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Supplemental Information

AKAP-Anchored PKA Maintains Neuronal L-type Calcium Channel Activity and NFAT Transcriptional Signaling

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Supplemental Material for Murphy et al., 2014 Cell Reports

Supplemental Figures

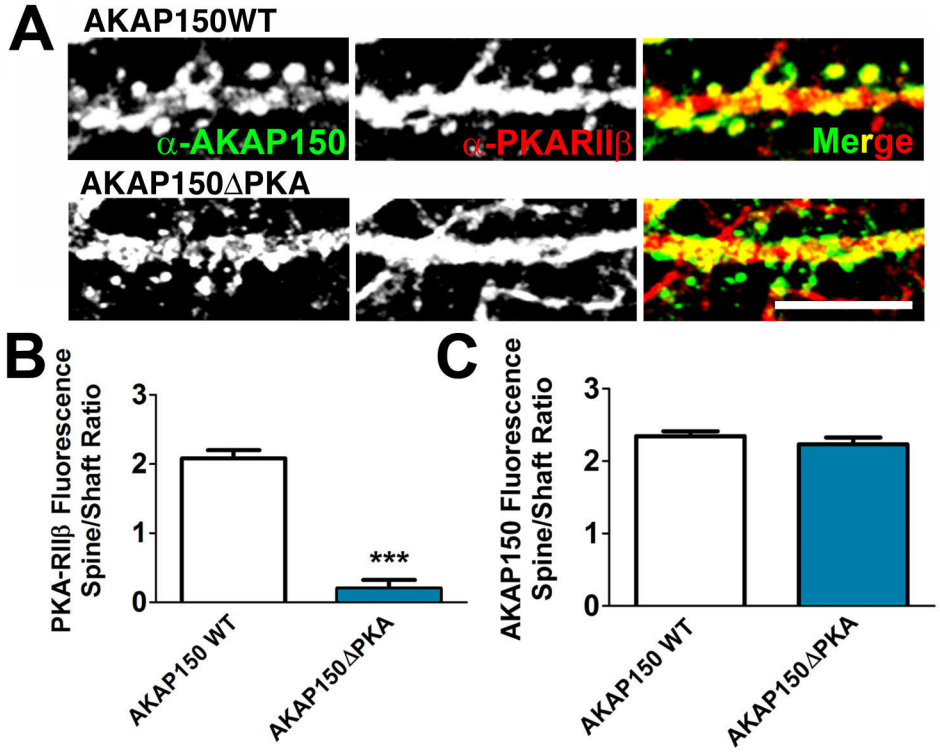

Figure S1 related to Figure 2

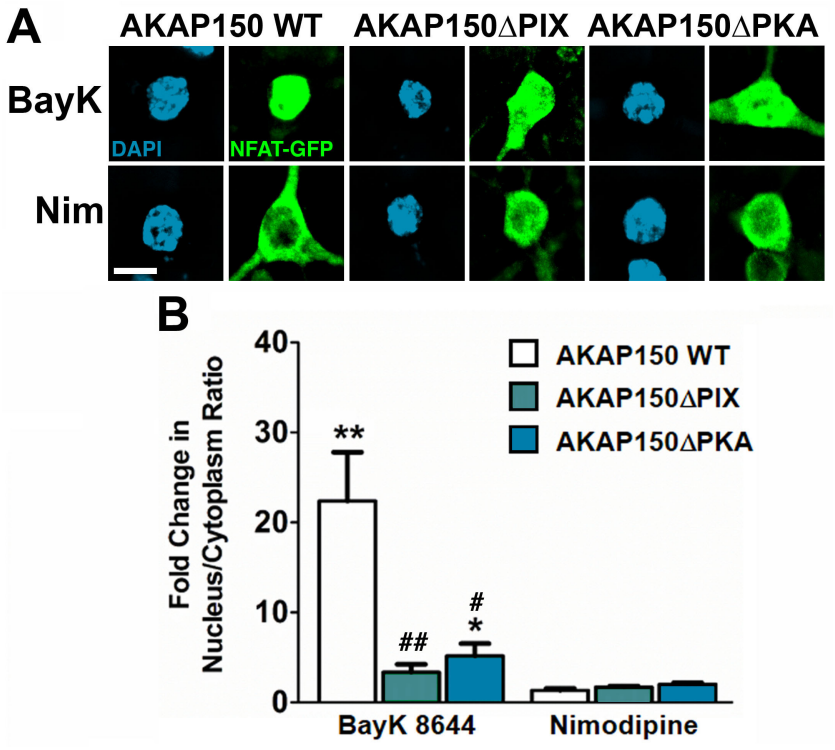

Figure S2 related to Figure 3

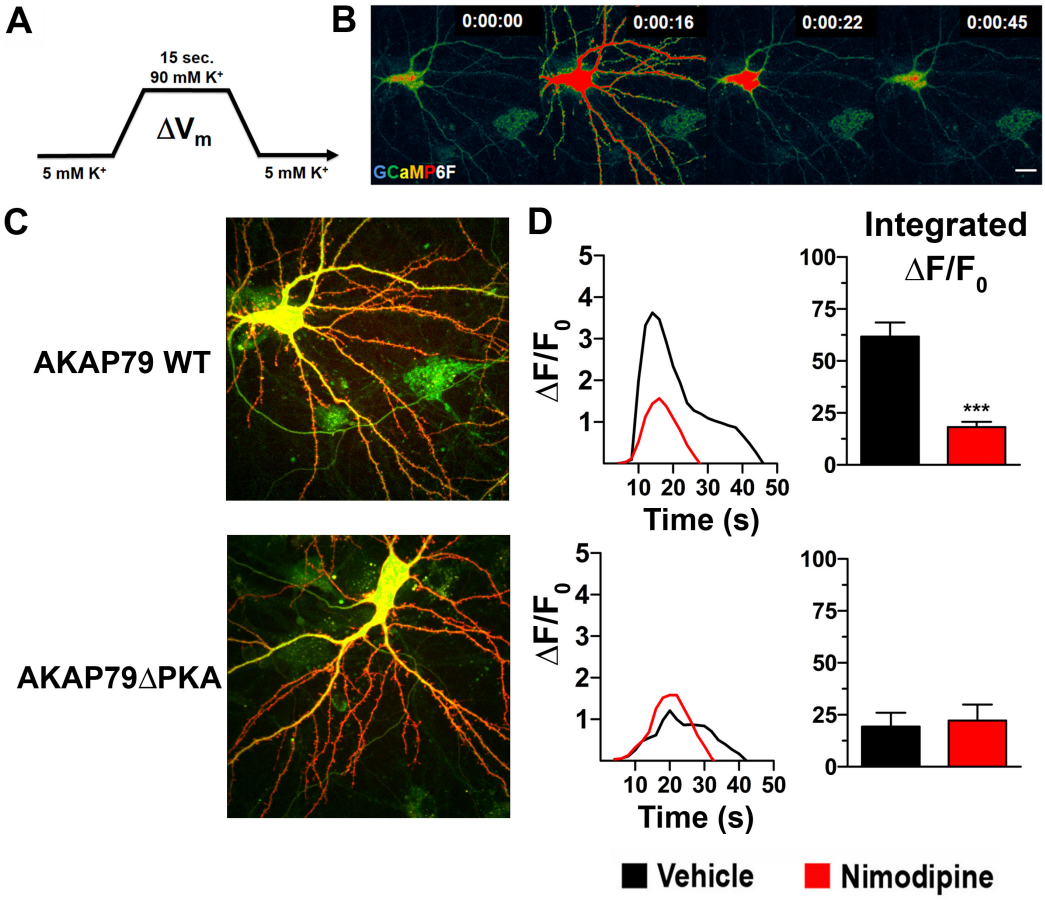

Figure S3 related to Figure 7

Supplemental Figure Legends

Figure S1 related to Figure 2: AKAP150 anchoring regulates PKA localization to dendritic spines. (A) Projection images of dendrite segments stained for AKAP150 (green) and PKA-RIIβ (red) for neurons from AKAP150 WT or ΔPKA knock-in mice showing reduced PKA-RIIβ localization in spines for AKAP150ΔPKA. (B) Quantification of PKA-RIIβ spine localization as a spine/dendrite shaft fluorescence intensity ratio for AKAP150 WT or ΔPKA knock-in mouse neurons. (C) Quantification of AKAP150 spine localization in AKAP150 WT or ΔPKA knock-in mouse neurons. Data expressed as mean ± SEM (***p<0.001by Student's t-test; n = 4-7). Scale bar = 10μm

