII binding to Shank3 also plays a key role in this pathway. Importantly, these interactions with Shank3 play a key role not only in depolarization-induced increases of CREB phosphorylation, but also for the downstream increase in expression of c-Fos, an important immediate early gene.

Discussion

We recently used a proteomics approach to show that synaptic CaMKII complexes immunoprecipitated from mouse forebrain contain many proteins, including substantial amounts of Shank3 (Baucum et al., 2015). In addition, we reported that Shank3-CaMKII complexes can be coimmunoprecipitated from HEK293T cells overexpressing Shank3 and CaMKII (Stephenson et al., 2017). However, coimmunoprecipitation of two proteins may arise from direct or indirect interactions of the proteins involved. Here, we explicitly demonstrate a direct interaction between purified Shank3 and purified CaMKII and define its regulation by CaMKII activation. Thr286-autophosphorylated CaMKIIα robustly and directly binds to a central domain in Shank3 with no previously defined biochemical or physiological function, and mutation of three basic residues within this domain to Ala (AAA mutation) largely prevents binding. *In vitro*, this Shank3 domain binds much more weakly to CaMKIIα activated only by Ca²⁺/CaM binding. The colocalization of CaMKII with Shank3 in intact cells is regulated by CaMKII activation and disrupted by the AAA mutation, concordant with our *in vitro* findings. Moreover, robust coimmunoprecipitation of CaMKIIα with Shank3 from mouse forebrain extracts is largely abrogated by a knock-in Thr286 to Ala mutation, showing that CaMKIIα Thr286 autophosphorylation is required for robust CaMKII-Shank3 interactions *in vivo*. From a functional perspective, we show that Shank3 expression is important for normal LTCC-dependent E-T coupling in cultured hippocampal neurons, as measured by CREB phosphorylation and the expression of c-Fos, a critical immediate early gene. Finally, our Shank3 shRNA rescue studies using WT and mutated Shank3 proteins indicate that interactions with both CaMKII and the Ca_v1.3 LTCC are important for normal E-T coupling. Together, these data substantially advance our understanding of the molecular mechanisms underlying a novel biological role for a major synaptic scaffolding protein.

CaMKII has diverse physiological functions, and the present findings add to a growing body of evidence that specific functions of CaMKII are controlled in part by interactions with CaMKAPs. Like Shank3, most CaMKAPs preferentially bind activated CaMKII conformations, induced by either Ca²⁺/CaM binding and/or Thr286 autophosphorylation. Therefore, CaMKII activation acts as a switch to stimulate binding to Shank3 and other CaMKAPs. Interestingly, Shank3 and the GluN2B subunit of the NMDAR exhibit different selectivity for distinct activated CaMKII conformations. Ca²⁺/CaM binding alone is insufficient for GluN2B binding but partially supports interactions with Shank3 and densin, relative to Thr286-autophosphorylated CaMKIIα (Robison et al., 2005a; Jiao et al., 2011). While the physiological significance of these differences is unclear, they presumably reflect subtle variations in how CaMKAPs interact with CaMKIIα. Consistent with this idea, the CaMKII binding domains of activation-dependent

CaMKAPs can be subclassified based on sequence homology. CaMKII binding domains in GluN2B subunits and LTCC β1/2 subunits share sequence homology with the CaMKIIα autoregulatory domain, surrounding the Thr286 autophosphorylation site (Strack et al., 2000; Grueter et al., 2008). However, the internal CaMKII binding domain in densin, another synaptic scaffolding protein, has a distinct amino acid sequence, resembling that of the naturally occurring CaMKII inhibitor protein CaMKIIN (Jiao et al., 2011). Furthermore, recently identified CaMKII binding domains in LTCC Ca_v1.2 and Ca_v1.3 α1 subunits and the mGlu5 metabotropic glutamate receptor contain critical tribasic residue motifs (Wang et al., 2017; Marks et al., 2018), similar to that in Shank3 identified here (Fig. 3). However, the tribasic residue motif alone is insufficient for CaMKII binding because Thr286-autophosphorylated CaMKIIα does not bind two GST-Shank3 proteins (#3 and #4a) that contain tribasic sequences (⁷⁰⁷Arg-Arg-Lys⁷⁰⁹ and ⁹¹⁹Lys-Arg-Arg⁹²¹, respectively) (Figs. 2B, 3B). Further studies will be needed to explore contributions of residues surrounding the tribasic binding motifs, and perhaps secondary or tertiary structure, to CaMKII binding with these CaMKAPs.

