Supporting Information

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SI Methods

Construction of Venus- and Amber-Tagged Calcium/Calmodulin-Dependent Kinase (CaMK) IIα Constructs and Venus Concatamers. Mouse CaMKIIα was PCR amplified by using *Pfu*Ultra (Stratagene) from a plasmid encoding the enzyme [a generous gift from D. Lovinger (National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD) and R. Colbran (Vanderbilt University, Nashville, TN)] and directionally cloned into Venus C1 (VC1), Venus N1 (VN1), or Amber C1 (AC1) (1, 2). Venus contained the A206K mutation, which prevents Venus dimer formation intrinsic to the wild-type fluorophore (3). For directional cloning into destination vectors, a SalI site (bold) and a BamHI (bold) site were incorporated into the sense 5′-GGCCT**GTCGAC**GCTACCATCACCTGCACCCGATTC-3′ and antisense 5′-GATC**GGATCC**TTCAATGCGGCAGGACGGAGGGCGCCCCAGA-3′ primers, respectively. The PCR product was subcloned into Zero Blunt II-TOPO (Invitrogen) and ligated into the SalI/BamHI-digested destination vectors and sequence verified.

Placement of CaMKIIα into VN1 was similarly accomplished by PCR amplification using XhoI and SacII restriction sites within the sense 5′-GATC**CTCGAG**GCCACCATGGCTACCATCACC-3′ and antisense 5′-GATC**CCGCGG**ATGCGGCAGGACGGA-3′ primers, respectively. The PCR product was subcloned into Zero Blunt II-TOPO and subsequently ligated into XhoI- and SacII-digested VN1. The construct was then sequence verified.

Introduction of the T286D into CaMKIIα in VC1 was accomplished through site-directed PCR mutagenesis by using *Pfu*Ultra polymerase and the sense 5′-CACAGACAGGAGGACGTGGACTGCCTGAAG-3′ and antisense 5′-CTTCAGGCAGTCCACGTCCTCCTGTCTGTG-3′ primers to generate aspartic acid in place of threonine at position 286 of the enzyme. The construct was then sequence verified.

Introduction of the T286A point mutation into CaMKIIα in VC1 was accomplished through site-directed PCR mutagenesis by using *Pfu*Ultra polymerase and the sense 5′-ATGCACAGACAGGAGGCCGTGGACTGCCTGA-3′ and antisense 5′-TCAGGCAGTCCACGGCCTCCTGTCTGTGCAT-3′ primers to generate an alanine in place of threonine at position 286 of the enzyme. The construct was then sequence verified.

Construction of CaMKIIα(1–315) was accomplished by placing a stop codon in V-CaMKIIα by using the sense primer 5′-ACTATGCTGGCCACCAGGAACTTCTCCGGATGAA**GTCGAC**GTAC-3′ and the antisense primer 5′-GTAC**GTCGAC**TTCATCCGGAGAAGTTCCTGGTGGCCAGATAGT-3′, which also placed a SalI site (bold) downstream from the stop codon to facilitate the screening of clones. The construct was sequence verified and found to have incorporated two stop codons; one correctly placed and another 3′ from position 316.

The construct Venus 5–Venus [VV(5)] was prepared as follows. First, a sense primer with a BglII site (underlined) 5′-GCAGATCTGTGAGCAAGGGCGAGGAGCTGTTCACC-3′ and an antisense primer with an EcoRI site (underlined) 5′-GCGAATTCCTTGTACAGCTCGTCCATGCCGAGAGTG-3′ were used to PCR amplify Venus from the C1 vector. The resultant fragment was cloned into Zero Blunt II-TOPO and sequenced. Full-length cDNA for Venus was excised by using BglII and EcoRI and cloned into VC1 to generate VV(5). Constructs were confirmed by restriction digest analysis.

The Venus 17–Venus (VV) clone was generated by amplifying the full-length Venus cDNA without the first two codons (ATG GTG) using oligonucleotides containing an Asp-718 site on the sense primer (underlined) 5′-AGTCGGTACCAGCAAGGGCGAGGAGCTGTT-3′ and a BamHI site on the antisense primer (underlined) 5′-AGTCTCGGATCCCTTGTACAGCTCGTCCATGCCGAGAGTGATC-3′. The insert was cloned into Asp-718/BamHI-digested VC1 to make VV.