Figure S2 related to Figure 3: AKAP79/150 anchoring of both CaN and PKA regulates NFAT movement to the nucleus in response to L-type Ca^{2+} channel opening driven by spontaneous neuronal activity. (A) Summed intensity projection images of NFATc3-GFP (green) and nuclei (DAPI, blue) in AKAP150 WT, AKAP150ΔPIX, and AKAP150ΔPKA mouse hippocampal neurons 10 min after TTX washout in the presence of BayK 8644 (BayK) to promote or nimodipine (Nim) to block LTCC activity. (B) Quantification of NFATc3-GFP translocation to nucleus 10 min after TTX washout measured as the fold-change in GFP fluorescence nucleus/cytoplasm ratio relative to non-stimulated conditions with no TTX washout (images not shown). Data expressed as mean ± SEM (*p<0.05, **p<0.01 compared to the corresponding Nim condition for that genotype by Student's t-test; $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared to AKAP150 WT BayK stimulation by ANOVA with Bonferonni's Multiple Comparison Test; n = 5-12). Scale bar = 10μm

Figure S3 related to Figure 7: LTCC Ca^{2+} signals imaged with GCaMP6F are reduced in the absence of AKAP79/150-PKA anchoring. (A) Diagram of the high K^+ depolarization protocol to trigger LTCC Ca^{2+} influx. (B) Time-course of GCaMP6F Ca^{2+}-indicator fluorescence change, shown in pseudocolor, in response to K^+ depolarization in rat hippocampal neurons co-expressing AKAP150 shRNAi and AKAP79WT-mCh. (C) Representative live-cell images of GCaMP6F (green) overlaid on AKAP79WT-mCh or AKAP79ΔPKA-mCh (red) fluorescence prior to Ca^{2+} imaging in rat neurons also expressing AKAP150 shRNAi. (D) Time-course (left panel) and quantification of area under the curve (right panel) for mean GCaMP6F fluorescence change over time in response to K^+ depolarization in rat neurons for the indicated conditions Data expressed as mean ± SEM. (***p<0.001 by Student's t-test; n = 5-6). Scale bars = 10μm

Supplemental Experimental Procedures

Animal Care and Use

All animal procedures were conducted in accordance with National Institutes of Health (NIH)-United States Public Health Service guidelines and with the approval of the University of Colorado Denver Institutional Animal Care and Use Committee.

Mutant Mouse Genotyping and Husbandry

AKAP150 KO and AKAP150ΔPIX mice were genotyped and cared for as described previously (Sanderson et al., 2012; Tunquist et al., 2008). For PCR genotyping of AKAP150ΔPKA mice, DNA was extracted from tail tissue using REDExtract-N-Amp Tissue PCR kit (Sigma) following manufacturer's recommendations. PCR with forward (5'-ACCGAGATCAGAAGAAAGCAAACG-3') and reverse (5'-CCTCGGAAACCATTTCATTAACCA-3') primers amplified nucleotides 2282–2559 of the coding sequence, giving a 277 bp fragment for the WT allele and a 247 bp fragment for the ΔPKA allele. For most experiments, AKAP150ΔPKA mice were maintained on a mixed C57Bl6/129 background as heterozygous breeding pairs to provide WT littermate controls; however, for neonatal cultured neuron preparations, WT and ΔPKA homozygous breeding pairs were used to provide litters of a single genotype. AKAP150ΔPKA mice have no obvious alterations in physical, behavioral, or breeding phenotype in the home-cage environment.