It is well established that activated CaMKII and Shank3 are highly enriched in dendritic spines (Shen and Meyer, 1999; Boeckers et al., 2005), and are also found in complexes containing NMDARs and other CaMKAPs (Naisbitt et al., 1999; Baucum et al., 2015). Since dendritic spines contain several CaMKAPs, it can be difficult to discern their individual contributions to modulating CaMKII localization and function. However, knock-in mutations of GluN2B in mice that block CaMKII binding to GluN2B and reduce the amount of CaMKII in postsynaptic densities also impair both synaptic plasticity and memory recall (Halt et al., 2012). While Shank3 knockdown has been shown to disrupt NMDAR-mediated postsynaptic currents and DHPG-induced LTD (Verpelli et al., 2011; Duffney et al., 2013), roles of specific protein interactions with Shank3 are poorly understood. We posit that one function of Shank3 is to target a subpopulation of CaMKII holoenzymes to postsynaptic LTCC complexes.

Our studies show that Shank3 has important roles in E-T coupling initiated by LTCC-dependent Ca²⁺ influx and CaMKII activation to stimulate CREB phosphorylation and c-Fos expression. Precise regulation of gene transcription is important for long-term, activity-dependent changes in synaptic properties and behavior. Diverse stimulation paradigms increase Ser¹³³ phosphorylation of the nuclear CREB transcription factor to stimulate immediate early gene transcription, and CREB is important for learning and long-term memory (for review, see Shaywitz and Greenberg, 1999; Alberini, 2009; Kandel, 2012; Kida and Serita, 2014). Plasma membrane LTCCs are major regulators of nuclear CREB phosphorylation and immediate early gene expression following depolarization (Dolmetsch, 2003; Flavell and Greenberg, 2008; Wheeler et al., 2008; Bading, 2013). Both major neuronal LTCC α1 subunits, Ca_v1.2 and Ca_v1.3, can initiate LTCC-CREB signaling, depending on the brain region (Hetzenauer et al., 2006) and the strength of depolarization (Zhang et al., 2006). The CTDs of Ca_v1.2 and Ca_v1.3 contain canonical binding motifs for Class 1 PDZ domains, and deletion of these motifs disrupts channel trafficking and clustering, as well as downstream signaling to increase CREB phosphorylation, although these mutations do not appear to affect global increases in Ca²⁺ following neuronal depolarization (Weick et al., 2003; Zhang et al., 2005). The CTD mutations might disrupt CREB signaling by interfering with the local LTCC nanodomain and/or with conformational changes in LTCCs that are required for E-T

coupling (Li et al., 2016). However, CTDs of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ interact with distinct proteins. The CTD of $\text{Ca}_v1.3$, but not $\text{Ca}_v1.2$, can interact with PDZ domains of Shank1 or Shank3 (Zhang et al., 2005), but the roles of Shanks in LTCC-CREB signaling have not been previously investigated. Our shRNA experiments showed that Shank3 expression is essential for maximal LTCC-CREB-c-Fos signaling, with little impact on global Ca^{2+} signals. Moreover, shRNA rescue experiments revealed that the Shank3 PDZ domain is required for E-T coupling, consistent with the idea that Shank3 PDZ domain binding to the $\text{Ca}_v1.3$ CTD is required, and significantly extending prior findings. Residual E-T coupling following Shank3 knockdown may be mediated by Shank3-independent $\text{Ca}_v1.2$ LTCC signaling, although our data cannot preclude contributions from low levels of residual Shank3 expression or from Shank1. Nevertheless, our observations strongly support a model in which Shank3 actions within the LTCC/ Ca^{2+} nanodomain are required for efficient E-T coupling to increase both CREB phosphorylation and c-Fos expression.