To generate Venus 5–Venus 6–Venus (VVV) Venus was amplified by using a sense primer with a SalI site (underlined) 5′-GCGTCGACGGGTGAGCAAGGGCGAGGAGCTGTTCACCG-3′ and an antisense primer with a BamHI site (underlined) 5′-AGTCTCGGATCCCTTGTACAGCTCGTCCATGCCGAGAGTGATC-3′. The resulting fragment was cloned and sequenced as described earlier. The Venus fragment was cloned into the SalI/BamHI-digested VV (5) to generate VVV.

VVVVVV was generated by the sequential addition of three Venus ORFs into VVV. First, a sense primer with the BspE1 site (underlined) 5′-AGTCTCCGGAAGGAGGTGGAAGCAAGGGCGAGGAGCTG-3′ and an anti sense primer with a BglII site 5′-AGTCAGATCTTCCACCTCCCTTGTACAGCTCGTCCATGCC-3′ were used to amplify Venus with *Pfu*Ultra (Stratagene). The DNA fragment was cloned into Zero Blunt II-TOPO, and the insert was sequenced. The insert was excised with BspE1 and BglII and cloned into VVV to generate V5V5V6V. Second, a sense primer with an EcoRI site (underlined) 5′-AGTCGAATTCGGAGGTGGAAGCAAGGGCGAGGAGCTG-3′ and a reverse primer with a SalI (underlined) and an EcoRV (underlined and bold) site 5′-AGTCGTCGACG**GATATC**CTTGTACAGCTCGTCCATGCC-3′ were used to amplify Venus. The insert was cloned into Zero Blunt II-TOPO and the insert was sequenced. The insert was excised and cloned into EcoRI/SalI-digested V5V5V6V to generate V5V5V5V5V. Finally, a sense primer with an EcoRV (underlined) site 5′-AGTCGATATCGGAGGTGGAAGCAAGGGCGAGGAGCTG-3′ and a SalI site (underlined) 5′-AGTCGTCGACCTCCACCCTTGTACAGCTCGTCCATG-3′ were used to amplify Venus and cloned into Zero Blunt II-TOPO, and the insert was sequenced. The insert was excised and cloned into an EcoRV- and SalI-digested V5V5V5V5V to generate VVVVVV.

Cell Culture. HeLa cells were cultured in medium containing 90% DMEM, 1 mM sodium pyruvate, 0.5 mM GlutaMAX (Gibco), 10% FBS, and nonessential amino acids (Gibco). Cells were dissociated with TryplE express (Invitrogen) and plated onto glass-bottom culture dishes. Transfection of cDNA was accomplished by using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Imaging took place the next day.

Sprague–Dawley rat pups (P0–P1) were killed according to National Institute on Alcohol Abuse and Alcoholism/National Institutes of Health animal regulations. Hippocampi were removed and dissociated in papain (PAP2; Worthington) for 30 min at 37 °C. Cells were subsequently washed with inactivation solution consisting of MEM (Gibco) and 5% FBS and mechanically dissociated. Cells were spun down, resuspended in inactivation medium, and plated at a concentration of 60,000 cells per dish. Glass-bottom dishes were precoated with poly-L-lysine (Sigma) overnight and washed once before plating. Three hours after plating, dishes were flooded with growth medium containing 90 mL of MEM, 5 mL of FBS, 2 mL of B-27 (Gibco), 100 μL of Mito Serum Extender (BD Biosciences), 1 mM sodium pyruvate, 1× penicillin/streptomycin, 2 mL of 30% dextrose, and 0.5 mM GlutaMAX (Gibco). Cultures were

pulsed with 5 μM cytosine 1-β-D-arabinofuranoside (AraC; Sigma) 3 days after plating. AraC was removed on days 5 and 6. Transfection of DNA constructs was accomplished by using Lipofectamine 2000 with cultures from 7 to 15 days in vitro. Imaging took place the day after transfection.

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3. Zacharias DA, Violin JD, Newton AC, Tsien RY (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296:913–916.