Immunoprecipitation and Immunoblotting

AKAP150 immunoprecipitations (IP) from the hippocampus of ~2 month-old male and female mice were performed as per Gomez et al (Gomez et al., 2002) with slight modifications. Hippocampi were homogenized in lysis buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 1 mM Benzamidine and 1 mM

AEBSF), then incubated on ice for 20 min in the presence of 1% Triton X-100. Lysates were spun for 20 min at 20,800g. 10% of supernatant was reserved for gel loading. The remaining supernatant was diluted to 0.5% Triton X-100 and split evenly into two samples, receiving either 5 µg rabbit anti-AKAP150 or 5 µg rabbit anti-IgG antibodies. Samples were incubated for 4 hours at room temperature with end-over-end shaking, followed by one hour in protein A Sepharose beads, prior to extensive washing.

The entire immunoprecipitates or 15 µg whole extract (WE/input) were resolved on Tris-SDS gels and transferred in 20% methanol to PVDF membranes. 40 µg of WE was loaded gel for parallel blotting with anti-myc and anti- AKAP150. Primary antibodies were incubated with the membranes for a minimum of 90 min as follows: rabbit anti-AKAP150 (1:2000); (Brandao et al., 2012), mouse anti-myc and anti-PKA-RIIα (1:1000; Santa Cruz Biotechnology), mouse anti-PKA-C (1:1000; BD-Transduction Labs), and mouse anti-CaNA (1:1000; Sigma-Aldrich). Detection was performed with horseradish peroxidase (HRP)-coupled secondary antibodies (Bio-Rad; 1:1000) followed by enhanced chemiluminescence (ECL) (WestPico or West Dura Chemiluminescent Substrate; Pierce). Chemiluminescence was imaged using an Alpha Innotech Fluorchem gel documentation system.

Quantification of Live-cell FRET Microscopy in tsA-201 Cells

YFP, CFP and CYFRET fluorescence were detected in single xy planes in living cells to capture three images: 1) YFPexcitation/YFPemission, 2) CFP excitation/CFPemission, and 3) CFPexcitation/YFPemission (raw FRET). After background subtraction, fractional image subtraction corrected for CFP bleed-through and YFP cross-excitation,

FRETc = rawFRET – (0.54 x CFP) – (0,02 x YFP)

to yield an image of corrected FRET (FRETc), which was then gated to the CFP donor channel to create a FRETc/CFP pseudocolor image of relative FRET intensity in the cell**.** Mean CFP,

YFP, and raw FRET fluorescence intensities were measured by mask analysis of membrane regions (or the cytoplasm for linked CFP-YFP) in Slidebook 4.0-5.5 as described previously (Oliveria et al., 2003, Oliveria et al., 2007; Li et al., 2012). Apparent FRET efficiency (FRETeff) values were calculated from these mean intensities using the equation,

FRETeff = FRETc / ((0.02 x YFP) x 10.6)

for a 1:1 complex, where FRETc is the emission from YFP due to FRET, 0.02 * YFP is the emission from YFP directly excited with the FRET filter cube, 10.6 is a factor relating CFP excited to YFP excited with the FRET filter cube, and (0.02 * YFP) * 10.6 is therefore the maximum sensitized YFP emission possible if every excited CFP transferred its excitation to the associated YFP (Erickson et al., 2001; Erickson et al., 2003; Li et al., 2012; Oliveria et al., 2007).

Transfection of Rodent Primary Hippocampal Neurons

DIV 9-13 neurons were transfected using Lipofectamine 2000 (Life Technologies). Each transfection reaction contained 4-8 μg total plasmid encoding cDNA/shRNAi. For each 18 or 25 mm coverslip, either 4 or 8 μl of Lipofectamine was added, respectively. Briefly, NB and Lipofectamine or NB and plasmid DNA were combined incubated for 5 min in separate tubes at room temperature. After 5min, the Lipofectamine and DNA tubes were combined at room temperature and incubated for 20 min to create Lipofectamine/DNA complexes. During the 20-min incubaton time, half of the medium was removed from the cultured cells and mixed with fresh NB (+B27, +Glutamax) and saved. Following the 20-min incubation, the Lipofectamine/DNA mixture was added dropwise to the cultured neurons and allowed to incubate for 1.5-2.0 h at 37ºC, 5% CO_2. After incubation, Lipofectamine-containing media was replaced with conditioned culture media. Neurons were then returned to the incubator at 37ºC, 5% CO_2, and imaged within 2 days at DIV 10-14.