The form of LTCC-dependent E-T coupling studied herein also requires direct interaction of CaMKII with the N-terminal domain of $\text{Ca}_v1.3$ $\alpha 1$ subunits in LTCC nanodomains (Wheeler et al., 2008; Wang et al., 2017). How can this observation be reconciled with the current finding that mutation of the tribasic residue motif in Shank3 to prevent CaMKII binding also disrupts E-T coupling? Since CaMKII holoenzymes can bind simultaneously to multiple CaMKAPs (Robison et al., 2005b), different subunits within a single dodecameric CaMKII holoenzyme may interact with $\text{Ca}_v1.3$ and Shank3, potentially providing a conformational constraint on LTCC cytoplasmic domains that could affect Ca^{2+} influx. Alternatively, $\text{Ca}_v1.3$ and Shank3 may recruit two different CaMKII holoenzymes to the LTCC nanodomain to facilitate a trans-holoenzyme autophosphorylation that appears to be required for shuttling Ca^{2+} /CaM to the nucleus to stimulate CREB phosphorylation (Ma et al., 2014; Cohen et al., 2018). Both hypotheses predict that loss of any one of these three proteins or disruption of any one of their mutual interactions would interfere with this form of E-T coupling.

Although mutual interactions of CaMKII, Shank3, and $\text{Ca}_v1.3$ LTCCs are required to initiate this form of E-T coupling, the precise role of these interactions remains unresolved. Knockdown of CaMKII or Shank3 expression has little effect on depolarization-induced global Ca^{2+} signals under our conditions (Fig. 7C) (Wang et al., 2017). However, it remains possible that CaMKII and/or Shank3 modulate Ca^{2+} concentrations within the LTCC nanodomain. Although CaMKII can potentiate net Ca^{2+} influx via $\text{Ca}_v1.3$ LTCCs by decreasing Ca^{2+} -dependent inactivation in heterologous cells (Jenkins et al., 2010), this modulation requires coexpression of densin, another synaptic CaMKAP containing a PDZ domain that binds to the $\text{Ca}_v1.3$ CTD. Like Shank3, densin has no direct effect on the biophysical properties of LTCCs (Zhang et al., 2005; Jenkins et al., 2010), but Shank3 presumably competes with densin for binding to the CTD of $\text{Ca}_v1.3$. Shank3 competition might be predicted to disrupt CaMKII- and densin-dependent facilitation of Ca^{2+} influx via $\text{Ca}_v1.3$, reducing overall Ca^{2+} influx, and it is unclear how this could enhance the initiation of E-T coupling. However, it is possible that Shank3 supports CaMKII modulation of $\text{Ca}_v1.3$ inactivation in a similar (or perhaps distinct) manner. CaMKII also was reported to mediate the effects of IGF1 to facilitate $\text{Ca}_v1.3$ currents and CREB phosphorylation at weaker depolarizing membrane potentials (Gao et al., 2006). Specific mechanisms underlying both of these effects of CaMKII on $\text{Ca}_v1.3$ remain poorly understood, but it is possible

that CaMKII phosphorylation of $\text{Ca}_v1.3$ $\alpha 1$ or β subunits or Shank3 is involved. A significant challenge in elucidating these mechanisms will be to assess their impact within the specific context of the LTCC/ Ca^{2+} nanodomain that initiates E-T coupling. Clearly, further studies will be needed to more precisely define these biochemical mechanisms.

SHANK3 mutations are strongly linked to ASD and other neuropsychiatric disorders (Gauthier et al., 2010; Herbert, 2011), and c-Fos expression is dysregulated in rodent ASD models (Orlandini et al., 1996; Williams and Umemori, 2014; Dubiel and Kulesza, 2015). Increases or decreases in Shank3 expression may be associated with distinct neuropsychiatric phenotypes (Bozdagi et al., 2010; Han et al., 2013; Uchino and Waga, 2013). Moreover, gain-of-function $\text{Ca}_v1.3$ mutations have been identified in patients with ASD (Pinggera et al., 2015, 2017) and a $\text{Ca}_v1.2$ point mutation associated with Timothy syndrome increases CREB phosphorylation (Li et al., 2016), perhaps indicating that hyperphosphorylation of CREB may be involved in some neuropsychiatric disorders. Diverse changes in E-T coupling may result from disruption of the LTCC nanodomain due to altered interactions between ASD-linked postsynaptic proteins (Bourgeron, 2009). Our recent work showed that an ASD-linked *de novo* CaMKII α -E183V mutation disrupts interactions with Shank3 and several other CaMKAPs (Stephenson et al., 2017), including the $\text{Ca}_v1.3$ -NTD (T.L.P., J.R.S., and R.J.C., unpublished observations). Thus, even though initial studies failed to detect gross changes in CaMKII expression following genetic disruptions of Shank3 (e.g., Peça et al., 2011), the present findings suggest that more detailed investigations of the role of CaMKII in animal models of neuropsychiatric disorders are warranted.

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