Quantification of AKAP79/150, CaNA, and PKA-RII Spine Enrichment

DIV12-14 cultured rat hippocampal neurons (24-48 hours post-transfection) were fixed for 10 min at room temperature with cold 3.7% paraformaldehyde in PBS and permeabilized for 10 min at room temperature in 0.1% TritonX-100 in PBS. Transfected rat neurons expressing AKAP-mCh, CaNAα-YFP, or PKA-RIIα-CFP were washed three times in PBS and mounted on glass slides with Pro-long Gold (Life Technologies). After fixation and permeabilization, cultured AKAP150 WT and ΔPKA knock-in mouse neurons were blocked in 3% BSA/PBS overnight at 4ºC. Primary antibodies (rabbit-anti-AKAP150 ((Brandao et al., 2012)) and mouse anti-PKA-RIIβ (R&D Systems)) were diluted 1:500 in 3% BSA/PBS, applied directly to coverslips, and incubated for 2 hrs at room temperature. After incubation with primary antibody, cells were washed 3x in PBS. Secondary antibodies (goat anti-rabbit Alexa Fluor 488 & goat anti-mouse Alexa Fluor 568, (Life Technologies)) were diluted 1:500 in 3% BSA/PBS, applied directly to coverslips, and incubated for 1 h at room temperature. Coverslips were then washed three times in PBS before mounting on glass slides with Pro-long gold. Measurements of spine fluorescence intensity were made by drawing masks using Slidebook 4.0-5.5 on all spines within the dendritic arbor in the field of view using a 63x, 1.4 NA Plan-apo objective and averaged for each neuron. Similarly, dendrite fluorescence intensity was measured by drawing masks over the dendrite shafts within the field of view and averaged into one number for each cell.

NFATc3-EGFP Translocation in Hippocampal Neurons

For rat neurons, DIV11-13 cultured hippocampal neurons (24-48 hours post-transfection) were placed in a Tyrode's salt solution including 1 μM TTX (Tocris) to dampen spontaneous activity and reduce basal levels of nuclear NFAT. Cells were incubated in Tyrode's + TTX at 37ºC, 5% CO_2, for 0.5 – 1.0 h. To block LTCCs, 10 μM nimodipine (Sigma-Aldrich) was applied 10 min prior to and throughout the TTX preincubation because of the use-dependence of

dihydropyridines. Subsequently, cells were depolarized for 3 min in isotonic 50 mM KCl Tyrode's solution to open LTCCs. Next, the cells were allowed to recover for 10 min in control Tyrode's solution containing 1 μM TTX at 37ºC, 5% CO_2. Cells then underwent immunocytochemistry to stain transfected NFATc3-GFP. After treatments, cultured hippocampal neurons were fixed, permeabilized, and stained as described above. Primary antibodies (mouse anti-GFP & rabbit anti-RFP, AbcaM) were diluted 1:500 in 3% BSA/PBS, applied directly to coverslips, and incubated for 2 hrs at room temperature. Cells were washed three times in PBS and secondary antibodies (goat anti-mouse Alexa Fluor 488 & goat anti-rabbit Alexa Fluor 568, Life Technologies) were diluted 1:500 in 3% BSA/PBS, applied directly to coverslips, and incubated for 1 h at room temperature. Coverslips were then washed three times in PBS before mounting on glass slides with Pro-long gold containing DAPI (Life Technologies).

Experiments in which spontaneous neuronal activity drives NFAT translocation were carried out as follows. After TTX pretreatment, neurons were washed two times over 5 min to remove residual TTX. Spontaneous activity in the neuron cultures then persisted for 10 min at 37°C in Tyrode's containing Vehicle (0.1% DMSO), BayK 8644 (10 μM), or nimodipine (10 μM). After treatments, cells were fixed, permeabilized, and stained as described above.

Measurement of NFAT Transcriptional Reporter Expression

NFAT transcriptional reporter assays were carried out essentially as described previously (Graef et al., 1999; Li et al., 2012). 12 DIV mouse hippocampal neurons were transfected with GL3NFAT firefly (Hedin et al., 1997) & RLSV40 *Renilla* luciferase plasmids. Alternatively, 12 DIV rat neurons were transfected with the 3xNFAT-AP1–CFP-NLS fluorescent reporter plasmid, pSilencer or AKAP150 shRNAi, and either YFP, AKAP79YFP WT or mutant plasmids as described above. 4h or 26 h after transfection (for 16 h or 6 h post-KCl conditions, respectively, to ensure in both cases that cDNA and RNAi constructs would be expressed for a total of ~2 days post-transfection before fixation) 1 μM TTX was added in conditioned NB culture medium for 24 h at 37ºC, 5% CO_2, to dampen spontaneous activity and decrease basal

CFP expression. After this TTX pretreatment, neurons were subjected to a 5 min wash in control Tyrode's followed by a 3 min depolarization with isotonic 50mM (mouse) or 90 mM (rat) KCl as above. Residual high K^+ was removed with an additional 5 min control Tyrode's wash and cells were returned to conditioned NB media containing 1 μM TTX for 6 or 16 h at 37ºC, 5% CO_2. Rat neurons expressing the 3xNFAT-AP1–CFP-NLS reporter were fixed in 3.7% formaldehyde in PBS, permeabilized with 0.05% Triton X-100 in PBS, stained with a 1 μM solution of propidium iodide (PI) to visualize the nucleus, and mounted onto glass slides as above. Mouse luciferase expression was measured in mouse cultures using the Dual Luciferase Reporter Assay System (Promega) and a GloMax Multi Microplate Reader (Promega). NFAT-dependent firefly luciferase signal was normalized to *Renilla* luciferase to correct for transfection and expression differences among cultures.

Phospho-Ca_v1.2 Western Blots

Primary hippocampal neurons from AKAP150 WT, ΔPIX, and ΔPKA mouse strains were cultured to DIV 12-16 in 6-well culture dishes and scraped into RIPA buffer (10.0 mM Tris pH 7.4, 100.0 mM NaCl, 5.0 mM EDTA, 5.0 mM EGTA, 1.0% Deoxycholate, 0.1% SDS, 1.0% Triton X-100) containing the protease inhibitors 1 μM AEBSF, 1 μM benzamidine, 1 μM calpain inhibitors I & II, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and the phosphatase inhibitors cyclosporine A, 1 μM microcystin, 5 mM NaF, and 2 mM Na_3VO_4. Each batch of cultured neurons were then lysed using a Dounce homogenizer. Extracts were cleared by centrifugation (20,800g) at 4°C for 20 min and stored at -80°C until immunoblotting. Prior to gel loading, samples were heated to 65°C for 20 min in sample buffer (62.5 mM Tris-HCl pH 6.8, 10.0 % glycerol, 5.0 % β-mercaptoethanol, 2.0% SDS, 0.025 % Bromophenol blue). 20μg of total extract protein was electrophoresed through Tris-SDS gels and transferred to PVDF membranes in 7.5% methanol. Blots were blocked for 1 hr at room temperature. Primary Ca_V1.2 antibodies were diluted 1:500 in 3% BSA/TBS-T (rabbit anti-Ca_V1.2 pS1700 (Fuller et al., 2010), rabbit anti-Ca_V1.2 pS1928 (De Jongh et al., 1996), and mouse anti-Ca_V1.2 (Neuromabs, clone

L57/46). Following 3x5min TBS-T washes, blots were incubated with goat α-mouse or goat α-rabbit horseradish peroxidase (HRP) conjugated secondary antibodies (Bio-Rad; 1:5000 in TBS-T) for 1 hr. After 4x5min TBS-T washes, detection was performed with chemiluminescence (WestPico Chemiluminescent Substrate; Pierce). Blots were imaged using an Alpha Innotech Fluorchem gel documentation system, and band intensities were analyzed using ImageJ software (NIH).

Supplementary References

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